Available online at www.sciencedirect.com
SCIENCE DIRECT®
Immunobiology 209 (2005) 661–668
Immunobiology
www.elsevier.de/imbio

Impairment of the splenic immune system in $P2X_2/P2X_3$ knockout mice

Robson Coutinho-Silva[a,b], Gillian E. Knight[a], Geoffrey Burnstock[a,*]

[a]*Autonomic Neuroscience Institute, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK*
[b]*Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

Received 28 June 2004; accepted 23 September 2004

Abstract

The isolated spleens from male and female mice lacking $P2X_2$ and $P2X_3$ receptors ($P2X_2/P2X_3$ knockout (KO) mice) and those from wild-type (WT) mice were investigated by flow cytometry, immunohistochemistry and functionally by organ-bath pharmacology. The spleens from the $P2X_2/P2X_3$ KO mice weighed significantly more than the corresponding WT mice. Flow cytometry was used to isolate the mononuclear cells, which were then phenotyped. T-lymphocytes, B-lymphocytes and macrophages were identified and counted. It was found that the increase in size of the spleens from the KO animals corresponded to an increase in the numbers of mononuclear cells present and that all three cell types (T-lymphocytes, B-lymphocytes and macrophages) increased in much the same proportion as those from the WT animals. Immunohistochemical localisation of $P2Y_1$, $P2Y_2$ and $P2X_1$ receptors revealed their presence on the spleen capsule and trabeculae. $P2X_1$ receptors were also present on blood vessels. There was no difference in the expression of these receptors between the WT and $P2X_2/P2X_3$ KO spleens. Functional studies revealed the presence of multiple P2 receptors inducing the contraction of the spleen capsule, from both WT and KO mice. There was no difference in the contractions induced by adenosine 5′-triphosphate (ATP), α,β-methylene ATP, 2-methylthio ADP or uridine triphosphate from WT and KO mice. It is concluded that mice lacking both $P2X_2$ and $P2X_3$ receptors have enlarged spleens and that this is correlated with an increase in the number of immune cells, perhaps as a consequence of a compromised immune system and chronic infection.

Keywords: Spleen; P2 receptors

Introduction

The spleen, in common with other immune organs such as the thymus, is innervated by sympathetic nerves, which release noradrenaline (NA; Elenkov and Vizi, 1991; Haskó et al., 1995a) and probably adenosine 5′-triphosphate (ATP) as a cotransmitter (Burnstock, 1990). Activation of α_1-, α_2- and β-adrenoceptors on immune cells regulate immunomodulatory functions such as the production of inflammatory mediators (nitric oxide and cytokines; Elenkov et al., 1995; Haskó

Abbreviations: ATP, adenosine 5′-triphosphate; α,β-meATP, α,β-methylene ATP; BSA, bovine serum albumin; BSS, buffered saline solution; DAB, 3, 3′-diaminobenzidine; IL-6, interleukin-6; KO, knock out; 2-MeSADP, 2-methylthio ADP; NA, noradrenaline; PBS, phosphate-buffered saline; NGS, normal goat serum; NHS, normal horse serum; UTP, uridine 5′-triphosphate; WT, wild-type

*Corresponding author. Tel.: +44 20 7830 2948; fax: +44 20 7830 2949.
E-mail address: g.burnstock@ucl.ac.uk (G. Burnstock).

0171-2985/$ - see front matter © 2005 Elsevier GmbH. All rights reserved.
doi:10.1016/j.imbio.2004.09.007

et al., 1995b, 1998a, b) and most aspects of cellular and humoral immunity (Elenkov et al., 2000; Haskó and Szabó, 1998). P1 and P2 receptors are also abundantly expressed on immune cells (Burnstock, 2001).

The splenic capsule of the guinea pig, rat and mouse spleen is known to contract to NA via α_{1B}-adrenoceptors (Eltze, 1994, 1996; Han et al., 1987), although the physiological significance of the capsular contraction is not clear. Agonists to other receptor types also initiate contraction of splenic capsular smooth muscle, for example, the rat spleen contracts in response to adenosine (Fozard and Milavec-Krizman, 1993) and the mouse spleen to bethanechol (Wong, 1990). *In vivo* infusions of both NA and α,β-methylene ATP (α,β-meATP) induced splenic capsular contraction of the pig as measured by an increase in venous blood flow (Lundberg et al., 1989), suggesting the presence of $P2X_1$ and/or $P2X_3$ receptors.

In the present study, the effect of P2 receptor agonists was examined on the splenic capsular smooth muscle of wild-type (WT) and mice that were genetically modified such that they lacked both $P2X_2$ and $P2X_3$ receptors. In addition to the functional study, the immunohistochemical expression of $P2Y_1$, $P2Y_2$ and $P2X_1$ receptors in the mouse spleen was investigated.

Materials and methods

General procedures

Adult male and female mice that were lacking the gene for $P2X_2$ and $P2X_3$ receptors were generated on an inbred (C57Bl6) and an outbred (MF1) genetic background (Cockayne et al., 2000, 2002). These mice are referred to as $P2X_2/P2X_3$ knockout (KO) mice and are compared to WT mice.

All mice were killed by CO_2 asphyxiation and death was confirmed by cervical dislocation according to Home Office (UK) regulations covering Schedule 1 procedures.

The spleen was dissected free and placed immediately in modified Krebs solution. The spleen was weighed and then prepared either for flow cytometry experiments, immunohistochemistry or functional studies.

Preparation of splenocytes for flow cytometry experiments

Spleens were collected from the WT and $P2X_2/P2X_3$ KO mice as described above and cells were gently removed by mechanical dissociation and then re-suspended in buffered saline solution (BSS). The erythrocytes were removed and mononuclear cells enriched by centrifugation on a Ficoll density-gradient Histopaque 1083 (Sigma, St. Louis, MO, USA). The cells were counted and cell viability following this procedure was greater than 95% in all cases, as measured by Trypan blue exclusion. The splenocytes were adjusted to 10^6 cells/sample, washed with BSS and incubated with BSS containing 5% normal goat serum (NGS), on ice, for 20 min. The cells were then incubated for 30 min with FITC-conjugated rat anti-mouse CD3 (1:100; PharMingen, UK), FITC-conjugated rat anti-mouse B220 (1:100; PharMingen, UK) or FITC-conjugated rat anti-mouse CD11b (1:100; PharMingen UK) to stain T-lymphocytes, B-lymphocytes and macrophages, respectively, diluted in BSS with bovine serum albumin (BSA). The negative controls for the isotypes used were: FITC rat IgG_{2b} (PharMingen, UK) for CD3 and CD11b antibodies and FITC rat IgG_{2a} (PharMingen, UK) for B220 antibodies control. The isotype controls were used at the same concentration as the antibody tested. Samples were then washed fixed in fresh 4% paraformaldehyde (Sigma) for 10 min on ice and then extensively washed in cold BSS. The cells were then re-suspended in phosphate-buffered saline (PBS) and analysed on a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA, USA). Post-analysis was carried out using WinMDI (Multiple Document Interface Flow Cytometry Application, V2.8) software.

Preparation of spleens for immunohistochemistry

Tissue handling

The spleens were removed, weighed and put in Hanks BSS solution, then embedded in OCT tissue compound (BDH Laboratory Supply, UK), progressively frozen in isopentane (pre-cooled in liquid nitrogen) and then stored in liquid nitrogen. Cryostat sections of the spleens were cut as a set of serial sections 10 μm thick. The sections were thaw-mounted on gelatine-coated slides and air-dried at room temperature. The slides were stored at −20 °C until use. Tissues were post-fixed for 2 min at room temperature in 4% formaldehyde (BDH) and 0.03% picric acid in PBS. Inactivation of endogenous peroxidase was carried out in 50% methanol and 0.3% H_2O_2 for 25 min. Blocking of non-specific binding sites was achieved by pre-incubation with normal horse serum (NHS; Harlan Sera-Lab, UK) in PBS containing 0.05% thimerosal (Methiolate; Sigma) at room temperature for 20 min, as described by Llewellyn-Smith et al. (1993).

Immunostaining

An indirect immunohistochemical method with three-layer amplification was used. Antibodies against $P2X_1$ (Roche bioscience, Palo Alto, CA, USA), $P2Y_1$ and $P2Y_2$ receptors (Alomone Labs. Ltd., Jerusalem, Israel) raised in rabbit were allowed to react with biotinylated