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Extracellular ATP induces P2X7 receptor activation in mouse Kupffer cells, leading to release of IL-1β, HMGB1, and PGE2, decreased MHC class I expression and necrotic cell death

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ABSTRACT

Kupffer cells, which are resident macrophages in liver, can produce various cytokines and chemokines that induce hepatitis and liver fibrosis. It is suggested that extracellular ATP-induced activation of macrophage P2X7 receptor plays an important role in inflammation via release of pro-inflammatory mediators, but the role of P2X7 receptor in Kupffer cells remains unclear. Here, we show that activation of P2X7 receptor in Kupffer cells causes multiple inflammatory responses, using the clonal mouse Kupffer cell line (KUP5) that we previously established. Treatment of LPS-primed Kupffer cells with 3 mM ATP induced Ca^{2+} influx, non-selective large pore formation, activation of MAPK, cell lysis, IL-1β release, prostaglandin E2 (PGE2) release, high mobility group box1 (HMGB1) release, and major histocompatibility complex (MHC) class I shedding. These events were significantly suppressed by pretreatment with P2X7 antagonist A438079, indicating involvement of P2X7 receptor activation in these inflammatory responses. Our results suggest that extracellular ATP-induced activation of P2X7 receptor of Kupffer cells plays multiple roles in the inflammatory response in liver. P2X7 receptor might be a new therapeutic target for treatment of liver diseases.

1. Introduction

Kupffer cells/liver macrophages are involved in immune response in the liver via phagocytosis, antigen-presenting ability and production of cytokines, which induce inflammation, necrosis, regeneration, and fibrogenesis [1]. They also participate in the acute and chronic responses of the liver to toxic agents, leading to hepatic damage through the release of various molecules [2–4], including pro-inflammatory cytokines [5]. In liver disease, Kupffer cells produce both pro-inflammatory and pro-fibrogenic factors [6–8]. Liver cirrhosis is an increasing public health issue associated with alcoholic hepatitis, non-alcoholic steatohepatitis, and infection with viruses such as hepatitis C virus and hepatitis B virus. Virus-associated liver damage is attributed to immune-mediated mechanisms. In the inflammatory microenvironment caused by infections, infiltration of immune cells and secretion of inflammatory cytokines may result in liver damage, and may indirectly induce cell death. Reconstruction of damaged liver cells induces hepatic fibrosis, and progression of hepatic fibrosis eventually leads to liver cirrhosis, which can be considered as a wound-healing response to chronic inflammation [9]. Thus, cirrhosis is a consequence of the interaction between liver damage and tissue repair processes.

P2X7 receptor is the seventh member of the P2X receptor subfamily, and is expressed in immune cells, such as monocytes/macrophages, T cells, and dendritic cells [10]. P2X7 receptor

Abbreviations: $[Ca^{2+}]i$, cytosolic Ca^{2+} concentration; EtBr, ethidium bromide; HMGB1, high mobility group box 1; LDH, lactate dehydrogenase; MHC, major histocompatibility complex; PGE2, prostaglandin E2.

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has two unique features among the P2X receptors, i.e., it requires high concentrations (millimolar range) of ATP to be activated and its C-terminus is over 200 aa longer than that of any other family member [11]. P2X7 receptor is up-regulated in response to lipopolysaccharide (LPS) and other inflammatory stimuli [10]. Activation of P2X7 receptor induces Ca^{2+} influx, non-selective large pore formation (allowing the passage of hydrophilic molecules of up to 900 Da in size), MHC class I shedding, CD62 ligand shedding, membrane blebbing, fibrosis, caspase-1 activation, IL-1β release, PGE2 release, and cell death [10–14].

Though it is suggested that P2X7 receptor contributes to liver injury in acetaminophen hepatotoxicity and carbon tetrachloride-mediated steatohepatitis [15,16], the involvement of P2X7 receptor of Kupffer cells has not been established. Since activation of P2X7 receptor in macrophages causes inflammation, cell death, and fibrosis, we hypothesized that activation of P2X7 receptor in liver macrophages/Kupffer cells would contribute to liver cirrhosis. Therefore, the objective of this study was to define the role of P2X7 receptor activation in Kupffer cells in inflammation. To address this question, we focused on P2X7 receptor-mediated inflammatory functions, such as necrotic cell death, release of IL-1β, HMGB1 and PGE2, and MHC class I shedding. Our findings indicate that activation of P2X7 receptor in Kupffer cells has multiple roles in inflammation, cell death, and tissue repair in liver.

2. Materials & methods

2.1. Cell culture

Kupffer cells were isolated from mixed primary culture of C57BL/6 mouse liver cells as described, and immortalized by retroviral transduction of human c-myc [17]. The resulting clonal Kupffer cell line (KUP5) was cultured in D-MEM (high glucose type, Wako Pure Chemical, Osaka, Japan) supplemented with 10% heat-inactivated FBS (Life Technologies), 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL insulin and 100 μM 2-mercaptoethanol at 37 °C in the presence of 5% CO_2. As required, cells were pre-incubated for 4 h with 1 μg/mL LPS.

2.2. Mobilization of intracellular calcium

Cells were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-4AM (Invitrogen, Carlsbad, CA) for 30 min at 37 °C, washed twice with Ca^{2+}-free buffer, and suspended in HBSS-HEPES buffer. Samples were analyzed using a fluorescence spectrometer (F-2500, Hitachi) with laser excitation at 495 nm and emission at 518 nm.

2.3. Analysis of pore formation

Cells were re-suspended in HBSS-HEPES buffer at 1.0×10^5 cells/mL and incubated with various concentrations of ATP (Sigma, St. Louis, MO) and 25 μM ethidium bromide (EtBr) at 37 °C. After incubation, each sample was analyzed using a flow cytometer (FACSCaliber cytometer, Becton, Dickinson and Co., Franklin Lakes, NJ) with laser excitation at 488 nm. Ethidium fluorescence was examined using an FL-2 filter.

2.4. Immunoblotting

Culture supernatant or an equal amount of cell lysate was mixed with 2× sample buffer (50% glycerin, 2% SDS, 125 mM Tris, 10 mM DTT) and incubated at 95 °C for 10 min. Aliquots were subjected to 10% SDS-PAGE and bands were transferred to PVDF membranes. The blots were incubated for 2 h at room temperature in TBST (0.1% Tween-20, 10 mM Tris–HCl, 0.1 M NaCl) containing 1% bovine serum albumin (BSA), then further incubated overnight at 4 °C with phosphor-p44/p42 MAPK (Thr202/Tyr204) rabbit monoclonal antibody (1:1000) (Cell Signaling Technology, Inc., Beverly, MA), p44/p42 MAP kinase antibody (1:1000) (Cell Signaling Technology), phosphor-p38 MAPK (Thr180/Thr182) rabbit monoclonal antibody (1:1000) (Cell Signaling Technology), p38α MAP kinase mouse monoclonal antibody (1:1000) (Cell Signaling Technology), anti-HMGB1 antibody (1:1000) (Cell Signaling Technology) or COX-2 (Cell Signaling Technology) to confirm equal loading. Blots were washed with TBST, incubated with goat horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:20,000) (Cell Signaling Technology) or goat horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:20,000) (Santa Cruz Biotechnology) for 1.5 h, and washed again with TBST. Specific proteins were visualized by using ImmunoStar®LD (Wako) Western blotting detection reagents.

2.5. Quantification of lactate dehydrogenase (LDH) release

Release of LDH into the cell culture supernatant was quantified with a Cytotoxicity Detection Kit (Roche Applied Science, Penzberg, Germany), according to the supplied instructions. The cells were incubated in a 96-well plate at 37 °C for the indicated times with ATP in HBSS-HEPES buffer. At the end of incubation, supernatants were collected and the LDH content was measured. LDH release is expressed as a percentage of the total content determined by lysing an equal amount of cells with 1% Triton X-100.

2.6. Determination of IL-1β and PGE2

Culture supernatant was harvested, and IL-1β was measured by ELISA, as described previously [18]. PGE2 production was determined with an enzyme immunoassay (EIA) kit (Cayman Chemical) according to the manufacturer's instructions.

2.7. Membrane MHC I quantification

Cells were re-suspended in HBSS-HEPES buffer at 1.0×10^6 cells/mL in 1.5 mL tube and washed with HBSS-HEPES buffer. To identify surface MHC I (H-$2K^b$), cells were stained with PE-conjugated anti-MHC I (H-$2K^b$) for 30 min at 37 °C and then washed with HBSS-HEPES. The fluorescence intensity of these samples were analyzed with a flow cytometer (FACSCaliber cytometer, Becton, Dickinson and Co.).

2.8. Statistics

Values are given as the mean ± SE. Comparison between two values was performed by means of the unpaired Student's t-test. The statistical significance of differences between control and other groups was calculated by using Dunnett's test with the Instat version 3.0 statistical package (GraphPad Software, San Diego, CA, USA). The criterion of significance was set at $P < 0.05$.

3. Results & discussion

It is known that activation of P2X7 receptor induces an increase of intracellular Ca^{2+} ($[Ca^{2+}]_i$). As shown in Fig. 1A, we examined the involvement of ATP in the elevation of $[Ca^{2+}]_i$. When KUP5 cells were stimulated with ATP, they showed an initial peak of $[Ca^{2+}]_i$ followed by a sustained increase. Pretreatment with A438079 (a P2X7 receptor antagonist) (Tocris Bioscience) suppressed the sustained phase, but not the initial peak of $[Ca^{2+}]_i$. These results indicate that the sustained phase of $[Ca^{2+}]_i$ elevation is induced by