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Expression of PGE_2 EP_3 receptor subtypes in the mouse preoptic region

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Abstract

Inflammatory-induced fever is dependent on prostaglandin E_2 (PGE_2) binding to its EP_3 receptor in the thermoregulatory region of the hypothalamus, but it is not known which EP_3 receptor isoform(s) that is/are involved. We identified the EP_3 receptor expression in the mouse preoptic region by *in situ* hybridization and isolated the corresponding area by laser capture microdissection. Real-time RT-PCR analysis of microdissected tissue revealed a predominant expression of the $EP_{3\alpha}$ isoform, but there was also considerable expression of $EP_{3\gamma}$, corresponding to approximately 15% of total EP_3 receptor expression, whereas $EP_{3\beta}$ was sparsely expressed. This distribution was not changed by immune challenge induced by peripheral administration of LPS, indicating that EP_3 receptor splicing and distribution is not activity dependent. Considering that $EP_{3\alpha}$ and $EP_{3\gamma}$ are associated with inhibitory and stimulatory G-proteins, respectively, the present data demonstrate that the PGE_2 response of the target neurons is intricately regulated.

Keywords: Prostaglandin E_2; Real-time RT-PCR; EP_3 receptor isoform; Fever; Laser capture microdissection; Mouse; Preoptic region

There is considerable evidence that fever is elicited by the binding of prostaglandin (PG) E_2, synthesized from arachidonic acid in response to an immune challenge, to its receptors in the preoptic area of the anterior hypothalamus [10–12]. Experiments in genetically modified mice have demonstrated that subtype 3 of the PGE_2-receptor (EP_3), which has been shown to be densely localized to the preoptic area [1], is critical for the febrile response, both to peripherally administered immunogens as well as when PGE_2 is directly injected into the brain [15,2,9].

The EP_3 receptor occurs in several isoforms that differ in their cytoplasmic carboxy-terminal tail. In rodents, three different isoforms have so far been identified, two of which (α and β) are exclusively coupled to inhibition of adenylate cyclase, whereas the third isoform (γ) is coupled to both stimulation and inhibition of adenylate cyclase [4,8,13]. While all three isoforms have been demonstrated in the brain [4,13], nothing is yet known about their distribution in discrete cell groups, such as the EP_3 receptor expressing nuclei in the preoptic area, and hence it is not clear which isoform(s) that may mediate the febrile response, or if isoform expression may vary during inflammation.

In the present study we aimed at characterizing the EP_3 receptor isoform distribution in the preoptic region of the mouse hypothalamus, by performing real-time RT-PCR analysis of the EP_3 receptor expressing area that had been isolated by laser capture microdissection, and to examine if isoform distribution was influenced by systemic immune challenge with lipopolysaccharide.

The study was performed on adult mice (C57Bl/6, and $EP_3^{-/-}$ on a C57Bl/6 background), which were housed at constant ambient temperature of 25 °C with food and water available *ad libitum*, and on a 12 h light/dark cycle (lights on at 8:00 h). All experiments were performed during the early phase of the light cycle. All experimental procedures were approved by the Animal Care and Use Committee at the Linköping University.

To identify the EP_3 receptor expressing region in the preoptic region, *in situ* hybridization histochemistry was used. In brief, mice were perfused transcardially with 4% paraformaldehyde in phosphate buffer for 20 min. Coronal frozen sections of the forebrain, 20 μm thick, were cut from rostral to caudal and mounted on slides in consecutive series. After treatment with 4% paraformaldehyde in phosphate buffer for 30 min, the slides were incubated with 0.001% proteinase K in 0.1 M Tris buffer with 0.05 M EDTA (pH 8.0) for 20 min at 37 °C. For hybridization [^{33}P]UTP or [^{35}S]ATP labeled riboprobes were used. The EP_3 probe was cloned into a pDrive vector (pDCV) using EP_3 specific primers (Table 1) generating a 905 bp cDNA. Following linearization with *Xba*1, the radioactive probe was synthesized using T7 polymerase. The hybridization solution

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Table 1
Primer sequences for *in situ* probes, standard curves (std) and real-time PCR (rt), fw = forward, rv = reverse

Primer	Sequence
EP_3 *in situ* fw	AGTCTTTCCTGCTGTGCATTG
EP_3 *in situ* rv	ATGGCCCTGGCTGTACTACTT
$EP_{3\alpha}$ std fw	GGATCATGTGTGTGCTGTCC
$EP_{3\alpha}$ std rv	CAGGTTGTTCATCATCTTTCCA
$EP_{3\beta}$ std fw	GGATCATGTGTGTGCTGTCC
$EP_{3\beta}$ std rv	TCGGTGTGTTTAATGGCAAG
$EP_{3\gamma}$ std fw	GATCTTGGATCCCTGGGTTT
$EP_{3\gamma}$ std rv	TTGGCTGCAAAAACTGTGTC
GAPDH std fw	CGTCCCGTAGACAAAATGGT
GAPDH std rv	TCTCCATGGTGGTGAAGACA
$EP_{3\alpha}$ rt fw	GCTTCCAGCTCCACCTCCTT
$EP_{3\alpha}$ rt rv	CATCATCTTTCCAGCTGGTCACT
$EP_{3\alpha}$ rt probe	6-FAM-TGCCCAGGCTCCT-MGB
$EP_{3\beta}$ rt fw	GGAAGTTCTGCCAGATGATGAA
$EP_{3\beta}$ rt rv	ATTCTCAGACCCAGGGAAACAG
$EP_{3\beta}$ rt probe	6-FAM-AAGTGGACTTTCATTGCAG-MGB
$EP_{3\gamma}$ rt fw	AGTTCTGCCAGGTAGCAAACG
$EP_{3\gamma}$ rt rv	GCCTGCCCTTTCTGTCCAT
$EP_{3\gamma}$ rt probe	6-FAM-TGTCTCCAGTTGCTCTAG-MGB

consisted of 50% formamide, 10% dextran sulphate, 2% Denhardt's solution, 0.3 M NaCl, 10 mM Tris, 1 mM EDTA, 0.025 M dithiothreitol, and tRNA (1 mg/ml), and was adjusted to an activity of 1×10^7 cpm/ml for the ^{33}P probe, and 5×10^6 for the ^{35}S probe. The sections were hybridized at 58 °C for 16 h, rinsed in 4× standard saline citrate (SSC) and incubated with 0.002% RNase A in 0.5 M NaCl, 10 mM Tris and 1 mM EDTA for 30 min at 37 °C. The slides were then rinsed in a series of SSC buffers (2× SSC, 2× SSC, 1× SSC, 0.5× SSC), heated at 72 °C in 0.1× SSC, and finally dehydrated, delipidized and dipped in autoradiographic emulsion (NTB2, Kodak, Rochester, NY). After 3 weeks of exposure the slides were developed, fixed (D-19, Kodak), and coverslipped. Hybridized sections were analyzed under darkfield illumination. Reconstruction of the preoptic area was done using the thionin-stained sections for the identification of anatomical landmarks in the adjacent hybridized sections. The position of the region showing EP_3 expression within the preoptic area was determined relative to the anterior segment of the third ventricle and the anterior commissure.

For subsequent real-time RT-PCR analysis, mice were given an i.p. injection of LPS (2 μg; Sigma, St. Louis, MO; 0111:B4) diluted in 100 μl saline ($n = 4$), or injected with saline only ($n = 4$), and killed by CO_2 asphyxiation 5 h later. The brains were rapidly dissected, covered with OCT freezing medium (Sakura Finetek, Zoeterwoude, NL) and frozen in isopentane on dry-ice. Eight-micrometer-thick coronal sections were cut on a cryostat, from caudal to rostral. The sections were stained using the HistoGene™ LCM Frozen Section Staining Kit (Arcturus, Mountain View, CA). The region corresponding to that displaying EP_3 expression in the *in situ* hybridization experiment was extracted by laser capture microdissection (Pix Cell II, Arcturus). Six adjacent sections were microdissected from each animal corresponding to approximately 240×10^{-6} mm^3 tissue per mouse. RNA from the microdissected tissue was isolated using PicoPure™ RNA Isolation Kit (Arcturus), and treated with DNAse using RNase-free DNase set (Qiagen, Hilden, Germany). The quality of the RNA was analyzed with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using RNA Pico II LabChip kit. cDNA was synthesized by SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA).

Due to differences in primer efficiency, the RNA was quantified using the relative standard curve method. A sequence of each EP_3 receptor isoform was amplified by HotStarTaq® DNA Polymerase kit (Qiagen) to prepare the standard curve using the primers listed in Table 1. The PCR products were separated by agarose gel electrophoresis and purified using E.Z.N.A.® Gel Extraction Kit (OMEGA Bio-Tek, Inc., Doraville, GA). The purified DNA-fragments were sequenced to ensure that the right product has been obtained (CyberGene AB, Huddinge, Sweden). Real-time primers and probes (Table 1) were designed using the Primer Express® oligo design software (Applied Biosystems UK, Warrington, UK) to specifically amplify each isoform with the probe containing a 5′ fluorescent reporter dye 6-FAM (6-carboxyfluorescein) and a non fluorescent 3′ quencher (MGB) (Applied Biosystems). The specificity of each set was tested by running the primer/probe set against the standard PCR product for each subtype. Care was taken to design primers specific for the $EP_{3\beta}$ isoform, without amplifying the non-translated β-sequence following the mRNA of the $EP_{3\alpha}$ isoform. As endogenous control, glucose-3 phosphate dehydrogenase (GAPDH) (TaqMan® Gene Expression Assay ID: Mm99999915_g1; Applied Biosystems) was used, with standard curve prepared using primers described in Table 1. PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System using TaqMan® Fast Universal PCR Master Mix according to the manufacturer's instructions. Standard curves were generated by amplifying the different PCR products corresponding to GAPDH and the α, β and γ EP_3 isoform, respectively, diluted from 10^{-16} to 10^{-20} g/μl. The correlation coefficient was higher than 0.99 for all standard curves. Concentration value was calculated from each standard curve, and the relative ratio of each gene correlated to the GAPDH value was adjusted to the lowest value ($EP_{3\beta}$ in LPS treated mice).

Data was analyzed by Minitab statistical software using a three way analysis of variance (ANOVA) with isoform and treatment as fixed factors and animals as a random factor nested within the treatment.

The *in situ* hybridization showed strong EP_3 expression in the preoptic area, surrounding the rostral end of the third ventricle. Specifically, the labeled region was localized to the rostral half of the part of the third ventricle that in frontal sections was situated anterior to the anterior commissure. This region, extending about 150 μm in length, involved the median preoptic and part of the medial preoptic nuclei (Fig. 1A and B.). The specificity of the EP_3 expression in this region was confirmed by the absence of signal in the knock-out mice (Fig. 1C and D).

The region identified above was microdissected mimicking the distribution pattern of EP_3 receptor mRNA expression (Fig. 2). Quantification of the EP_3 receptor isoforms α, β and γ showed that they were expressed at a ratio of 220:1:34 (α:β:γ) in the saline treated mice. LPS-treated mice displayed a similar