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REVIEW

Purinergic signaling in liver disease: calcium signaling and induction of inflammation

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Abstract

Purinergic signaling regulates many metabolic functions and is implicated in liver physiology and pathophysiology. Liver functionality is modulated by ionotropic P2X and metabotropic P2Y receptors, specifically P2Y1, P2Y2, and P2Y6 subtypes, which physiologically exert their influence through calcium signaling, a key second messenger controlling glucose and fat metabolism in hepatocytes. Purinergic receptors, acting through calcium signaling, play an important role in a range of liver diseases. Ionotropic P2X receptors, such as the P2X7 subtype, and certain metabotropic P2Y receptors can induce aberrant intracellular calcium transients that impact normal hepatocyte function and initiate the activation of other liver cell types, including Kupffer and stellate cells. These P2Y- and P2X-dependent intracellular calcium increases are particularly relevant in hepatic disease states, where stellate and Kupffer cells respond with innate immune reactions to challenges, such as excess fat accumulation, chronic alcohol abuse, or infections, and can eventually lead to liver fibrosis. This review explores the consequences of excessive extracellular ATP accumulation, triggering calcium influx through P2X4 and P2X7 receptors, inflammasome activation, and programmed cell death. In addition, P2Y2 receptors contribute to hepatic steatosis and insulin resistance, while inhibiting the expression of P2Y6 receptors can alleviate alcoholic liver steatosis. Adenosine receptors may also contribute to fibrosis through extracellular matrix production by fibroblasts. Thus, pharmacological modulation of P1 and P2 receptors and downstream calcium signaling may open novel therapeutic avenues.

Keywords Liver disease · Steatosis · Purinergic receptors · P2X7 receptor · Inflammasome · Calcium signaling · Inflammation

Liver cell types in healthy tissue, disease, and danger signaling

The functional unit of the liver, known as the hepatic lobule, comprises a central vein and hexagonal or polygonal portal triads, consisting of the portal vein, hepatic artery, and bile duct. Sinusoids connect the central vein to the portal triads, traversing the hepatic plates. Although hepatocytes share similar morphology, their functions vary depending on their position along the porto-central axis within the hepatic lobule. Hepatocytes near the portal region specialize in oxidative liver processes such as gluconeogenesis, fatty acid oxidation, and cholesterol synthesis, while those closer to the central vein play a crucial role in glycolysis, lipid synthesis, and cytochrome P450-mediated drug metabolism. Metabolic zonation is established through a gradient of Wnt/ß-catenin signaling. Recent research has elucidated the involvement of LGR4/5 receptors and RSPO ligands in enhancing Wnt/ß-catenin signaling and regulating liver zonation [1]. Beyond hepatocytes, the liver houses stellate cells, cholangiocytes, endothelial and smooth muscle cells, and Kupffer cells, with the latter serving as the resident macrophages [2].

Liver metabolism plays a crucial role in maintaining the body's functional homeostasis. It oversees the regulation of blood glycemia; synthesis and degradation of glucose and glycogen; and metabolism of fatty acids, lipids, and

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cholesterol, as well as detoxification, among numerous other functions. The predominant liver cells are hepatocytes, which constitute 70–85% of the cell mass and are responsible for the metabolic and detoxification functions of the liver. In addition, in response to metabolic dysfunction or infection, hepatocytes release a variety of signaling molecules that elicit responses from the innate immune system [3], including the release of endogenous molecules into the extracellular space. These molecules, termed damage-associated molecular patterns (DAMPs) or "alarmins," are recognized by receptors on cell surfaces as signals of danger. DAMPs are released passively by dying cells following events such as injury, trauma, ischemia, or necrosis induced by infection, particularly prevalent in damaged hepatocytes in the liver. They can also be actively secreted by immune cells or stressed parenchymal and non-parenchymal cells.

Once released, DAMPs are sensed by pattern recognition receptors (PRRs) and proteins including NOD-like receptor protein 3 (NLRP3), which activates the inflammasome. This triggers the release of chemokines and other mediators, initially promoting a beneficial proinflammatory response that aids in combating infections and repairing cellular damage. However, prolonged release of DAMPs can have adverse effects, particularly in chronic liver disease, exacerbating inflammation and tissue damage.

Chronic release of DAMPs can be further exacerbated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) released during injury and inflammation, creating a cycle of increasing damage and inflammation. In the liver, this leads to the activation of immune cells such as Kupffer cells and monocyte-derived macrophages, which release cytokines like tumor necrosis factor-alpha (TNF-α). These cytokines activate pathways such as NF-kappaB in hepatocytes, further aggravating liver damage.

Therefore, while DAMPs and associated molecules are crucial for maintaining liver homeostasis at normal levels, their uncontrolled release and activation can significantly contribute to chronic liver injury and the progression of liver disease. The immune response of the liver is orchestrated by both resident Kupffer cells and non-resident immune cells, as comprehensively reviewed by Jain and Jacobson [4].

Liver disease is a major health problem worldwide as the 10th major cause of death (The World Health Organization 2023). It can develop as an inflammatory disease based on chronic dysregulation of hepatic homeostasis, which may be related to obesity (non-alcoholic fatty liver disease), excessive alcohol consumption (alcoholic fatty liver disease), viral infections, or responses to drug treatment. Steatosis, characterized as the accumulation of triglyceride in intracellular lipid droplets resulting from increased uptake of circulating lipids and sugar with an imbalance of fatty acid synthesis and oxidation, promotes inflammation, which then leads to fibrosis as a structural disruption of the liver tissue and can proceed to liver cirrhosis [5].

In alcoholic fatty liver disease, the release of DAMPs into the extracellular environment varies depending on the induced type of cell death. Apoptosis is typically associated with DAMP release from hepatocytes [7]. In cases of severe acute alcoholic hepatitis, necrosis predominates, characterized by hepatocyte swelling, autolysis, and death without significant signal transduction [8]. Necroptosis, a regulated form of necrotic cell death mediated by the RIPK1-RIPK3 heterodimer scaffold complex, also leads to the release of intracellular contents similar to necrosis. In both necrosis and necroptosis, multiple DAMPs are discharged into the extracellular space, initiating an inflammatory response [2].

The released DAMPs can vary according to the type of liver disease. For instance, in alcoholic liver disease, mitochondrial DNA, uric acid, ATP, and LCN2 induce proinflammatory signals through activation of the receptors: TLR9, NLR, P2X7, and LCN2R, respectively [6–8]. In NASH, mitochondrial DNA, single-stranded RNA, advanced glycation end products (AGE), mitochondrial ROS, biglycan, galectin-3, fibrinogen, and cholesterol crystals promote monocyte-derived macrophage activation, IFN and TNF-α production, T-cell recruitment, fibrosis, and hepatocyte ballooning, through activation of TLR9 and TLR7, the receptor for advanced glycation end products (RAGE), NLRP3, and the TLR2/4 [9–13]. In fibrosis, HMGB1 and HSP90 induce hepatic stellate cell activation by RAGE and TLR2/TLR4 activation, while ATP and adenosine, through P2X7, A2A, and A2B receptors, promote release of IL-1β and HMGB1 by monocyte-derived macrophages [14–16].

In liver cancer, specifically hepatocellular carcinoma, HMGB1 plays an important role in tumor initiation and progression through the activation of RAGE and TLR9, which can also control tumor growth and metastasis by different stimuli, including S100A1, S100A4, S100A8, S100A9, and mitochondrial DNA. Extracellular ATP can also induce hepatocellular carcinoma cell migration through P2Y11 receptor activation [9].

In the context of chronic inflammation, liver cells continuously generate growth factors, fibrogenic cytokines, and chemokines, all of which contribute to fibrogenesis. For example, TGF-ß, produced by immune cells, directly triggers fibrogenesis by stimulating the transcription of collagen types I and III via the Smad signaling pathway [17]. Conversely, IL-1β and TNF-α, while not directly activating hepatic stellate cells (HSCs), support the survival of activated HSCs, thereby promoting liver fibrosis [18]. Recent research highlights IL-33, a member of the IL-1 family, in liver fibrosis. Damaged hepatocytes release IL-33, which stimulates type 2 innate lymphoid cells (ILC2) to produce IL-13, further driving HSC activation through STAT6 activation [19].

Chemokines also play a significant role in liver fibrosis, affecting both immune and non-immune liver cells. Interactions between CXCL12 (SDF1) and its receptors, CXCR4 and CXCR7, modulate the balance between liver regeneration and fibrosis post-injury by influencing the hepatic vascular niche [20]. Expression dynamics of CXCR4 and CXCR7 in liver sinusoidal endothelial cells (LSECs) under various liver conditions determine the outcome [1]. For instance, increased CXCR7 levels promote liver regeneration by inducing factors like Wnt2 and HGF, while heightened CXCR4 expression under chronic hepatitis conditions shifts the vascular niche towards a pro-fibrotic state. Additionally, MCP-1 (CCL2), secreted by Kupffer cells and HSCs, recruits $CCR2^+$ $Ly6C^+$ monocytes into the liver. These monocytes differentiate into proinflammatory and pro-fibrotic Ly6Chi macrophages, which produce various cytokines and growth factors, thus bolstering the survival, activation, and proliferation of myofibroblasts and ultimately contributing to liver fibrogenesis [21].

However, it is crucial to acknowledge the dual role of hepatic macrophages in extracellular matrix (ECM) resolution. Restorative macrophages, characterized by diminished Ly6C expression, exhibit pro-resolution traits, including heightened expression of matrix metalloproteinases (MMPs) and growth factors, facilitating ECM resorption post-acute inflammation [21]. Hence, the delicate balance between proinflammatory and restorative macrophages, along with the intricate interplay between immune and non-immune cells in response to persistent inflammatory cues, determines the direction towards hepatic regeneration or fibrosis in chronic hepatitis.

Liver metabolic functions and purinergic receptor–induced calcium signaling

Calcium (Ca^{2+}) serves as a primary second messenger, overseeing various metabolic and cellular functions such as proliferation, differentiation, apoptosis, etc. Ca^{2+} enters cells either through receptor- or voltage-gated ion channels or is released from intracellular stores, most importantly from the endoplasmic reticulum (ER). In hepatocytes, physiological Ca^{2+} signaling depends primarily on the inositol 1,4,5-trisphosphate (IP_3) second messenger system and mobilization of intracellular Ca^{2+} stores and is characterized by frequency-modulated oscillations of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) [22, 23]. Hormone-induced $[Ca^{2+}]_c$ oscillations in hepatocytes play a pivotal role in the regulation of glycogenolysis, gluconeogenesis, oxidative metabolism, the balance between lipolysis and lipogenesis, and bile secretion pathways [22, 24–27]. Moreover, alterations in $[Ca^{2+}]_c$ concentration play a key role in controlling the regeneration, proliferation, and cell death of hepatocytes and cholangiocytes [27].

Only the liver is capable of synthesizing purine and pyrimidine rings necessary for nucleotide synthesis and exporting them through the blood [28]. Purinergic receptors provide a ubiquitous mechanism for signal transmission through the plasma membrane and maintain communication between cells of the organ and the body. Purinergic receptors consist of P1 receptors activated by adenosine and P2 receptors stimulated by ATP and other nucleotides. ATP-stimulated P2X ion channels are assembled from P2X1-P2X7 subunits [29]. Eight metabotropic P2Y G protein–coupled receptor subtypes were identified in mammalian tissue (P2Y1,2,4,6, 11–14) [30]. P2Y1,2,4,6,11 receptors act through the Gq protein [31] to induce cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C-β, generating IP_3, which then activates IP_3 receptors in the ER to release stored Ca^{2+} and generate $[Ca^{2+}]_c$ oscillations [32]. P2Y12,13,14 receptors preferentially couple to Gi/0 proteins [31]. In common with other IP_3-linked hormones [22], P2Y1, 2, and 4 receptors are suggested to activate glycogen phosphorylase and inhibit glycogen synthase [33]. The P2Y2 receptor participates in the proliferation and survival of oval cells, which are progenitors of hepatocytes and biliary epithelial cells [34]. Transcription of P2Y6 receptors was enhanced in response to rat stellate cell activation, and this was followed by pre-collagen expression and cell contraction [35]. P2X receptors promote the influx of Ca^{2+} from the extracellular space. Hepatocytes express P2X1-4 as well as P2X7 subunits, as shown by gene expression analysis, but in contrast to the $[Ca^{2+}]_c$ oscillations elicited by IP_3-linked P2Y receptors, hepatic P2X receptors elicit sustained $[Ca^{2+}]_c$ increases [32, 36]. The P2X receptor agonist Bz-ATP, acting through P2X4 receptors, was reported to cause Ca^{2+}-mediated activation of glycogen phosphorylase and glycogenolysis [36].

Purinergic receptor–mediated calcium signaling in hepatocyte proliferation and injury

Hepatocyte volume changes, commonly referred to as "swelling," are a normal part of physiological processes and play a crucial role in regulating hepatocellular metabolism and gene expression. These changes respond to various environmental factors, such as changes in osmolarity, oxidative stress, accumulation of intracellular substrates, and hormones like insulin [37].

However, abnormal cellular swelling is a key indicator of cellular injury and is termed oncosis. In liver pathology, this condition is known as "ballooning degeneration." During ballooning degeneration, hepatocytes become significantly

enlarged, often containing clumped cytoplasmic proteins and clear intracellular spaces. This phenomenon results from severe cellular injury, marked by ATP depletion and an increase in $[Ca^{2+}]_c$ [37]. These changes lead to loss of plasma membrane volume regulation and disruption of the hepatocyte's intermediate filament network. In severe cases, cell death may ensue. Ballooning degeneration is not exclusive to alcoholic liver disease but is also observed in conditions such as non-alcoholic steatohepatitis (metabolic dysfunction-associated steatotic liver disease, MASLD), ischemic liver injury, cholestasis, and various other forms of hepatic toxicity [38].

Calcium signaling contributes to both hepatocyte proliferation and liver injury (reviewed in [27]). Hepatocytes slowly divide under homeostatic conditions, with a complete turnover of hepatocytes lasting more than one year [39]. This process is largely enhanced upon liver injury and involves initial inflammation, which is then followed by a mitogen-induced proliferation phase [40]. It makes sense that extracellular ATP accumulation provides inflammatory signals such as through P2X7 receptor activation in Kupffer cells, resulting in IL-6 and TNF-α release and proinflammatory stimulation. IL-6 mediates proliferative actions in hepatocytes [41]. The importance of IL-6 in this process was recognized, as IL-6-deficient mice showed impaired hepatocyte regeneration [41]. IL-6 binds to its soluble receptor and then complexes with gp130, which is expressed by hepatocytes [42]. Soluble IL-6 receptors induce STAT3 signaling in hepatocytes for cytoprotection [43]. Calcium signaling for proliferation induction is also promoted in hepatocytes by mitogens including hepatocyte growth factor (HGF) resulting in the activation of nuclear phospholipase C-γ and liberation of calcium into the nucleoplasm [44] and activation of proliferation-inducing transcription. P2Y2 receptor stimulation has also been shown to promote hepatocyte proliferation following partial hepatectomy [45]. The downstream signaling pathway was mediated by activation of AP-1, serum response factor, which primes hepatocyte proliferation, and is also induced by other growth factors.

Chronic liver disease may result from intrinsic events, such as metabolic dysfunction, or from extrinsic factors, such as excessive alcohol consumption, infection by pathogens, or other events that induce oxidative stress and liver injury [16]. Dysfunctional purinergic signaling mediated by P2X and P2Y receptors has been reported in liver pathogenesis, such as in metabolic dysfunction-associated steatotic liver disease (MASLD), also known as non-alcoholic liver disease, and liver fibrosis [4].

Cells transport ATP into the extracellular space via pannexin 1 to support various functions. In this process, ATP is converted into AMP and adenosine. Extracellular ATP activates macrophages (MFs) through the P2X7 receptor, leading to the release of IL-1β and HMGB1, which promote inflammation and fibrogenesis. Adenosine in the extracellular environment interacts with G protein–coupled receptors, such as A2A (A2AR) or A2B (A2BR) receptors, stimulating fibroblasts to produce extracellular matrix (ECM) and thereby promoting fibrosis [16].

The participation of P2 receptors was indicated in diethylnitrosamine- or CCl_4-induced fibrosis models, where the unspecific P2 receptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS) inhibited fibrotic marker expression [46]. Under physiological conditions, nanomolar extracellular ATP concentrations maintain normal liver function by hepatocytes. In injured hepatocytes, intracellular ATP leaks out of the cells and can elevate local extracellular ATP concentrations to the near mM range [47]. These high ATP concentrations hyperactivate purinergic P2X receptors and elevate $[Ca^{2+}]_c$ by Ca^{2+} influx to cytotoxic concentrations, promoting further cell death [4]. High extracellular ATP concentrations are countered by CD39 and CD73, which hydrolyze ATP into AMP and adenosine [48]. However, tissue injury results in the unbalanced release of ATP and other damage-associated molecular patterns (DAMPs), which then activate resident immune cells in the liver and attract peripheral immune cells (T lymphocytes, neutrophils, eosinophils, among others) [49, 50]. Metabolic dysfunction-associated steatohepatitis (MASH, previously referred to as non-alcoholic steatohepatitis, NASH) in mice induced by feeding with methionine- and choline-deficient diet activates the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome [51] and pro-apoptotic caspase in the liver, inducing fibrogenesis and fibrosis [52]. As reviewed for alcohol-induced liver disease [53], elevated $[Ca^{2+}]_c$ levels trigger pro-caspase-1 processing into caspase-1 and NLRP3 inflammasome activation. High ATP concentrations released by dying hepatocytes induce P2X7 receptor-dependent Ca^{2+} influx and K^+ efflux in Kupffer cells, required for activation of the inflammasome and caspase-1, leading to programmed cell death [53]. The imbalance of ATP and adenosine ratios towards high ATP concentrations triggers the DAMP response, which attracts immune cells to the site of injury, such as cytotoxic T, NKT and T helper cells, neutrophils, Tregs, dendritic cells, and macrophages. Each of these cells expresses the P2X7 receptor (reviewed in [54]), which is cytotoxic when activated by high extracellular ATP concentrations (> 0.3 mM). P2X7 receptor activation in immune cells, such as macrophages [55], promotes pore formation for promoting proinflammatory cytokine release and cytotoxicity and cell death. This process involves pannexin channels. The pannexin Panx1 expressed in parenchymal and non-parenchymal liver cells [56] acts as an undocked gap junction hemichannel associated with the P2X7 receptor [57, 58], responding to extracellular ATP with the formation of a cation-selective ion channel. At high extracellular ATP concentrations

(> 0.3–0.5 mM), a DAMP characteristic process occurs, which triggers pore formation allowing the efflux of ATP, cytokines, and other molecules up to 900 Da [59]. The P2X7 receptor is linked to several disease states and has crucial roles in fatty liver disease, including hypertrophy of adipocytes and liver inflammation [60]. P2X7 receptor inhibition reduced ERK1/2 phosphorylation and mitigated alcoholic steatosis [61]. In macrophages, resembling Kupffer cells, p38 activation, and apoptosis were largely inhibited in the presence of P2X7 receptor antagonists [62]. These pathways leading to proinflammatory signaling and reactive oxygen species (ROS) production depend on initial Ca^{2+} influx mediated by the P2X7 receptor (see Fig. 1).

P2X7 receptor-mediated inflammation leading to IL-1β release, which is independent from NLRP3 inflammasome activation, has also been detected. Different from NLRP3 inflammasome activation, this mechanism does not depend on K^+ efflux, nor the participation of serine, cysteine, and aspartic proteases. Bockstiegel et al. [63] studied this phenomenon in human macrophages, which are often used as a model for inflammation. P2X7 receptor-induced IL-1β release was partially independent of caspase cleavage. The authors concluded that pro-IL-1β cleavage did not occur by serine, cysteine, and aspartic proteases, but through an unknown mechanism [63]. NLRP3 inflammasome-independent pathways are also suggested for the liver, where

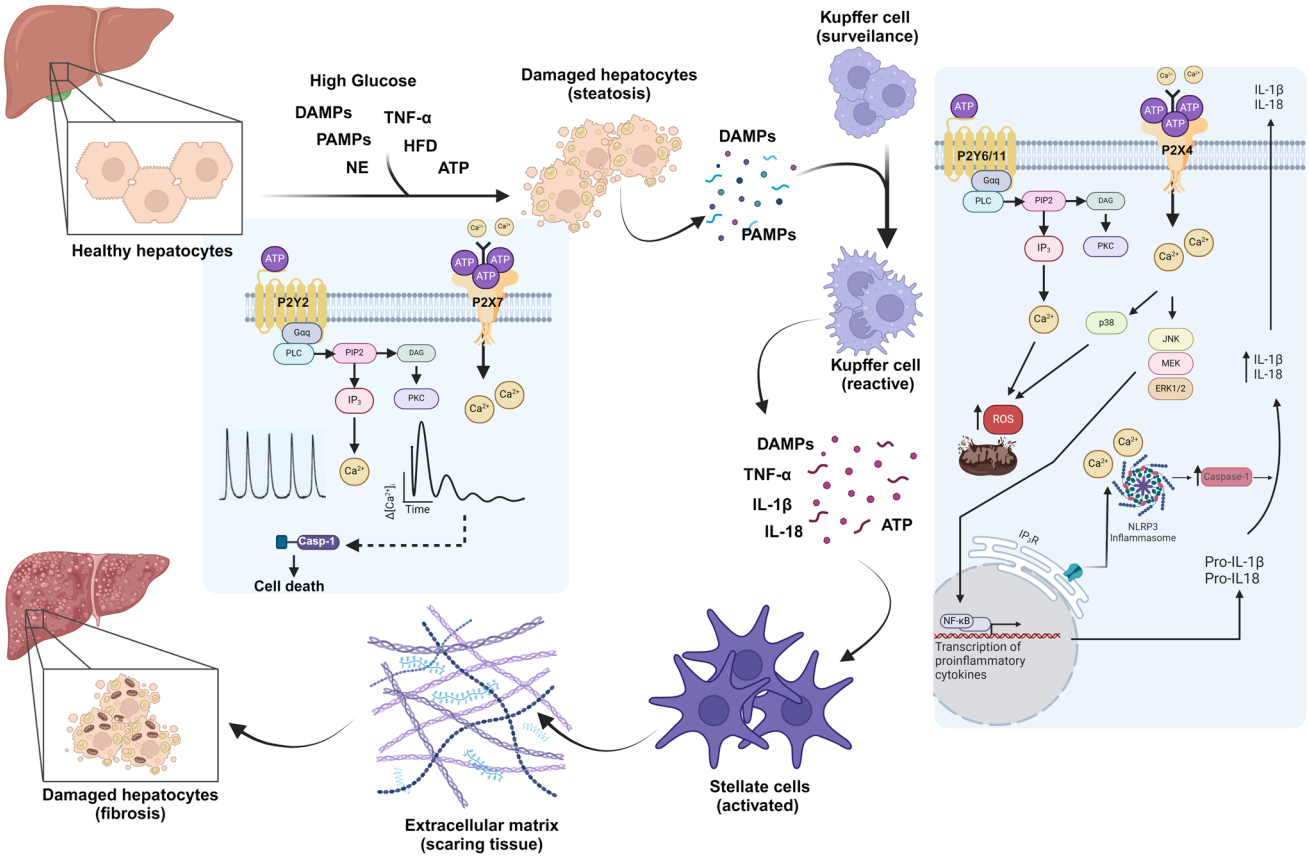

Fig. 1 Roles of purinergic receptors in liver fibrosis development. A healthy liver receives cues from the blood flow to control a plethora of physiological homeostasis processes, such as detoxification, metabolism of glucose, and lipids. This is controlled by a distinct pattern of $[Ca^{2+}]_c$ signals evoked by purinergic and other types of receptors. Chronic exposure of the liver to harmful signaling molecules, such as high glucose, tumor necrosis factor-alpha (TNF-α), high-fat diet, excess noradrenaline, damage-associated molecular patterns (DAMPs, ex. ATP), or pathogen-associated molecular patterns (PAMPs, ex. lipopolysaccharides, virus), leads to aberrant $[Ca^{2+}]_c$ signals that result in the activation of proinflammatory cytokine release and cell death. Dying hepatocytes extrude DAMPs, such as ATP, which activates P2X and P2Y receptors, including P2X4, P2X7, P2Y2, P2Y6, and P2Y11 subtypes, as emphasized in the figure. Aberrant Ca^{2+} signaling in Kupffer cells induces increased reactive oxygen species production, ERK1/2, and NF-kB activation leading to proinflammatory cytokine production. Ca^{2+} can also induce the assembling of NLRP3 inflammasome, which can favor the processing and release of interleukins 1β and 18 (IL-1β and IL-18). TNF-α is also released by reactive Kupffer cells. This change in extracellular milieu in the liver induces stellate cells to overproduce and secrete extracellular matrix proteins that in turn causes the scar tissue characteristic of fibrosis. GTP-binding protein alpha q (Gαq), phospholipase C-β (PLC), phosphatidylinositol 4,5-bisphosphate (PIP_2), inositol trisphosphate (IP_3), diacylglycerol (DAG), protein kinase C (PKC), c-Jun N-terminal kinase (JNK), mitogen-activated protein/extracellular signal-regulated kinase (MEK), extracellular-regulated kinase, noradrenaline (NE)

inflammatory cytokine release precedes mitogen-induced proliferation.

The P2X7 receptor is crucial for activation of the NLRP3 inflammasome, triggered by extensive K^+ outflow, which is needed for sustained Ca^{2+} entry into the cell [64]. Excessive Ca^{2+} influx as a consequence of P2X7 receptor pore formation results in Ca^{2+}-induced mitochondrial damage and activation of the NLRP3 inflammasome [65]. This process results in the production (processing and maturation) of the potent proinflammatory interleukins IL-1β and IL-18, as well as the cell death program involving the caspase cascade and activation of TNF-α. Importantly, IL-1β and TNF-α have been shown to activate TGF-β and fibrosis [66]. Consequently, liver fibrosis induced by excessive deposition of extracellular matrix, predominantly collagen, has been suggested as a consequence of P2X7 receptor-induced NLRP3 inflammasome activation in stellate cells [67, 68]. In agreement with a crucial role of the P2X7 receptor in liver damage and fibrosis, antagonism of this receptor by Brilliant Blue G resulted in diminished expression levels of IL-6, IL-1β, and TNF-α in a rat model of liver cirrhosis induced by bile-duct ligation [69]. Consistent with this, P2X7 receptor deficiency resulted in a reduction of hepatocyte death and inflammation in a CCl_4-mediated steatohepatitis model in obese mice [70].

NLRP3 inflammasome-independent mechanisms for P2X7 receptor liver damage have also been described for macrophage actions. As Kupffer cells are specialized macrophages of the liver, we suggest that such mechanisms are also valid for these immune cells. In disease, Ca^{2+} influx may be mediated by TRPA-1 channels [71], which are co-localized with the P2X7 receptor in macrophages and activated following P2X7 receptor stimulation. TRPA-1 channel activation would induce ROS formation and increase the inflammation process (reviewed by [72]). Further, P2X7 receptors upon pore formation promote proinflammatory functions by releasing the DAMP ATP. The P2X7-NADPH axis has also been shown to induce redox-mediated Kupffer cell activation upon treatment of hepatocytes with CCl_4. This pathway depends on P2X7 receptor-mediated Ca^{2+} influx [70].

While P2X7 receptor actions are generally proinflammatory, anti-inflammatory effects exerted by this receptor have also been suggested [72], which are important for the understanding of its function in macrophage/Kupffer cell polarization. P2X7 receptor stimulation in macrophages may release anti-inflammatory proteins, such as annexin, and is not associated with proinflammatory responses [73]. Further, ATP through its pyrophosphate chains may block ROS production and inflammasome activation through intracellular actin clustering [72]. Due to ambivalent actions of high ATP concentration and P2X7 receptor stimulation, clinical tests with P2X7 receptor antagonists in various therapeutic contexts may have failed [21]. Extracellular ATP accumulation may activate Ca^{2+} fluxes through further P2X receptors detected in the liver, such as P2X1, P2X2, P2X3, and P2X4 receptor channels (reviewed in [74]).

The P2X4 receptor has major roles in liver homeostasis and pathology. On the one hand, the receptor contributes to liver regeneration. The P2X4 receptor is expressed in the pericanalicular lysosomes of hepatocytes. Through Ca^{2+} influx the receptor controls HCO_{3^-} concentrations in the bile with a less alkaline bile pH. By these characteristics, this receptor contributes to liver regeneration following a partial hepatectomy [75]. On the other hand, the P2X4 receptor by inducing Ca^{2+} flux has a major function in liver pathology, maintaining the activated fibrogenic phenotype of myofibroblasts during liver fibrosis [76]. Further, P2X4 receptor inhibition was also beneficial in attenuating alcohol-related liver fibrosis as studied in a liver fibrosis model [77]. The authors of this manuscript concluded that P2X4 receptor inhibition blocks the PI3K/AKT pathway and upregulation of α-SMA and collagen 1 or collagen 3 gene expression, resulting in the blockade of hepatic stellate cell activation. In addition to this direct action, an indirect pathway was proposed by which P2X4 receptor inhibition diminishes the activation of stellate cells through the reduction of TGF-β release by liver macrophages [77]. P2X4 receptor inhibition was also efficient in experimental autoimmune hepatitis, as reported in [78]. Immune-related hepatitis induced by concanavalin A treatment was reduced in P2X4 receptor knockout mice when compared to wild-type animals. P2X4 receptor deletion also resulted in a reduction of liver cytokine levels, such as IL-1β, IL-6, IL-17A, and TNF-α, and less apoptosis and autophagy. Furthermore, intraperitoneal injection of the autoimmune hepatitis model with the P2X4 receptor antagonist 5-BDBD showed beneficial effects as reported by diminished serum alanine and aspartate aminotransferase levels [78].

P2Y receptors are important mediators in innate immune system activation acting on phagocytes (macrophages and neutrophils), dendritic cells, mast cells, NK cells, eosinophils, basophils, and eosinophils. We will focus here on P2Y1,2,4,6 and 11 receptors, which promote transient increases in $[Ca^{2+}]_c$; see Le Duc et al. [79] for a comprehensive review. P2Y1 receptors are expressed by eosinophils, neutrophils, macrophages, and dendritic and mast cells and contribute to immune functions by inducing chemotaxis and ROS formation, while P2Y2 receptors in these cells trigger chemotaxis, neutrophil activation, granule, and histamine release. P2Y4 receptors are expressed by neutrophils, macrophages, dendritic cells, and eosinophils, but besides the activation of eosinophils, further functions of this receptor in the innate immune system are not known. The functions of the P2Y6 receptors lie in chemokine release by eosinophils and the induction of chemotaxis in neutrophils,

macrophages, and eosinophils. The P2Y11 receptor is known to promote granulocytes, cytokine release, migration, chemotaxis, and inhibition of neutrophil apoptosis (reviewed in [79]).

Neutrophils are present at low numbers in liver sinusoids and the healthy liver and can be rapidly recruited in conditions of infection or inflammation. They function as a double-edged sword, as these eliminate pathogens in an initial innate immune response, but on the other hand, contribute to liver inflammation and promote the innate immune response and initiate chemotaxis, phagocytosis, degranulation, ROS production, cytokine production, and neutrophil extracellular trap formation [80]. P2Y receptors are also functional in liver cells. We have shown that P2Y2 receptor activation in hepatocytes elicits $[Ca^{2+}]_c$ oscillations with much wider spike widths than the brief periodic $[Ca^{2+}]_c$ transients in hepatocytes stimulated through P2Y1 receptor activation [32]. Significantly, P2Y2 receptor knockout mice fed with a high-fat diet revealed reduced insulin resistance and decreased steatosis, suggesting that the P2Y2 receptor may play a role in the etiology of MASLD [81]. This study showed decreased lipid biosynthesis and increased fatty acid oxidation mediated by AMP kinase and PGC-1α pathways, both potential targets of P2Y receptor-mediated Ca^{2+} signaling. Further functions for the P2Y2 receptor in ameliorating liver disease were suggested, such as induction of neutrophil invasion and hepatocyte apoptosis [82]. In addition, the upregulation of P2Y2 receptor-mediated responses in CCl_4-treated mice points to functions of this receptor in fibrosis induction [74].

In Kupffer cells, the P2Y6 receptor apparently contributes to chronic inflammation. Kupffer cell P2Y6 receptor levels were elevated in a mouse model of chronic ethanol feeding, and alcoholic steatohepatitis was reduced by P2Y6 receptor inhibition [83]. Genomics data in human subjects progressing from MASLD to MASH also showed increased P2Y6 receptor expression, suggesting that this receptor plays a role in human liver pathology [84]. However, P2Y6 receptor knockout did not improve outcomes in a MASH mouse model when compared to wild-type mice and indeed resulted in increased serum aspartate aminotransferase levels and augmented CCL2 gene expression, indicating liver damage [84]. Overall, P2Y6 receptor signaling in chronic liver disease is ripe for further investigation and determination of its underlying mechanisms.

As noted above, physiological regulation of hepatic glucose production and oxidative metabolism by catabolic hormones involves periodic $[Ca^{2+}]_c$ transients, known as $[Ca^{2+}]_c$ oscillations in hepatocytes [85]. Disruptions in these $[Ca^{2+}]_c$ oscillations have been implicated in the development of liver disease [22, 25, 86]. Brumer et al. [87] demonstrated that short-term high-fat diet feeding of mice for as little as 1 week led to suppression of noradrenaline-stimulated $[Ca^{2+}]_c$ oscillations in both isolated hepatocytes and intact liver, and these effects may be important in switching from lipolytic to lipogenic metabolism. Longer term high-fat diet and genetically obese mice demonstrated decreased activity of the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), which leads to reduced ER Ca^{2+} accumulation and ER stress, effects that can be reversed by SERCA overexpression with improvements in glucose homeostasis [88, 89]. In addition, obesity has been reported to be associated with increased IP_3 receptor type-1 expression [90, 91], reduced expression of IP_3 receptor type-2 [91, 92], and enhanced coupling between ER Ca^{2+} release and mitochondrial Ca^{2+} uptake [93, 94]. These changes may reflect adaptation to the earlier suppression of IP_3-dependent Ca^{2+} signaling, but at the same time have maladaptive effects on metabolic regulation and oxidative stress.

Intercellular calcium signaling and the role of connexins in liver disease

The transmembrane proteins connexins (Cx) and pannexins (Panx) connect the extracellular space with the interior of the cell. Different from pannexins, connexins can connect two cells with each other allowing intercellular transport [95]. Cx play an important role in purinergic signaling, with intercellular Ca^{2+} waves transmitted through gap junction channels assembled by paired connexons each formed by six Cx subunits, and unpaired Cx hemichannels at the plasma membrane functioning as nucleotide permeation pathways. Twenty-one and 20 Cx types have been described in humans and mouse, respectively [96–98]. All liver cell types express Cx, with the major isoforms being Cx26, Cx32, and Cx43 isoforms [99]. Cx32 and Cx26 are abundantly expressed in hepatocytes, and cholangiocytes express Cx32 and Cx43. Cx43 is the predominant connexin type in stellate and Kupffer cells [100]. Kupffer cells also express Cx26 and Panx1. The latter was shown to be important for association with the P2X7 receptor to induce pore activity and ATP release [101–103].

In the short term, the regulation of these channels involves changes in intracellular $[Ca^{2+}]_c$ and membrane voltage, while transcriptional regulation occurs in the long term, as extensively reviewed by Willebrords and co-workers [104]. Cx hemichannels, serving as ATP-release channels, play a role in facilitating purinergic signaling and aggravating liver inflammation. In line with this, Cx32 and Cx43 antagonism alleviated non-alcoholic liver steatosis in mice [105]. Diet-induced ER stress induced Cx43 expression, mediating hepatocyte coupling. Further, specific knockout of Cx43 in the liver protected against ER stress and steatosis [106].

Hepatocytes are highly coupled by gap junctions and in terms of Ca^{2+} signaling function almost as a syncytium, able to transmit $[Ca^{2+}]_c$ waves from cell to cell over long distances [26, 107]. In the intact liver perfused with IP_3-linked hormones, $[Ca^{2+}]_c$ waves propagate across entire hepatic lobules without losing amplitude or frequency, and this is likely important in coordinating the metabolic responses of hepatocytes. Moreover, this intercellular Ca^{2+} signaling is disrupted in the presence of alcohol and after a high-fat diet [86, 87]. Connexins not only facilitate intercellular Ca^{2+} waves between hepatocytes but may also play a role in Kupffer cells. Following treatment with LPS and interferon-γ, Cx43 expression increased and there was demonstrable coupling between Kupffer cells [108]. These authors suggest that gap junctions between Kupffer cells might coordinate immune responses during liver inflammation.

Cx43 also plays an important role as a hemichannel, mediating both paracrine and autocrine effects of extracellular ATP. A macrophage-specific Cx43 gene knockout, as well as its pharmacological inhibition, resulted in reduced cytokine secretion by peritoneal macrophages in a sepsis model, leading to increased mouse survival and improved sepsis outcomes [109]. These findings highlight the detrimental effects of extracellular ATP accumulation. Notably, the P2X7 receptor subtype co-localizes with Cx43 and Panx1, promoting innate immunity inflammatory processes, as shown in the gut [110]. Consistent with the crucial role of Cx43 in inflammation, this connexin exhibits high expression in inflamed and necrotic spots, as observed in the acute-on-chronic liver failure rat model [111]. The co-localization of Cx43 with caspase 3 further supports its involvement in inducing hepatocyte cell death [112]. Proinflammatory IL-1β induces the expression of Cx43, as demonstrated in fibroblasts [113], and intercellular Ca^{2+} wave propagation in astrocytes [114]. Although these interactions with IL-1β have not been demonstrated in the liver, they may be proposed for the synchronization of Kupffer cells in innate and pathogen-induced immune responses in the liver. Several other P2 receptor subtypes interact with connexins, including the P2X4 receptor with Panx1 in hepatitis C virus-infected hepatocytes [115].

The P2Y1 receptor is known to have a close association with intercellular Ca^{2+} signaling in astrocytes through connexins [116], and the expression levels of P2Y1 receptors were diminished in Cx43 knockout mice [117]. The overactivation of macrophages following ATP release by Cx43 activates P2Y1 receptors in mouse peritoneal and liver macrophages [109]. Cx43 interactions with P2Y2 receptors and other purinergic receptor subtypes, such as the P2Y6 subtype, through ATP release and subsequent induction of the inflammasome, appear to be plausible. It is possible that aberrant IP_3 receptor signaling induced through P2Y receptors overloads stellate and Kupffer cells with Ca^{2+}, resulting in mitochondrial dysfunction and ROS production.

Figure 1 schemes the scenario of purinergic signaling through Ca^{2+} signaling promoting liver damage and fibrosis. At the physiological level of agonists, including sub-micromolar ATP, periodic Ca^{2+} oscillations drive normal hepatic functions. Exposure of hepatocytes to damaging molecules and aberrant Ca^{2+} signals in hepatocytes results in the induction of steatosis and the release of DAMPs, such as ATP, and PAMPs, which stimulate the innate immune system resulting in Kupffer and stellate cell activation. Hepatocyte injury leads to ATP release, activating P2 receptors. Augmented Ca^{2+} signaling in Kupffer cells promotes the production of reactive oxygen species, ERK1/2, and NF-kB activation and promotes the proinflammatory signaling cascade. Augmented $[Ca^{2+}]_c$ levels as a consequence of P2X7 receptor activation induce the assembly of the NLRP3 inflammasome, favoring the processing and release of proinflammatory cytokines, such as interleukins (IL) 1β and 18. TNF-α is also released by reactive Kupffer cells. As a further consequence of liver inflammation and damage, stellate cells overproduce and secrete extracellular matrix proteins promoting fibrosis.

Conclusion

This manuscript has explored the intricate relationship between purinergic receptors (P2X7, P2X4, P2Y1, P2Y2, P2Y11, A2A, A2B) and calcium signaling within the context of liver physiopathology. The main findings highlight the following:

P2X7 receptor Demonstrated to be crucial in mediating inflammatory responses and fibrosis in liver diseases. Its activation leads to significant changes in intracellular calcium levels, promoting hepatocyte apoptosis and contributing to liver injury.

P2X4 receptor Important for physiological functions in liver homeostasis and liver regeneration. However, this receptor also contributes to liver fibrosis, and the inhibition of this receptor is beneficial in alcohol-related and autoimmune hepatitis.

P2Y1 receptor Physiological regulation of hepatic metabolism at low levels of extracellular ATP and ADP.

P2Y2 and P2Y11 receptors Both receptors play essential roles in liver regeneration and repair mechanisms. Their activation influences calcium signaling pathways, which are vital for cell proliferation and migration, key processes in liver regeneration.

A2A and A2B receptors These adenosine receptors are involved in modulating liver inflammation and fibrosis. Their interaction with calcium signaling pathways can either exacerbate or mitigate liver damage, depending on the receptor subtype and the pathological context.

Further research is needed to elucidate the precise molecular mechanisms by which these purinergic receptors regulate calcium signaling in different liver cell types under various pathological conditions. In particular, there is limited understanding of how P2Y11 receptor-mediated calcium signaling affects liver stellate cell activation in fibrosis. Moreover, available data are insufficient regarding the crosstalk between P2X7 and A2A receptors in hepatocyte survival and death pathways.

Considering the therapeutic potential of modulating these receptors to treat liver diseases, selective agonists or antagonists could be developed to fine-tune calcium signaling pathways, thereby ameliorating liver damage or enhancing regeneration. This includes exploring potential synergistic or antagonistic effects that could influence disease outcome.

In conclusion, while significant progress has been made in understanding the role of purinergic receptors and calcium signaling in liver disease, there remain substantial gaps and opportunities for future research to develop novel therapeutic strategies.

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Data availability No datasets were generated or analysed during the current study.

Compliance with ethical standards

Ethical approval Not applicable.

Conflicts of interest The authors declare no competing interests.

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