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Purinergic Signaling in Liver Disease

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Abstract

Adenosine triphosphate (ATP) is essential for the myriad of metabolic processes upon which life is based and is known widely as the universal energy currency unit of intracellular biologic reactions. ATP, adenosine diphosphate, adenosine, as well as other purines and pyrimidines also serve as ubiquitous extracellular mediators which function through the activation of specific receptors (viz. P2 receptors for nucleotides and purinergic P1 receptors for adenosine). Extracellular nucleotides are rapidly converted to nucleosides, such as adenosine, by highly regulated plasma membrane ectonu-cleotidases that modulate many of the normal biological and metabolic processes in the liver - such as gluconeogen-esis and insulin signaling. Under inflammatory conditions, as with ischemia reperfusion, sepsis or metabolic stress, ATP and other nucleotides can also act as ‘damage-associated molecular patterns’ causing inflammasome activation in innate immune cells and endothelium resulting in tissue damage. The phosphohydrolysis of ATP by ectonucleotidases, such as those of the CD39/ENTPD family, results in the generation of immune suppressive adenosine, which in turn markedly limits inflammatory processes. Experimental studies by others and our group have implicated purinergic signaling in experimental models of hepatic ischemia reperfusion and inflammation, transplant rejection, hepatic regeneration, steatohepatitis, fibrosis and cancer, amongst others. Expression of ectonucleotidases on sinusoidal endothelial, stellate or immune cells allows for homeostatic integration and linking of the control of vascular inflammatory and immune cell reactions in the liver. CD39 expression also identifies hepatic myeloid dendritic cells and efficiently distinguishes T-regulatory-type cells from other resting or activated T cells. Our evolving data strongly indicate that CD39 serves as a key ‘molecular switch’ and is an integral component of the suppressive machinery of myeloid, dendritic and T cells. Increased understanding of mechanisms of extracellular ATP scavenging and specifically conversion to nucleosides by ectonucleotidases of the CD39 family have also led to novel insights into the exquisite balance of nucleotide P2-receptor and adenosinergic P1-receptor signaling in inflammatory and hepatic diseases. Further, CD39 and other ectonucleotidases exhibit genetic polymorphisms in humans which alter levels of expression/function and are associated with predisposition to inflammatory and immune diseases, diabetes and vascular calcification, amongst other problems. Development of therapeutic strategies targeting purinergic signaling and ectonucleotidases offers promise for the management of disordered inflammation and aberrant immune reactivity.

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The authors have no conflicts of interest to disclose.

Keywords

Ectonucleotidases; P2 receptor; CD39; CD73; T lymphocytes; Liver disease

Introduction

Purinergic signaling is the mechanism in which extracellular nucleotides such as adenosine triphosphate (ATP) and derivatives act as signaling molecules. Initially proposed by Burnstock [1] in 1972, this has now become a widely recognized pathway involved in basic cellular mechanisms in multiple organ systems, in both health and disease, and is intimately related to liver functionality under these conditions.

ATP and adenosine bind to distinct sets of receptors (P1 and P2, respectively) first recognized in the late 1970s and cloned and characterized in the 1990s. ATP receptors are separated into P2X ion channel and P2Y G-protein-coupled receptors [2]. These types of purinergic receptors are present on many cells in the liver: including hepato-cytes, Kupffer cells, cholangiocytes, immune cells, endothelial and smooth muscle cells. There are four types of adenosine receptors, viz. A1, A2A, A2B and A3 subtypes, which are all G-protein-coupled receptors and widely expressed in the liver sinusoidal cells and parenchyma.

The scavenging and catalysis of extracellular nucleotides are essential to the proposed purinergic model with ATP, nucleotide derivatives and adenosine serving as extracellular signaling molecules. Others and we have shown that ectonucleotidases of the ectonucleoside triphosphate diphosphohydrolase (ENTPD) CD39 family are the dominant factors responsible for the hydrolysis of extracellular nucleotides to ultimately generate the respective nucleoside derivatives and uniquely regulate purinergic signaling in the vasculature and immune systems.

Many normal functions of the liver such as gluconeo-genesis and insulin responsiveness are modulated by extracellular nucleotides. While these mechanisms play a role in normal homeostasis, certain biologic stressors can alter the release of these nucleotides, as well as modulate ectonucleotidase ectoenzymatic functions [3].

Substantial recent data that we will summarize here have resulted in development of increased understanding into mechanisms of purinergic signaling in acute toxic liver injury and in those chronic and increasingly common hepatic diseases, characterized by steatosis, fibrosis and malignancy. This short review will briefly explore the role of purinergic signaling in hepatic physiology and metabolism as well as developing in depth our understanding of both the acute and chronic pathophysiology of liver disease. Lastly, we will briefly describe and speculate on potential future clinical applications of established drugs that impact purinergic signaling as well as new developments in this area.

Hepatic Physiology

Carbohydrate Metabolism—In health, purinergic signaling has a role in many normal hepatic functions such as glycogenolysis, gluconeogenesis and glycolysis. Glycogenolysis is predominately mediated by the actions of glucagon, although noradrenaline and ATP

released from the splanchnic nervous system contribute. However, adenosine is inferior to glucagon at increasing glucose production. This difference may be, at least in part, related to adenosine-mediated antagonism of the actions of glucagon [4]. Extracellular ATP arises not only from the splanchnic nervous system but also from hepatocytes and activated platelets [4]. In vitro the addition of exogenous ATP to rat hepatocytes stimulates both glycogenolysis and glucose release from the cell [5]. Additionally, in hepatocytes and perfused livers, extracellular ATP stimulates glycogenolysis [6–9]. Furthermore, the addition of P2X-selective agonists, such as BzATP, decreases the content of glycogen in isolated human hepatocytes [10]. Thus, extracellular ATP mediates glycogenolysis predominately through stimulation. The mechanism of regulation appears to be via modulation of glycogen phosphorylase. Glycogen phosphorylase catalyzes the rate-limiting step in glycogenolysis and is directly activated, in both rat and human hepatocytes, by activation of $P2Y_X$ receptors [11, 12]. The mechanism of activation relies on the increase of intracellular calcium and additionally the activation of phospholipase D.

Gluconeogenesis is increased in response to ATP and to a lesser extent adenosine. Similarly to glycogenolysis, this effect appears to be mediated through increases in intracellular calcium [13, 14]. High concentrations of ATP, however, will inhibit gluconeogenesis from certain glucose sources: specifically gluconeogenesis from pyruvate and lactate are inhibited whereas glycerol and fructose are not [15]. Mechanisms such as this may be responsible for alterations in glucose metabolism in disease states when extracellular ATP may be more abundant.

Lastly, ATP attenuates glycolysis in cultured hepatocytes. This effect is through inhibition of phosphofructokinase-2 [16]. The actions of mTOR via P2Yx and $P2Y_2$ purinergic signaling may regulate many of these functions [17]. In sum, through regulation of extracellular ATP, glucose production can be mediated through glycogenolysis, gluconeogenesis and glycolysis.

Lipid Metabolism and Fatty Acids—Extracellular ATP can act to regulate fatty acid synthesis through modulating the activity of acetyl-CoA carbox-ylase. ATP inhibits acetyl-CoA carboxylase likely through an increase in intracellular calcium. Other consequences of ATP signaling such as the inhibition of carnitine O-palmitoyltransferase may be inhibited via PKC-depen-dent mechanisms [18]. Despite these actions, A1 receptors do not appear to regulate lipogenolysis to any extent [19].

Extracellular nucleotides may have a role in regulating the movement of cholesterol from the peripheral tissue back to the liver (i.e. reverse cholesterol transport). The process of cholesterol movement from the periphery to stool is dependent on an intact purinergic signaling pathway [20]. $P2Y_{13}$-deficient mice have decreased hepatic HDL cholesterol uptake, decreased overall hepatocyte cholesterol content, and decreased biliary output [21]. Notably in these $P2Y_{13}$-deficient mice, while reverse cholesterol transport is impaired, plasma lipids, including HDL, are normal [20]. This suggests the purinergic signaling modulates reverse cholesterol transport independently of plasma HDL.

Acute Liver Injury in Ischemia Reperfusion—Vascular injury can occur to a native liver during inflammation or in shock or shock-like states. Additionally, ischemic liver damage can be an acute consequence of ischemia reperfusion injury following transplantation. Despite the dual blood supply to the liver, vascular compromise can be catastrophic. Vascular injury induces a wide array of inflammatory responses including the release of adenine nucleotides, which drives inflammation and platelet activation [22]. Infusion of ATP under these conditions improves hepatic function, as well as survival following reperfusion. Such interventions are associated with decreased levels of the inflammatory cytokines TNF and IL-6 [23].

Vascular CD39 (ENTPD1) ectonucleotidase action seems to have a protective role in hepatic ischemia reperfusion injury, potentially by generating adenosine in concert with CD73/ecto-5'-nucleotidase. Unfortunately, after hepatic ischemia and reperfusion injury, the hepatic vascular ectonucleotidase activity is lost. Additionally, the deletion of Entpd1 in mice leads to significant increase in vascular injury and decreased survival under these conditions. However, wild-type and CD39-deficient mice that receive adenosine are protected from reperfusion injury [24],

Pharmacologic preconditioning is a potential mechanism to protect against hepatic ischemic reperfusion injury. Stimulation of adenosine receptors has been associated with protection of the liver from ischemia. Interestingly, hepatic ischemic preconditioning is associated with upregulation of CD39. This is likely mediated by the transcription factor Sp1, making it a potential therapeutic target for the treatment of liver ischemia [25],

Curiously, deletion of CD39 in natural killer (NK) cells attenuates hepatic ischemia/reperfusion injury in mice, suggesting that ATP modulates innate cell functions during liver injury and consequent regeneration. In this respect, NK cells that lack the CD39 gene produce less interferon-γ in response to inflammatory mediators [26]. CD39 expression on conventional liver myeloid dendritic cells limits their pro-inflammatory activity and confers protective properties on these important innate immune cells against liver transplant ischemia/reperfusion injury [27],

Acute Toxic Liver Injury—Hepatic inflammation with stimulation of immune cells contributes to acetaminophen (APAP) hepatotoxicity in mice, and is triggered by P2X7 activation. In this model, Entpd1 null mice exhibit enhanced P2X7 signaling and show increased APAP-induced hemorrhage and mortality. The use of soluble ectonucleotidases, e.g. apy-rase, also decreases APAP-induced mortality, suggesting a potentially future therapeutic role based on purinergic principles [28].

Autoimmune Chronic Active Hepatitis—Imbalance between effector and regulatory T cells results in loss of tolerance towards liver autoantigens with consequent development of autoimmune hepatic damage.

In autoimmune hepatitis (AIH), a severe hepatopathy characterized by hypergammaglobulinemia, seropositivity for circulating autoantibodies and interface hepatitis on histology, liver damage is mediated by CD4 and CD8 T lymphocytes [29, 30].

Effector lymphocyte overreaction is permitted by defective CD4+CD25+Foxp3+ regulatory T cells (Tregs) [31–33], a subset maintaining immune system homeostasis [34, 35]. Numerical and functional Treg impairment in AIH results in massive recruitment of inflammatory cells, which invade the portal tracts and spread towards the parenchyma.

Tregs are identified by high CD25 fluorescence ($CD25^{high}$), expression of glucocorticoid TNFR (GITR), CD62L, CTLA-4, Foxp3 and CD39 [34–36]. Expression of CD39 confers Tregs the ability to control effector cell overreaction through generation of immunomodulatory adenosine [36]. Upon binding to A2A receptor, adenosine modulates T-effector cell function, by decreasing IL-2 production and proliferation and by limiting differentiation into T-helper 1 (Th1) and T-helper 17 (Th17) lineages [37],

We have recently shown that there are multiple defects associated with CD39+ Tregs in AIH [38]. In addition to being numerically decreased, CD39+ Tregs display reduced ability to control production of IL-17, a pro-inflammatory cytokine elevated in the serum of patients suffering from this condition. Defective ability of CD39+ Tregs to contain IL-17 production was previously observed in other autoimmune conditions, such as multiple sclerosis [39, 40]. The mechanism enabling IL-17 control by CD39+ Tregs has not been elucidated yet, though it has been postulated that CD39 might diminish IL-17 levels via ATP removal [40].

Tregs isolated from AIH patients display impaired hydrolysis of pro-inflammatory nucleotides compared to control cells and are skewed towards a pro-inflammatory phenotype (i.e. elevated CD127 levels and IFN-γ production), an observation which has led us to postulate that the Treg defect in AIH might also derive from an increased rate of conversion or dedifferentiation into effectors. The reasons for CD39 downregulation on Tregs in AIH are unknown, though low levels of TGF-β, an inhibitory cytokine that promotes upregulation of CD39 on human leukocytes [41], may account for this phenomenon. Expression of CD39 by memory T cells has been linked to acquisition of immunoregulatory properties: whether lack of CD39 upregulation by Th17 as a mechanism to auto-limit effector cell potential might contribute to immune system dysregulation and to perpetuation of liver damage in AIH is currently being evaluated.

Natural killer T cells (NKT) have also been implicated in AIH pathogenesis. NKT cell function is regulated upon interaction of extracellular ATP with P2X7 receptor as well as by the CD39 ectoenzyme expressed by these cells [42]. Interestingly, genetic deletion of CD39, which negatively impacts Treg function and consequently exacerbates adoptive immune responses in transplant rejection models [43], results in an increase in NKT cell apoptosis in AIH models. Thus, concanavalin-A hepatitis induction in Cd39 null mice leads to enhanced loss of NKT cells and paradoxical protection from liver injury. This unexpected experimental finding indicates the complexity of purinergic signaling in affecting diverse immune cell types (Treg vs. NKT cells) and in governing opposing outcomes in the immune liver injury.

Alloimmune Liver Transplant Injury—Xenograft rejection occurs secondary to vascular inflammation and thrombosis which is in part mediated by extracellular nucleotides [44, 45]. Loss of ectonucleotidase activity has been noted in the vasculature of the xenograft

[45]. As expected then, upregulation of NTPDase1 activity is associated with graft survival [46]. In addition, increased NTPDase activity via exogenous administration into the blood or upregulation of CD39 after adenoviral infection is associated with prolonged transplant graft survival.

In addition to the vascular effects, purinergic signaling is also associated with liver regeneration. ATP has been shown to activate cell cycle progression of hepatocytes, thus inducing proliferation. This occurs in vitro and in vivo and is thought to be through $P2Y_2$ receptor modulation of various growth factors [47].

Small-for-size liver transplantation is a unique scenario, but has the potential to alleviate some burden given the limited number of available organs. Unfortunately, in addition to the technical difficulties of small-for-size liver transplantation, the small liver can create an excess of reactive oxygen species when administered a large blood flow creating an inflammatory response in the donor liver [48]. It has been reported that A2A receptor activation can protect a small-for-size donor liver after transplantation [49]. A2A receptor activation resulted in downregu-lation of pro-inflammatory cytokines and adhesion molecules. In rats, this was associated with improved liver function [50].

In addition to the potential therapeutic effects of modulating purinergic signaling in the acute transplant setting and beyond, monitoring graft function is another area of research linked to extracellular nucleotides. Im-muKnow assay is an experimental assay to predict the graft function and risk of rejection in patients after solid organ transplant and is a measure of peripheral blood CD4+ total cellular ATP.

In one study, patients who received a living donor transplant had a correlation with ImmuKnow assay results and immune response or required immune suppression with tacrolimus [51]. Additionally it may be beneficial to measure early rejection. Significantly higher levels of ATP were found in CD4+ cells of patients who developed acute rejection compared to those who did not [52]. This test has the potential to be a biomarker for early development of rejection.

Additionally this technique has been used outside of transplant rejection in the setting of liver transplantation for hepatitis C, differentiating recurrent hepatitis C or rejection can be difficult. Often a biopsy is required, although despite that the diagnosis can be confusing. The ImmunoKnow assay was tested in a group of patients to assess the ability to detect acute cellular rejection from recurrent HCV. The immune response was significantly lower in the patients with recurrent HCV compared to those with acute cellular rejection [53].

Hepatic Regeneration—Liver regeneration is important for restoration of parenchymal volume and return of adequate hepatic functions. After partial hepatectomy, there is a rapid decrease of total nucleotides in the remnant liver, presumably related to the need for cellular growth. Thirty seconds after partial hepatectomy, the ATP content of the remaining liver decreases up to 50% as compared to controls [54]. Beyond increased energy demands, these changes in the remnant liver may act as an early stress signal to promote regeneration.

As stated above, extracellular ATP can activate cell cycle progression and hepatocellular proliferation in vivo and in vitro likely through $P2Y_2$ receptors [47]. Whereas there are certainly increased energy demands in the setting of partial hepatectomy, it has been suggested that ATP may be a 'sensor' allowing the rapid signal for the remnant liver to start regenerating. In fact, $P2Y_2$ receptor knockout mice display impaired proliferation [55]. It has also been shown that regulated catalysis of extracellular nucleotides via vascular CD39 is required for both hepatocyte and endothelial cell proliferation (sinusoidal angiogenesis) during liver regeneration [56]. Therefore, extracellular ATP may have a trophic role for normal hepatocyte regeneration and additionally act as the first signal after injury to stimulate hepatocytes to regenerate.

Hepatic Steatosis/Steatohepatitis and Alcoholic Liver Disease—Metabolic diseases related to the liver are linked to purinergic signaling in many ways. Extracellular nucleotides and nucleosides can be thought of as 'meta-bolokines'. This term suggests a pathophysiologic link between inflammation and metabolisms. A1 receptors on adipocytes are activated by adenosine, decreasing adenylate cyclase activity and inhibiting lipolysis. Modulation of A1A receptor thus has implications on the regulation of lipolysis in many pathologic conditions where free fatty acids play a role, including insulin resistance, diabetes and dyslipidemia. In fact, deletion of CD39/Entpd1 has been shown to worsen insulin resistance and impact hepatic glucose metabolism via aberrant extracellular nucleotide signaling [57]. Additionally, metabolism of ethanol generates adenosine, and the effects of ethanol-induced hepatic steatosis appear to be dependent on A1 and A2B receptors. Pharmacologic inhibition of this pathway could one day be a potential preventative therapy for alcohol-induced steatosis [58].

Fibrotic Liver Disease and Cirrhosis—Caffeine is widely known as the most common ingested pharmacologically active agent worldwide. Unsurprisingly, this has major effects as an adenosine receptor antagonist. Clinically it has been noted that consumption of coffee is associated with protection against cirrhosis [59, 60]. In rats, carbon tetrachloride-induced cirrhosis is partially reduced by caffeine and collagenolytic activity can reverse some micronodular cirrhosis while stimulating hepatocyte proliferation [61]. Stellate cells express A2A receptors and P2Y receptors and activated stellate cells express various P2Y subreceptors. As these cells are the predominate source for the extracellular matrix and integral to the deposition of collagen leading to fibrosis, they are an attractive pharmacologic target to modulate during times of inflammation [62]. Other forms of cirrhosis, such as biliary cirrhosis, have also been implicated through downregulation of ectonucleotidases on portal myofibroblasts [63].

Portal Hypertension, Hepatorenal Syndrome and Hepatic Encephalopathy—Purinergic signaling might also be responsible for some of the extrahepatic complications of cirrhosis. Adenosine is a key vascular regulator in hepatic artery vascular resistance. In cirrhosis, the hepatic artery vascular resistance decreases. Exposure to adenosine in this state leads to an exaggerated dilatory response [64]. Purinergic signaling is not limited to the hepatic vasculature but may play a role in hepatorenal syndrome as well. Intrahepatic caffeine administered to the portal veins of rats increases urine output; however, this effect is

ameliorated by denervating the liver or administering the caffeine into the systemic (not portal) system [65]. Others have noted similar diuretic effects in rat models of cirrhosis apparently mediated through hepatic A1 adenosine receptors [66]. Finally, purinergic signaling may also be involved in hepatic encephalopathy as adenosine can modulate the uptake of glutamine and aspartate, both of which have been implicated in the disease [67],

Cancer

Liver cancer is typically associated with chronic inflammatory states, which are linked to immune dysregulation, disordered metabolism and aberrant cell proliferation. In established or disseminated metastatic malignancy however, ATP also plays an important role as an early danger signal to the immune system. The catalytic properties of CD39, expressed by either immune suppressive Treg or endothelium, appear key in abrogating this process.

In generating adenosine, CD39 ectonucleotidase activities inhibit T-cell proliferation, impact immune responses while promoting angiogenesis, thereby being permissive for the growth of transplanted tumors [68].

Both ATP and adenosine in concert alter the balance of apoptosis and proliferation deviating cells towards malignancy. ATP infusions into the intraperitoneal space of a two-stage rat model of hepatocarcinogenesis increase numbers of preneoplastic foci in the liver. These manifestations are comparable to what has been observed with Entpd5 knockout mice [69]. ENTPD5/CD39L4 is a related ectoenzyme to CD39 and is a soluble endoplasmic reticulum UDPase involved in intracellular purine metabolism which promotes glycolysis as well as proliferation in cancer cells via the PTEN signaling pathway.

Interestingly, there are contrasting roles of this ecto-nucleotidase as well as CD39 in the suppression of liver cancer development versus the promotion of transplanted tumor growth in mice [69]. Recent work by Wu and colleagues [70] has shown development of liver cancer in CD39 null mice. Lack of CD39 resulted in higher concentrations of extracellular nucleotides, increase in hepato-cyte proliferation and suppression of autophagy, a mechanism controlling cell growth through stress-induced degradation of cellular components. Loss of CD39 was found to alter the bioenergetic metabolism of hepatocytes by deviating them towards aerobic glycolysis and was also associated with activation of Ras-mitogen-activated protein kinase and mammalian target of rapamycin-S6K1 pathways.

Liver Transplantation—Assessment of graft viability and post-transplant instability using a combination of liver ATP levels and serum hyaluronic acid has been proposed [71]. Although the human liver has been successfully maintained under hypothermic conditions with high concentrations of adenosine as in University Wisconsin preservation solutions for up to 10–14 h, fully overcoming ischemic damage is a major obstacle to liver transplantation.

In this respect, infused ATP promotes cellular recovery after ischemic injury; this action is enhanced by the synergistic effect of superoxide dismutase [72],

Genetic Polymorphisms

CD39 and other ectonucleotidases, e.g. of the NPP family, have described genetic polymorphisms, which alter levels of expression/function and are associated with predisposition to inflammatory and immune diseases, diabetes and vascular calcification, amongst other problems.

Gene expression profiling studies in the context of Crohn's disease have revealed a single nucleotide polymorphism (SNP) in the proximity of the CD39 promoter. Thus, the presence of AA genotype at rs10748643 was found to be associated with low levels of CD39 mRNA expression and with increased susceptibility to the disease in accordance with animal studies in which CD39 deletion resulted in heightened susceptibility to dextran-sodium-sulfate-induced colitis [73].

Another study by the same authors reported an association between a two-SNP haplotype within ENTPD1/ CD39 and susceptibility to type 2 diabetes and end-stage renal disease in African-Americans. Interestingly, determination of ENTPD1 expression levels in HapMap cell lines, derived from African subjects, revealed that lines homozygous for the two-SNP risk haplotype expressed 39% more ENTPD1 mRNA than lines with protective haplotype, supporting the association between CD39, diabetes and diabetic nephropathy [74]. Later on, mutations of NFTE5, which encodes for CD73 - the ectoen-zyme that works in tandem with CD39 converting AMP to adenosine - have been described in members of families with symptomatic arterial and joint calcifications, indicating a role for this enzyme in the inhibition of ectopic tissue calcification [75].

Conclusions and Future Directions

This review has focused on the developing role of pu-rinergic signaling in the pathophysiology of liver diseases and we have proposed potential future clinical applications. Clearly, there are now substantial data implicating extracellular nucleotides and nucleosides in a variety of normal metabolic liver functions. Aberrant or disordered purinergic signaling are also components of many disease states of the liver independent of metabolic disruption per se.

In terms of therapeutic strategies, modulation of pu-rinergic signaling via changes in nucleotide fluxes, inducing or inhibiting ectonucleotidase actions or otherwise scavenging nucleosides may prove useful for limiting and controlling deleterious immune responses, as in the case of AIH. In this instance, induction of CD39 by pharmacological or other modalities may enhance Treg function, while auto-limiting effector cell activation. Further, unravelling the role of adenosine in ischemia reperfusion injury may lead to therapies for the donor liver and/or recipient that result in prolonged allograft survival.

Finally, development of selective agonists and antagonists for purinoceptor subtypes that are orally bioavailable and stable in vivo would have application for hepatic inflammatory and fibrotic conditions, in addition to other pathological conditions.

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