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Myoendothelial gap junction frequency does not account for sex differences in EDHF responses in rat MCA

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Abstract

Previous findings from our laboratory have shown that dilations to endothelium-derived hyperpolarizing factor (EDHF) in rat middle cerebral artery (MCA) are less in females compared to males. Myoendothelial gap junctions (MEGJs) appear to mediate the transfer of hyperpolarization between endothelium and smooth muscle in males. In the present study, we hypothesized that MEGJs are the site along the EDHF pathway which is compromised in female rat MCA.

Membrane potential in endothelium was measured using the voltage-sensitive dye di-8-ANEPPS and in smooth muscle using intracellular glass microelectrodes in the presence of L-NAME (3×10^{-5} M) and indomethacin (10^{-5} M). Electron microscopy was used to assess MEGJ characteristics. In endothelial cells, the di-8-ANEPPS fluorescence ratio change to 10^{-5} M UTP was similar in males ($-2.9\pm0.5\%$) and females ($-3.2\pm0.2\%$), indicating comparable degrees of endothelial cell hyperpolarization. However, smooth muscle cell hyperpolarization to 10^{-5} M UTP was significantly attenuated in females (0 mV hyperpolarization; -31 ± 1.5 mV resting) compared to males (8 mV hyperpolarization; -28 ± 1.7 mV resting). Ultrastructural evidence suggested that MEGJ frequency and area of contact were comparable between males and females. Taken together, our data suggest that in rat MCA, MEGJ frequency does not account for the reduced EDHF responses observed in females compared to males. We conclude that reduced myoendothelial coupling and/or homocellular coupling within the media may account for these differences.

Keywords: Endothelium-derived hyperpolarizing factor; Gap junctions; Sex; Smooth muscle

Introduction

The mechanism defining endothelium-derived hyperpolarizing factor (EDHF)-mediated dilations in the vasculature still remains largely unresolved. As a complicating factor, the mechanism appears to be specific to the vascular bed (Dong et al., 2000), age (Fujii et al., 1993), species (Triggle et al., 1999), and pathological condition (Fukao et al., 1997; Golding et al., 2001). Nonetheless, there are certain hallmark characteristics of EDHF that stand alone (Busse et al., 2002; Feletou and Vanhoutte, 2006). Namely, it is independent from both nitric oxide (NO) and prostacyclin, it requires activation of endothelial cell (EC) calcium-sensitive potassium (K_{Ca}) channels, and it culminates in smooth muscle cell (SMC) hyperpolarization (Golding et al., 2002a,b). Many 'factors' have been ascribed to the actions of EDHF including K^+ (Edwards et al., 1998), 11,12 epoxyeicosatrienoic acid (Campbell et al., 1996; Fisslthaler et al., 1999), hydrogen peroxide (Matoba et al., 2000) and C-type natriuretic peptide (Chauhan et al., 2003). However, in some arteries, EDHF does not appear to be solely attributed to a diffusible endothelial factor, leading to speculation that direct electrical communication between the endothelium and smooth muscle may exist (Sandow and Hill, 2000). Indeed, recent studies in rat middle cerebral artery (MCA) have implicated a role for intercellular channels called gap junctions in mediating EDHF responses (Sokoya et al., 2006; McNeish et al., 2006). In

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this way, EC hyperpolarization may be communicated directly to the underlying SMC via myoendothelial gap junctions (MEGJs) (Sandow et al., 2002).

To add to the heterogeneity of the EDHF response, we have shown that sex is a contributing factor. In the rat MCA, vasodilations to EDHF are less in females than in males (Golding and Kepler, 2001). The reasoning behind this is largely unknown although previous studies suggest that intermediate KCa channels are not involved (Sokoya et al., 2007) and the site has been localized to being downstream from EC calcium and upstream from SMC hyperpolarization (Golding et al., 2002a,b).

In the present study, we hypothesized that MEGJs are the site along the EDHF pathway which is compromised in female rat MCA. To address this hypothesis, we assessed both function and structure of MEGJs in male and female rat MCAs by measuring EC and SMC hyperpolarization during EDHF stimulation and examining MEGJ frequency, respectively. Our findings revealed that while EC hyperpolarization was comparable, SMC hyperpolarization was attenuated in females compared to males. MEGJ area and frequency were comparable between males and females. We conclude that reduced myoendothelial coupling and/or SMC homocellular coupling may account for the attenuated EDHF-mediated SMC hyperpolarization in females.

Materials and methods

Experiments were carried out in accordance with the NIH guidelines for the care and use of laboratory animals and were approved by the Animal Protocol Review Committee at Baylor College of Medicine. Rats were housed under a 12 h light/12 h dark cycle with unrestricted access to food and water. Experiments were performed on age-matched (70–90 days old) male ($n=17$) and female ($n=17$) Long–Evans rats.

Harvesting and mounting cerebral vessels

Animals were allowed to spontaneously breathe isoflurane in an anesthetic chamber and were then decapitated. The brain was removed and placed in cold physiological salt solution (PSS). The MCA was harvested, cleaned of surrounding connective tissue and cannulated with micropipettes in a vessel chamber. PSS was circulated abluminally through a heat-exchanger in order to maintain the bath temperature at 37 °C. Intraluminal pressure was monitored via in-line transducers, which were connected to two strain gauge panel meters (Omega, Stamford, CT). Once mounted, vessels were tested for leaks and those that did not maintain a steady pressure were discarded. The vessel chamber was mounted on the stage of an inverted microscope. Transmural pressure was set at 85 mm Hg with a flow of 150 μl/min through the lumen, and the vessels were allowed to equilibrate for 1 h. During this time the vessels constricted from their fully dilated diameter at initial pressurization. Vessels that did not develop this 'spontaneous tone' were deemed non-viable and discarded.

Measurement of endothelial cell membrane potential changes

Following the development of spontaneous tone in pressurized MCAs, the luminal and abluminal compartments were exposed to L-NAME (3×10^{-5} M) and indomethacin (10^{-5} M) for 30 min to inhibit nitric oxide synthase and cyclooxygenase, respectively. EC membrane potential changes were measured using a voltage-sensitive dye, 4-{2-[6-(dioctylamino)-2-naphthalenyl]ethenyl} 1-(3-sulfopropyl)-pyridinium (di-8-ANEPPS) (Beach et al., 1996; Marrelli et al., 2003). The ratio of the 560 and 620 emissions changes with endothelial membrane potential thereby offering a qualitative measurement of EC membrane potential changes. In order to reduce the potential artifact of changes in fluorescence intensity due to vasodilation, MCAs were pre-dilated using verapamil (3 μM, abluminally). Dye was selectively loaded into the endothelium by perfusing the lumen of the MCAs with di-8-ANEPPS (10 μM/0.1% pluronic) for 20 min followed by a 20 min washout period. Using this method, previous studies using confocal microscopy have shown that the dye is confined to the endothelium (Marrelli et al., 2003). UTP (10^{-5} M) was specifically applied to the lumen for 5 min and then washed out with PSS. Prior work has shown that this concentration of UTP produces a maximum dilation of male rat MCA (You et al., 1997).

Measurement of smooth muscle cell membrane potential

The MCA was isolated, cut open longitudinally and pinned to the base of a vessel chamber with the smooth muscle facing upwards. The preparation was superfused with PSS. The vessels were exposed to L-NAME (3×10^{-5} M) and indomethacin (10^{-5} M) for 30 min. Membrane potential was recorded according to the protocol previously described (Sandow et al., 2002). Briefly, intracellular glass microelectrodes with resistances of 100 to 150 MΩ were filled with 2% Lucifer Yellow and 1 M KCl. During impalement, the Lucifer Yellow diffused into the cell, thereby confirming identification of the impaled cell. Membrane potential changes to UTP (10^{-5} M) or KCl (15 mM) were then recorded in the smooth muscle.

Electron microscopy

MCAs were processed for electron microscopy as previously described (Sokoya et al., 2006). Briefly, anesthetized male and female rats were perfusion fixed with Sorenson's PBS containing 3% glutaraldehyde. The brain was removed and immersed in this fixative overnight at 4 °C. The following day, the MCA was dissected from the brain and placed in PBS at 4 °C. Tissue samples were post-fixed in 1% tannic acid (5 min) followed by 1% osmium tetroxide (1 h) and then aqueous uranyl acetate (1 h). Samples were subsequently dehydrated in a graded ethanol series, embedded in Araldite resin and ultrathin serial sections (~100 nm) were obtained using an ultramicrotome (RMC 7000, RMC, AZ) equipped with a diamond knife. Sections were stained with uranyl acetate and lead citrate before viewing with a JEOL 200CX electron microscope.

Myoendothelial gap junctions (MEGJs) were identified as a breach in the basal laminae of both the EC and SMC through which cell protrusions extended such that the distance between the EC and SMC plasma membranes was ≤3.5 nm. MEGJs displayed the characteristic pentalaminar membrane structure where the central region had a higher electron opacity than the inner leaflets. MEGJ area was determined as the length of membrane contact multiplied by the thickness (100 nm) of the section multiplied by the number of sections in which it appeared. The maximum diameter of the MCAs was calculated from circumference measurements (using the public domain NIH Image J Program) made on thick (0.5 μm) sections stained with toluidine blue and viewed on a light microscope.

En face whole mounts

Male and female rats were perfused via the left ventricle with 0.2% silver nitrate solution. The MCA was then dissected from the brain, cut open longitudinally with the endothelium facing upwards and placed on a microscope slide which was then coverslipped. EC borders, revealed by silver nitrate deposition, allowed measurements of EC area, perimeter, length and maximum width in *en face* whole mounts using MetaVue software (Universal Imaging Corporation, PA).

Drugs and solutions

Stock solutions of UTP (10^{-2} M) and L-NAME (3×10^{-2} M) were prepared in distilled water, aliquotted and then frozen. A stock solution of indomethacin (10^{-2} M) was prepared in a solution of Na_2CO_3 and distilled water (1:1 by weight).

Data analysis and calculations

All data are presented as means±SEM. For the EC and SMC membrane potential data, statistical significance was tested using a repeated measures