Evaluating crosstalk between epoxyeicosatrienoic acids (EETs) and neuropeptide signaling in neurogenic vasodilation

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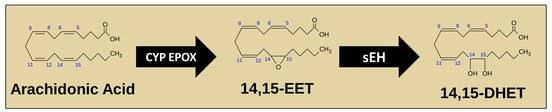
Background

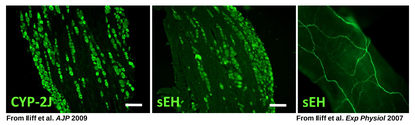
• **Epoxyeicosatrienoic acids (EETs)** are products of **cytochrome P450 (CYP) epoxygenase** metabolism of arachidonic acid. Cellular EETs levels, release and function are importantly regulated by their metabolism to dihydroxyeicosatrienoic acids (DHETs), which is catalyzed by the enzyme **soluble epoxide hydrolase (sEH).**



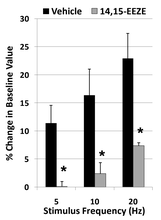
• **EETs** are potent **vasodilators** in the cerebral circulation produced both in the cerebrovascular endothelium and by cortical **astrocytes**.

• The cerebral surface vasculature is innervated by **extrinsic perivascular nerves**, including **parasympathetic** vasodilator fibers from the **sphenopalatine ganglia (SPG)** and vasodilator **sensory afferents** that project to the **trigeminal ganglia (TG)**.

• We have recently reported the expression of **EETs-synthetic CYP-2J** and **EETs-regulatory sEH** proteins in the rat **SPG** and **TG**, in addition to parasympathetic and trigeminal **perivascular fibers** innervating the **middle cerebral artery (below)**.



From Iliff et al. *AJP* 2009



From Iliff et al. *Exp Physiol* 2007

• Neurogenic cerebral vasodilation is blocked by the putative EETs antagonist, 14,15-EEZE (right). **These findings suggest that neurogenic EETs participate in the regulation of cerebral blood flow by perivascular vasodilator nerves.**

In this study, we evaluate two mechanistic explanations that account for the expression of EETs-related enzymes in perivascular vasodilator fibers and the functional effect of the EETs antagonist upon neurogenic CBF regulation:

1. **Neurogenic EETs act on the cerebral vasculature to modulate the vasomotor action of the parasympathetic and sensory neuropeptides VIP and CGRP.**
2. **EETs facilitate the release of the vasoactive neuropeptide CGRP from trigeminal neurons.**

Methods

Rat Primary Trigeminal Neuron Culture

Trigeminal ganglia were dissected from newborn Sprague Dawley rat pups, digested with trypsin and collagenase, and purified on a 35%-60% Percoll gradient. Neurons were plated in 24-well plates coated with poly-D-lysine and laminin in Neuralbasal A media supplemented with fetal bovine serum (2.5%), B27, glutamax, penistrep and nerve growth factor (50 ng/ml). Neurons remained in culture for four days prior to experimentation.

Immunofluorescence Double Labeling

Trigeminal neurons were initially plated on glass coverslips. After four days in culture, cells were washed with PBS, fixed with 4% paraformaldehyde (20 min), blocked for one hour at room temerature (5% normal donkey serum, 1% BSA, 0.5% Triton-X), and incubated at 4C overnight with primary antibody. After washing, secondary detection was conducted for 1 hr at room temperature. Coverslips were mounted with Prolong Antifade Gold and imaged by conventional fluorescence microscopy (Leica). Primary Abs: rabbit anti human CYP-2J2 (1:500, a gift from Dr. Darryl Zeldin, NIEHS), rabbit anti-sEH (1:500, Cayman Chemical), goat anti-rat CGRP (1:1000, Serotec), guinea pig anti-substance P (1:400, Millipore). Secondary Abs: Donkey anti-rabbit ALEXA Fluor 488, donkey anti-goat ALEXA Fluor 488, donkey anti-guinea pig ALEXA Fluor 488 (all at 1:800, Invitrogen)

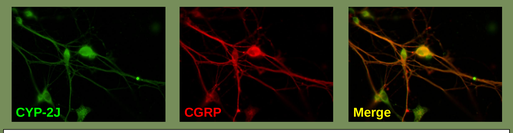
CGRP ELISA

After washing, cells were pre-treated for 30 min with stimulation buffer (Hepes-buffered saline) or drug, then stimulated for 60 min with buffer (control), 100 nM capsaicin, or 60 mM K⁺. Conditioned buffer was collected and assayed for CGRP content by ELISA (SPI-Bio). All release rates were normalized to control wells pre-treated and stimulated with buffer alone (representing basal release). The effect of drug upon CGRP release was analyzed by ANOVA with Bonferroni's post hoc test (Graphpad Prism).

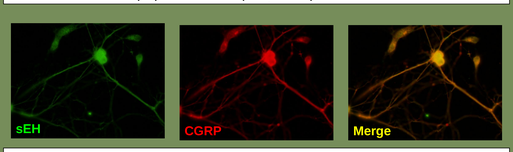
Closed Cranial Window

250-350 g male Sprague Dawley rats were anesthetized with chloralose and urethane (50 and 500 mg/kd, respectively), mechanically ventilated, equipped with a closed cranial window constructed from dental acrylic and a glass coverslip. Blood pressure was monitored via an arterial catheter. CBF was monitored by laser Doppler flowmetry (Moor Instruments). Vehicle (.0325% EtOH in artificial CSF) or 14,15-EETs (100 nM, Cayman Chemical) were perfused within the window for 30 min, after which cumulative concentration response curves were constructed for VIP and CGRP (Sigma). Each concentration of neuropeptide was perfused for 10 min and the peak change in cortical perfusion was measured. Concentration response curves were compared by two-way repeated-measures ANOVA and EC_{50} values were calculated (Graphpad Prism).

CYP-2J and sEH Expression in Trigeminal Neurons

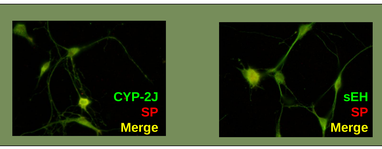


Rat trigeminal neurons (TNs) were cultured for four days. Immunoflourescent labeling revealed dominant CYP-2J expression in neuronal soma, with less intense labeling extending through dendritic processes. Double labeling revealed that CYP-2J (green) co-localized in TNs with the neuropeptide CGRP (red, n = 3).



Trigeminal neurons express sEH both in the soma and dendrites. Double labeling demonstrated that sEH immunoreactivity (green) co-localized with the neuropeptide CGRP (red, n = 3).

Both CYP-2J- and sEH-IR was observed in all trigeminal neurons observed.



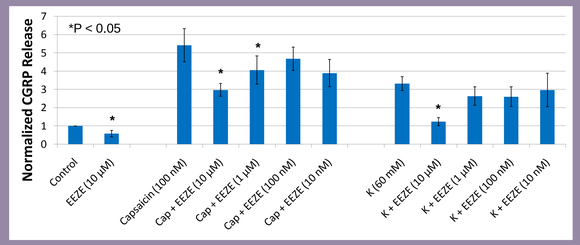
Double labeling further revealed that both CYP-2J (left, green) and sEH (right, green) immunoreactivity is evident in TNs expressing the neruopeptide substance P (SP, red; n = 3).

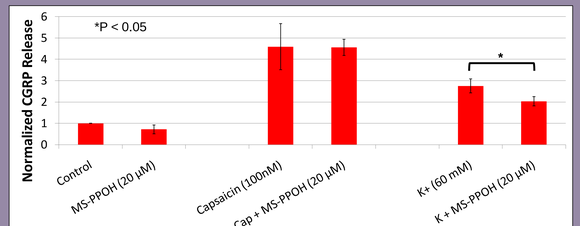
Regulation of Neuropeptide Release from Trigeminal Neurons by EETs

Trigeminal neurons were cultured for four days, then stimulated for 60 min with the TRPV1 agonist capsaicin (100 nM) or depolarizing K^+ (60 mM). Resulting CGRP release was assayed by ELISA. **We tested the hypothesis that inhibition of the EETs signaling pathway would impair CGRP release.**

Upper: 30 min pre-treatment with the putative EETs antagonist 15,15-EEZE attenuated basal (control) CGRP release, in addition to capsaicin- and K^+ -stimulated CGRP release (n = 8)

Lower: 30 min pre-treatment with the P450 epoxygenase inhibitor MS-PPOH (20 μM) significantly reduced K^+ -stimulated CGRP release. However, MS-PPOH did not significantly alter capsaicin-evoked CGRP release (n = 3).

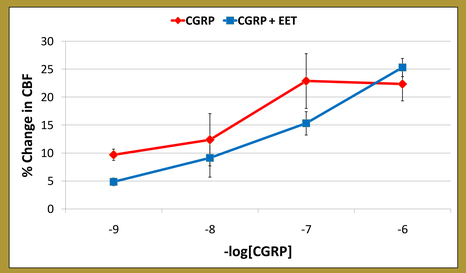




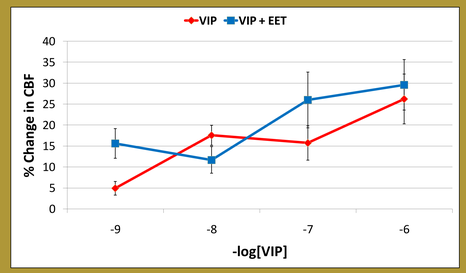
Post-Junctional Modulation of Vasodilator Effects of Neuropeptide by EETs

A rat closed cranial window was utilized to assess the effect of vasoactive neuropeptide administration to the cortical surface upon CBF, as measured by laser Doppler flowmetry.

We tested the hypothesis that EETs act post-junctionally to potentiate the vasomotor effects of the neuropeptides VIP and CGRP.



Administration of CGRP to the cortical surface produced a concentration-dependent increase in cortical blood flow. This effect (EC_{50} = 6.64 μM) was not altered by 30 minute pre-treatment with exogenous 14,15-EET (100 nM, n = 5).



Administration of VIP to the cortical surface evoked a concentration-dependent hyperemia. This effect (EC_{50} = 475 μM) was not altered by 30 minute pre-treatment with exogenous 14,15-EET (100 nM, n = 5).

Conclusion

In the present study, we report the following findings:

1. **Cultured rat trigeminal neurons express both CYP-2J epoxygenase and sEH protein.**

   These findings confirm that TNs express the biochemical machinery necessary for EETs synthesis and regulation.

2. **Exogenous EETs do not alter the in vivo cerebrovascular responses to the vasoactive neuropeptides VIP and CGRP.**

   These findings suggest that neurogenic EETs do not act post-junctionally to modulate the cerebral vasomotor response to neuropeptides released from extrinsic perivascular nerves.

3. **Inhibition of the EETs signaling pathway attenuated stimulus-evoked CGRP release from culture trigeminal neurons.**

   These findings suggest that neurogenic EETs act intracellularly to mediate neuropeptide release from perivasuclar neurons.

The regulation of neuropeptide release from trigeminal sensory afferents suggests an important contribution of EETs to the function of extrinsic perivascular vasodilator fibers innervating the cerebral surface vasculature.

Based upon this role, the epoxyeicosanoid pathway may represent a novel therapeutic target in neurovascular disorders characterized by trigeminovascular dysfunction, such as migraine.

Acknowledgements

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