Expression of P2Y Nucleotide Receptors and Ecto-Nucleotidases

In Quiescent and Activated Rat Hepatic Stellate Cells

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ABSTRACT

Background. Extracellular nucleotides regulate a variety of cellular activities, including proliferation of fibrogenic cells outside of the liver. However, the expression of receptors for extracellular nucleotides in hepatic stellate cells (HSC) is unknown. Thus, our Aims were to investigate the expression of mediators of nucleotide signaling in HSC and to determine whether extracellular nucleotides regulate HSC function. Methods. Confocal video microscopy was used to observe nucleotide-induced changes in cytosolic Ca\(^{2+}\) (Ca\(^{2+}\text{,i}\)) in live HSC. P2Y receptor subtype expression and ecto-nucleotidase expression in quiescent and activated HSC were determined using RT-PCR, Northern blot, immunoblot, and confocal immunofluorescence. Functional ecto-nucleotidase activity was assessed using a colorimetric method. Nucleotide-sensitive procollagen-1 mRNA expression in activated HSC was assessed using real-time RT-PCR. Results. Extracellular ATP increased Ca\(^{2+}\text{,i}\) in HSC; this was inhibited by the P2 receptor inhibitor suramin. Quiescent HSC expressed the P2Y subtypes P2Y\(_2\) and P2Y\(_4\) and were activated by ATP and UTP, whereas activated HSC expressed the P2Y subtype P2Y\(_6\) and were activated by UDP and ATP. Activated, but not quiescent, HSC expressed the ecto-nucleotidase NTPDase2. Extracellular UDP tripled procollagen-1 mRNA expression in activated HSC, and this was inhibited by the P2Y receptor inhibitor suramin. Conclusions. HSC express functional P2Y receptors and switch the expression of P2Y receptor subtypes upon activation. Moreover, HSC differentially regulate NTPDase expression after activation. Since activation of P2Y receptors in activated HSC regulates procollagen-1 transcription, P2Y receptors may be an attractive target to prevent or treat liver fibrosis.
Background.

Hepatic stellate cells (HSC) are the primary fibrogenic cells of the liver. HSC in the normal liver are rich in vitamin A, with low levels of proliferation and extracellular matrix deposition, and are termed “quiescent.” In the setting of chronic liver disease, or when cultured on plastic in vitro, they undergo a transdifferentiation into highly proliferative and fibrogenic myofibroblasts that are responsible for the bulk of abnormal matrix deposition in liver fibrosis and are termed “activated.” While many of the signaling systems that regulate HSC activity have been elucidated in recent years (Brenner et al. 2000; Friedman 2000), a number of questions remain. Identification of novel signals regulating matrix deposition and HSC proliferation is of particular importance, since this may lead to novel prophylactic and therapeutic approaches to liver fibrosis.

Extracellular nucleotides act as signaling molecules through activation of specific receptors. Receptors for nucleotides are known as P2 receptors. These are further divided into ligand-gated P2X receptors and G protein coupled P2Y receptors (Ralevic and Burnstock 1998). P2 receptors have been shown to regulate a variety of cell functions, such as gene transcription (Pines et al. 2003), growth (Burnstock 2002), apoptosis (Sellers et al. 2001), secretion (Hwang et al. 1996), contractility (Kitamura et al. 1991), and chemotaxis (Honda et al. 2001). Cloning of individual P2 receptor subtypes and detailed pharmacologic experiments have begun to define the specific roles of these receptors in cell physiology. Ecto-nucleotidases have now been identified as critical regulators of
P2 receptor activation. The ecto-nucleotidase NTPDase1 is a critical regulator of platelet activity (Enjyoji et al. 1999). NTPDase2 is expressed in portal fibroblasts in normal rat liver, suggesting a role in regulation of bile ductular signaling (Dranoff et al. 2002). Thus, there are both specific receptors for extracellular nucleotides and enzymatic regulators of those receptors.

Extracellular nucleotides are important regulators of proliferation and apoptosis in fibrotic mesangial cells of the kidney (Harada et al. 2000). Moreover, a prior report has suggested that cultured HSC express functional nucleotide receptors (Takemura et al. 1994). We hypothesized that both quiescent and activated HSC express specific P2Y receptors for extracellular nucleotides and ectonucleotidases, and that these nucleotides are important regulators of activated HSC function. We report here that both quiescent and activated HSC express the necessary cellular machinery for nucleotide-mediated signaling, that HSC undergo a shift in expression of P2Y receptors and ecto-nucleotidases as they activate, and that activation of P2Y receptors in activated HSC regulates transcription of the matrix component $\alpha(1)$-procollagen.

**Methods.**

**Materials and reagents.** ATP, apyrase, imidazole, ammonium molybdate, collagenase, Malachite green hydrochloride, suramin, alkaline phosphate-conjugated anti-rabbit secondary antibody, and mouse monoclonal anti-smooth muscle actin antibody were obtained from Sigma Chemical (St. Louis, MO). Alexa Fluor 488 anti-rabbit antibody and Alexa Fluor 594 were purchased
from Molecular Probes (Eugene, OR). Pronase protease was obtained from Calbiochem (San Diego, CA) and Roche Molecular Biochemicals (Chicago, IL). M199 medium was purchased from Invitrogen (Carlsbad, CA). All other chemicals were of the highest quality commercially available.

**Laboratory animals.** Adult male Sprague-Dawley rats were used for all experiments. Treatment of animals was within the prescribed guidelines of the Yale, Harvard, and University of Pennsylvania Institutional Animal Care and Use Committee.

For CCl₄ treatment, rats (180-250 g) were provided drinking water containing 1% Phenobarbital starting two weeks prior to and during the entirety of CCl₄ treatment, in order to upregulate cytochrome P₄₅₀ enzyme levels(Hashimoto et al. 1999). Rats were injected subcutaneously with CCl₄ (0.1 ml, CCl₄ diluted 1:6 in olive oil) three times per week for 6-8 weeks. For control experiments, rats were injected in identical fashion with olive oil alone (CCl₄-free vehicle). Livers were harvested and fixed for immunofluorescence as described below.

**Isolation of HSC.** HSC were isolated from retired breeder rats (500-700g) by *in situ* pronase/collagenase perfusion followed by density gradient centrifugation, as described(Liu et al. 2003). Primary cells were greater than 95% pure. Cells were grown on standard tissue culture plastic dishes in M199 medium with 10% fetal calf serum and antibiotics. Primary cells were used at either 1 or 7 days after isolation, or were passaged after 7 days and used at passages 1-4. HSC at day 1 after isolation are phenotypically quiescent, while cells at day 7 are phenotypically activated(Liu et al. 2003).
Reverse-transcriptase polymerase chain reaction (RT-PCR). RNA was isolated from HSC using chaotropic methods. HSC cDNA was produced using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) on DNase-treated RNA. Specific oligonucleotides primers designed based on the cloned rat P2Y subtypes P2Y1, P2Y2, P2Y4, and P2Y6 (Dranoff et al. 2001) were used to amplify Day 1 and Day 7 HSC cDNA using the following thermal cycling parameters: 94°C x 5 min; 30 cycles (94°C x 30 sec, 60°C x 1 min (63°C for P2Y4), 72°C x 1 min); 72°C x 5 min. Control reactions were performed with reverse transcriptase-treated RNA that was not subjected to DNase treatment. Products were evaluated using agarose gel electrophoresis.

In separate experiments, specific oligonucleotide primers designed based on the published sequence of rat NTPDase1 (GenBank Accession # NM_022587), NTPDase2 (NM_172030), NTPDase3 (NM_178106), NTPDase5 (NM_007647), and NTPDase6 (NM_053498) were used to amplify Day 1, Day4, and Day 7 HSC cDNA using the following thermal cycling parameters: 94°C x 5 minutes; 30 cycles of 94°C x 30 seconds, 62°C x 1 minute, and 72°C x 1 minute; and 72°C x 10 minutes.

Real-time RT-PCR. Alterations in expression of α(1)-procollagen (procollagen-1) mRNA in activated HSC were determined using real-time RT-PCR. HSC were treated with either vehicle alone (control), suramin (50 µM), UDP (100 µM), or UDP + suramin every 24 hr (for a total of two treatments) in a 48 hr period. Total RNA was isolated as described above and subjected to real-time PCR using the-ABI-PRISM 7700 (Applied Biosystems, Foster City, CA). Detection of
procollagen-1 was accomplished by labeling with 6-FAM normalized to a VIC-labeled GAPDH probe. PCR was performed using the following cycling parameters: reverse transcription at 48°C x 30 min; activation of AmpliTaq polymerase (Applied Biosystems) at 95°C x 10 min; PCR cycling 40 cycles of 95°C x 15 sec (denaturation) and 60°C x 1 min (annealing/extension). Experiments were performed in triplicate and expressed in µg procollagen-1 mRNA/mg total RNA.

**Western blot.** Alterations in expression of NTPDase2 was determined by Western blot using a rabbit polyclonal antibody directed against NTPDase2/CD39L1 (Dranoff et al. 2002; Sevigny et al. 2002). Protein was isolated from HSC at 1, 4, and 7 days after isolation after osmotic lysis. Equal amounts of protein for each group were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Immobilon; Millipore Corp., Bedford, MA). The membrane was blocked with nonfat milk (5% in PBS + 0.05% Tween), hybridized to the anti-NTPDase2 antibody then anti-rabbit secondary antibody, and developed using enhanced chemiluminescence.

**Confocal immunofluorescence.** Changes in distribution of NTPDase2 after CCl4 treatment were determined using confocal immunofluorescence. Tissue was fixed by perfusion with 2% (w/v) paraformaldehyde in 0.075 M sodium phosphate, pH 7.3, cryopreserved overnight in 30% (w/v) sucrose, and frozen in isopentane/liquid nitrogen. After quenching with 50 mM NH₄Cl and 3% (v/v) goat serum in PBS, 5 µm-thick sections were labeled with a 1:25 dilution of anti-NTPDase2 antibody and a 1:800 dilution of anti-smooth muscle actin antibody for 45 min at 37°C, then washed and incubated with Alexa 488 conjugated
anti-rabbit secondary antibody and Alexa 598-conjugated anti-mouse secondary antibody. Negative controls were stained with Alexa 488-conjugated anti-rabbit secondary antibody plus Alexa 598-conjugated anti-mouse secondary antibody without primary antibodies. Specimen fixation plus all washes and incubations included pepstatin (2 \( \mu \)M), PMSF (0.2 mM), and benzamidine (0.5 mM) to inhibit proteolysis.

Specimens were examined using a Zeiss LSM 510 confocal imaging system equipped with both a Krypton/Argon and Helium/Neon laser at 400x magnification. Double-labeled specimens were serially excited at 488 nm and observed at > 515 nm to detect Alexa 488, then excited at 568 nm and observed at > 585 nm to detect Alexa 598 using only the Krypton/Argon laser.

**Assay of ecto-nucleotidase activity.** Functional ecto-nucleotidase activity was determined in live HSC by colorimetric detection of inorganic phosphate (Pi) (Sevigny et al. 2000). Rat HSC were isolated as described above and plated in equal amounts on 24-well plastic plates. Cells were washed with nucleotide- and Pi-free incubation medium (150 mM NaCl, 1.5 mM CaCl\(_2\), 50 mM Tris, and 50 mM imidazole, pH 7.4) x 2 before use. Enzyme activity was determined in incubation medium containing 0.3 mM nucleotide (ATP, ADP, or AMP). The reaction was incubated at 37\(^\circ\) for exactly 8 min and reactions were terminated by the addition of 0.25 ml of Malachite Green reagent (Baykov et al. 1988). Malachite green color change in response to Pi was detected by measuring the absorbance at 620 nm using a multiplate spectrometer (PowerWare 340, Bio-Tek Inst., Winooski, VT).
**Confocal video microscopy.** Confocal video microscopy was performed using HSC grown on glass coverslips. HSC were loaded with the Ca\(^{2+}\)-sensitive fluorophore fluo-4/AM (Molecular Probes) and mounted on a specially designed stage for use on a confocal microscope. Cells were perifused initially with HEPES buffer, then with buffer containing ATP, ADP, UTP, or UDP (100 µM). Changes in fluo-4 fluorescence were monitored using a Bio-Rad MRC 600 confocal imaging system. Fluo-4 fluorescence was excited using a Kr/Ar laser at 488 nm; emitted fluorescence > 515 nm was collected. Changes in fluorescence over time were expressed as peak fluorescence (f) divided by initial fluorescence (f\(_0\)). Separate experiments were performed in the presence P2Y inhibitor suramin (50 µM) in the initial buffer and the ATP-containing buffer and in Ca\(^{2+}\)-free media.

**Statistical analysis.** Data are expressed as mean ± standard deviation where appropriate. Comparisons between individual groups were made with two-tailed t tests.

**Results.**

**HSC express functional P2Y receptors.** Functional expression of P2Y receptors in activated, passaged HSC was determined using confocal video microscopy to image changes in cytosolic Ca\(^{2+}\) (Ca\(^{2+}\)_i) in response to extracellular nucleotides. P2Y receptors induce downstream effects via inositol trisphosphate (IP3)-mediated Ca\(^{2+}\)_i increases (Dranoff and Nathanson 2000). As seen in the representative micrograph in Figure 1, perifusion of passaged HSC with ATP (100 µM) induced a rapid and sustained increase in Ca\(^{2+}\)_i. Both sustained and
oscillatory increases in Ca\(^{2+}\) were noted in separate experiments (Figure 1B). These separate patterns of Ca\(^{2+}\) signals may be due to the distribution of different inositol trisphosphate receptors in HSC (although this remains to be elucidated)(Bezprozvanny et al. 1991; Hagar et al. 1998)

These Ca\(^{2+}\) increases were due to activation of P2Y receptors, since they were inhibited by the P2Y receptor inhibitor suramin, but were unaltered by removal of extracellular Ca\(^{2+}\) even when measured at time points up to 5 minutes (and thus were not dependent on Ca\(^{2+}\) influx) (Figure 2). Taken together, these findings demonstrate that HSC express functional P2Y receptors coupled to Ca\(^{2+}\) signals. These findings also demonstrate that nucleotide-mediated Ca\(^{2+}\) signals in HSC are triggered by P2Y receptors and not P2X receptors, since they occur in the absence of extracellular Ca\(^{2+}\).

**P2Y-mediated Ca\(^{2+}\) signals in HSC are simultaneous and not polarized.**

Because downstream effects of hormone-mediated Ca\(^{2+}\) signals in epithelia result in part from special and temporal gradients in Ca\(^{2+}\) (Nathanson 1994), we investigated whether there was evidence for Ca\(^{2+}\) gradients or waves in HSC in response to extracellular nucleotides. To do this, we performed post-hoc analysis of Ca\(^{2+}\) signals in regions within HSC activated by extracellular nucleotides as demonstrated in Figure 1. Because HSC lack tight junctions and thus do not have apical and basolateral plasma membrane domains(Friedman and Roll 1987; Friedman 1996), regions of interest were determined empirically. As seen in Figure 3, ATP-induced changes in Ca\(^{2+}\) occurred simultaneously throughout the cell, suggesting that Ca\(^{2+}\) signals are not localized to the nucleus(Leite et al. 2003) or
other specific regions of HSC.

**HSC alter their expression of P2 receptor subtypes upon activation.** The expression of specific P2Y receptor subtypes by day 1 (quiescent) and day 7 (activated) HSC was determined using molecular and functional methods. Four P2Y subtypes have been cloned in rat: P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub>. In rats, the ligand specificity for each of these is as follows: P2Y<sub>1</sub> (ADP), P2Y<sub>2</sub> and P2Y<sub>4</sub> (ATP = UTP), and P2Y<sub>6</sub> (UDP) (Ralevic and Burnstock 1998).

The molecular expression of P2Y receptor subtypes in day 1 and day 7 HSC was demonstrated using RT-PCR with oligonucleotide primers specific to the cloned rat P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub> receptors (Dranoff et al. 2001). As seen in Figure 4A, day 1 HSC expressed the P2Y subtypes P2Y<sub>2</sub> and P2Y<sub>4</sub>, whereas day 7 HSC expressed the P2Y subtypes P2Y<sub>6</sub> (intense band) and P2Y<sub>1</sub> (faint band).

The functional expression of P2Y receptor subtypes in day 1 and day 7 HSC was determined using confocal video microscopy (Figure 4B). HSC were grown on glass coverslips, loaded with fluo-4/AM, mounted in a special perifusion chamber, and stimulated with a variety of nucleotides. Changes in fluo-4 fluorescence were plotted over time. Consistent with their expression of the triphosphate-specific receptors P2Y<sub>2</sub> and P2Y<sub>4</sub>, day 1 HSC were activated by ATP and UTP, but not ADP or UDP. Consistent with their expression of the UDP-sensitive receptor P2Y<sub>6</sub>, day 7 HSC were activated by UDP. However, day 7 HSC were not activated by the P2Y<sub>1</sub> agonist ADP, suggesting that they either do not express appreciable quantities of P2Y<sub>1</sub> protein or that these receptors are not tightly coupled to HSC Ca<sup>2+</sup><sub>i</sub> signals. Interestingly, day 7 HSC were activated by ATP, suggesting that
they may express an ATP-sensitive P2Y receptor distinct from P2Y2 or P2Y4.

Taken together, these data demonstrate that both quiescent and activated HSC express functional P2Y receptors and that they switch expression of these receptors upon activation.

**Expression of ecto-nucleotidases varies upon activation of HSC.** Because ecto-nucleotidases of the nucleoside triphosphate diphosphohydrolase (NTPDase) class are critical regulators of P2Y receptors (Enjyoji et al. 1999; Goepfert et al. 2000; Goepfert et al. 2001), we investigated expression of cloned NTPDases in quiescent and activated HSC.

Because antibodies for immunoblot that detect rat NTPDase1 are not available, molecular expression of the five cloned rat NTPDases in HSC was determined using RT-PCR. RT-PCR was performed using oligonucleotide primers designed to correspond to the published sequence of rat NTPDase1 (GenBank Accession # NM_022587), NTPDase2 (NM_172030), NTPDase3 (NM_178106), NTPDase5 (NM_007647), and NTPDase6 (NM_053498) with RNA from HSC at 1, 4, and 7 days after isolation. As seen in Figure 5A, day 1, 4, and 7 HSC express NTPDase1 and NTPDase2 mRNA. Day 4 HSC also express NTPDase6 mRNA. This ecto-nucleotidase may be less likely to contribute to hydrolysis of extracellular nucleotides by HSC, because it is primarily a Golgi-associated ecto-nucleotidase (Braun et al. 2000) although a fraction of this protein is localized to the plasma membrane.

Immunoblot analysis of relative NTPDase2 levels in quiescent and activated HSC was performed using polyclonal antibodies to mouse NTPDase2 in
day 1, day 4, and day 7 rat HSC (Figure 5B). While no NTPDase2 protein was detected in day 1 HSC, Day 4 HSC had the greatest expression of NTPDase2, and NTPDase2 expression persisted in day 7 HSC.

Functional expression of ecto-nucleotidases in HSC was determined using a functional assay for the detection of inorganic phosphate ($P_i$) from ATP or other nucleotides (Figure 5C)(Dranoff et al. 2002; Baykov et al. 1988). Both day 1 and day 7 had ecto-nucleotidase activity, and were roughly equal in hydrolysis of ATP and ADP, suggesting that NTPDase1 may be an important mediator of nucleotide hydrolysis by HSC. However, the upregulation in NTPDase activity may be due to upregulation of NTPDase2. No hydrolysis of AMP was observed, which is consistent with the fact that NTPDases do not hydrolyze AMP.

Taken together, these data demonstrate that activated, but not quiescent HSC express the ecto-nucleotidase NTPDase2. Both quiescent and activated HSC express may NTPDase1, although the lack of available antibodies for immunoblot limits this conclusion. Furthermore, activated HSC express approximately twice as much ecto-nucleotidase activity as quiescent HSC.

**NTPDase2 co-localizes with activated HSC in CCl4 cirrhosis.** To determine whether induction of NTPDase2 was observed in experimental cirrhosis, rats were treated with CCl4 for 6 weeks. Confocal immunofluorescence was used to determine whether NTPDase2 co-localized with $\alpha$-SMA positive activated HSC. As seen in Figure 6A, NTPDase2 was expressed along several bridging fibrous bands between central areas of the liver. As seen in Figure 6B, $\alpha$-SMA was expressed in the same region. Figure 6C demonstrates co-localization of
NTPDase2 and $\alpha$-SMA. Figure 6D demonstrates that no NTPDase2 or $\alpha$-SMA fluorescence was detected in liver sections from control animals treated only with olive oil (CCl$_4$ vehicle). These findings are representative of 10 experimental and 5 control animals and demonstrate that NTPDase2 co-localizes with activated HSC in CCl$_4$ cirrhosis.

**Extracellular UDP regulates transcription of procollagen-1 transcription by HSC via P2Y receptors.** The role of P2Y receptors in regulation of procollagen(1) mRNA expression in Day 7 HSC was assessed using real-time RT-PCR. Since Day 7 HSC express the P2Y receptor P2Y$_6$ and respond to extracellular UDP (Figure 4), HSC were stimulated with either control vehicle, suramin alone, UDP alone, or UDP + suramin (Figure 7). UDP roughly tripled transcription of procollagen-1 after 24 hr, and this effect was partially blocked by suramin, suggesting that the effect was mediated via P2Y receptor activation. Since suramin alone had no effect, it is likely that either HSC do not secrete large amounts of extracellular nucleotides or that they do not secrete enough endogenous extracellular nucleotides to overcome ecto-nucleotidase activity to act in an autocrine fashion.

**Discussion**

Hepatic stellate cells are important targets of liver disease therapy, since they are the chief fibrogenic cells of the liver. Although excellent work has been done elucidating signal transduction in these cells over recent years(Brenner et al. 2000; Friedman 1996; Bataller and Brenner 2001), there is still no effective
treatment for liver fibrosis. Thus it is critical to search for new signaling mechanisms that regulate HSC activity. We now demonstrate that HSC express functional P2Y purinoceptors that regulate cytosolic Ca\(^{2+}\) signals in both quiescent and activated HSC. The patterns of Ca\(^{2+}\) signals observed in HSC include both sustained Ca\(^{2+}\) increases that are thought to be important in exocytosis and Ca\(^{2+}\) oscillations that may be important for cell volume autoregulation following exocytosis (Nathanson 1994; Nathanson et al. 1996). These findings are consistent with those of Takemura et al (Takemura et al. 1994), in which extracellular nucleotides were found to link to inositol trisphosphate accumulation, cytosolic Ca\(^{2+}\) increases, and contractility of cultured HSC. We have now established that the activation of these same receptors regulate procollagen-1 transcription by HSC, suggesting that P2Y receptors are a potentially new target in the treatment of liver fibrosis.

Why might HSC be stimulated by extracellular nucleotides? Although P2Y receptors are distributed widely throughout the body, their role in mediating response to injury might be expected. Regulated release of nucleotides may occur through a variety of mechanisms, including channel-mediated release and exocytosis. However, non-regulated release of nucleotides occurs after cell lysis, as may occur in hepatitis. Thus, released nucleotides could act as trophic factors for cells that produce scar matrix, although this remains hypothetical in the liver. Similar findings have been noted, however, in fibrogenic kidney mesangial cells (Rost et al. 2002).

The relative roles of the ecto-nucleotidases NTPDase1 and NTPDase2 in
HSC have only been determined in part based on the findings of this study. It is clear that NTPDase2 (CD39L1) is expressed in HSC activated by prolonged culture and in experimental fibrosis, but not expressed in quiescent HSC. However, this ecto-nucleotidase has much greater activity for triphosphate nucleotides than for diphosphate nucleotides. Because both quiescent and activated HSC express relatively equipotent ecto-nucleotidase activity, NTPDase1 may be the more important ecto-nucleotidase regulating net ecto-nucleotidase activity of HSC. However, it is possible that HSC may express newer NTPDases that have not been defined on a molecular basis. In any case, the likely physiologic role of these proteins is to minimize P2Y activation to prevent receptor desensitization, as occurs in the NTPDase1 null mouse (Enjyoji et al. 1999). The outcome of NTPDase1 and NTPDase2 blockade or knockout in liver fibrosis, however, remains to be addressed.

In summary, both quiescent and activated HSC express the necessary cellular machinery for nucleotide-mediated signaling: nucleotide-mediated cytosolic Ca\textsuperscript{2+} signals, P2Y receptors, and ecto-nucleotidases that may regulate those receptors. Furthermore, extracellular UDP may regulate fibrogenesis by activated HSC via regulation of procollagen-1 transcription.
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FIGURE LEGENDS

Figure 1. Extracellular ATP induces Ca^{2+}_{i} signals in HSC. A. Confocal micrographs of HSC perifused with ATP (100 µM) over time. HSC were loaded with the Ca^{2+}-sensitive dye fluo-4/AM and observed at 0.6 sec intervals using a confocal microscope. HSC were perifused with HEPES buffer containing ATP (100 µM). An increase in fluo-4 fluorescence is seen first at 20 sec, and markedly at 40 sec after addition of ATP in several HSC. B. Graphic representation of Ca^{2+}_{i} signal in several individual HSC over time. Two patterns of Ca^{2+}_{i} signals are observed from separate HSC: sustained Ca^{2+} increases (seen in the bottom two tracings) and Ca^{2+} oscillations (upper two tracings). Oscillations were less frequently seen than sustained Ca^{2+} increases (seen in 4 of 28 experiments). Changes in fluo-4 fluorescence were monitored using the narrowest possible pinhole settings, ensuring that changes in cell morphology would not affect fluorescence changes significantly. Note that the pixel intensities were shifted to avoid overlap of tracings.

Figure 2. ATP-induced Ca^{2+}_{i} signals in HSC are due to activation of P2Y receptors. ATP-induced Ca^{2+}_{i} increases in HSC were investigated in the presence of the P2Y inhibitor suramin and in the absence of extracellular Ca^{2+}. Since the P2 receptor antagonist suramin inhibited ATP-induced Ca^{2+} increases, these increases were likely due to P2Y activation. Since removal of extracellular Ca^{2+} had no effect on ATP-induced Ca^{2+}_{i} increases, this suggests that these increases
are due to mobilization of intracellular stores after activation of P2Y receptors rather than due to influx of extracellular Ca\textsuperscript{2+} after activation of P2X receptors. (* p < 0.05 vs. ATP)

**Figure 3. ATP-induced Ca\textsuperscript{2+} signals occur uniformly across HSC. A. Microscopic display of regions of interest within an individual cell.** A blow-up of the central cell seen in Figure 1 is shown. Regions of interest were drawn on three different areas within the cell. **B. Graphical representation of Ca\textsuperscript{2+} signals within regions of interest depicted in Figure 3A.** fluo-4 fluorescence increases were plotted for the entire cell and for the three regions of interest identified in Figure 3A. Unlike in some cell types, the fluorescence increases occur uniformly across the cell.

**Figure 4. Quiescent HSC express the P2Y receptor subtypes P2Y\textsubscript{2} and P2Y\textsubscript{4}, whereas activated HSC express the P2Y receptor subtype and P2Y\textsubscript{6}. A. RT-PCR.** RNA was isolated from HSC at 1 and 7 days after isolation and used for RT-PCR with oligonucleotides corresponding to the published sequences of the rat P2Y\textsubscript{1}, P2Y\textsubscript{2}, P2Y\textsubscript{4}, and P2Y\textsubscript{6}(Dranoff et al. 2001). Day 1 HSC express the ATP and UTP receptors P2Y\textsubscript{2} and P2Y\textsubscript{4}, whereas day 7 HSC express the UDP receptor P2Y\textsubscript{6}. No bands were seen in reverse transcriptase negative controls (not shown). **B. Confocal video microscopy.** Changes in fluo-4 fluorescence in HSC over time were calculated for the nucleotides ATP, ADP, UTP, and UDP in day 1 and day 7 HSC. Data are expressed as maximal fluorescence (f) divided by initial
fluorescence ($f_0$). In day 1 HSC, ATP and UTP induced a 2-fold increase in fluo-4 fluorescence. In day 7 HSC, UDP induced a 3-fold increase in fluo-4 fluorescence, and ATP induced a 2-fold increase in fluo-4 fluorescence.

**Figure 5. NTPDase2, but not NTPDase1, is upregulated in activated HSC.**

**A. RT-PCR.** RNA was isolated from day 1, day 4, and day 7 HSC and used for RT-PCR with oligonucleotides corresponding to the published sequence of the five cloned rat ecto-NTPDases (NTPDase1, NTPDase2, NTPDase3, NTPDase5, and NTPDase6) (GenBank). HSC express mRNA for both NTPDase1 and NTPDase2. Only day 4 HSC express the Golgi-associated UDPase NTPDase6 (Braun et al. 2000).

**B. Immunoblot for expression of NTPDase2.** Total protein fractions were isolated from day 1 (quiescent), day 4 (intermediate), and day 7 (activated) HSC and subjected to PAGE, then immunoblotted with a polyclonal antibody to the mouse NTPDase2 (Dranoff et al. 2002). Bands are absent from day 1 HSC, but are strongest at day 4 and remain present at day 7. Spleen protein was used as a positive control. Bands from the same column represent separate experiments.

**C. Ecto-nucleotidase assay.** Changes in ecto-nucleotidase function between day 1 and day 7 HSC were tested using the method of Baykov (Baykov et al. 1988). Since ecto-nucleotidase function is equal between ATP and ADP, NTPDase1 is likely an important mediator of ecto-nucleotidase function in HSC. Upregulation in nucleotide hydrolysis, however, may be mediated by the observed upregulation in NTPDase2.
Figure 6. NTPDase2 is co-localized with \(\alpha\)-smooth muscle actin in fibrous bands bridging from central areas in CCl\(_4\)-induced cirrhosis. To determine whether NTPDase2 was localized with activated HSC in liver injury, rats were treated with CCl\(_4\) for 6 weeks. Confocal immunofluorescence was used to determine the expression of NTPDase2 and \(\alpha\)-smooth muscle actin (SMA).

A. NTPDase2. NTPDase2 fluorescence is seen in green in a region of bridging fluorescence with several collapsed vessels.

B. SMA. SMA fluorescence is seen in red in the same region.

C. Colocalization. Simultaneous red/green fluorescence demonstrates that NTPDase2 and SMA are expressed in the same region.

D. Negative control. Rats treated with olive oil alone (CCl\(_4\) vehicle) show no NTPDase2 or SMA fluorescence.

Figure 7. Extracellular UDP regulates transcription of a(1)-procollagen in activated HSC via P2Y receptor activation. Real-time RT-PCR was used to determine procollagen-1 mRNA content in Day 7 HSC treated under the following conditions: control (vehicle alone), suramin (50 mM), UDP (100 mM), or UDP + suramin. Suramin had no significant effect on procollagen-1 mRNA content. However, addition of UDP roughly tripled procollagen-1 mRNA, and this was partially inhibited by suramin addition. (* \(p < 0.01\) vs. control; ** \(p < 0.05\) vs. UDP, \(p < 0.05\) vs. control; \(\dagger p = NS\) vs. control; \(n = 3\) for all groups).
FIGURE 1

A

0 sec  20 sec

40 sec  60 sec

B

Pixel Intensity (arbitrary)

0 100 200 300 400 500 600 700

0 30 60 90 120 150 180

Time (sec)
**FIGURE 2**

![Graph showing relative fluorescence (f/f₀) for different conditions.](image)

- **ATP**
- **ATP + Suramin**
- **ATP (Ca²⁺-free)**

Relative fluorescence (f/f₀) ranges from 1 to 3. **ATP** and **ATP (Ca²⁺-free)** show higher fluorescence compared to **ATP + Suramin**.
FIGURE 3

A

HEPES

ATP 100µM

B

pixel intensity

0 50 100 150 200 250

time [sec] 0 30 60 90 120 150 180

Entire Cell
Region 1
Region 2
Region 3

ATP 100 µM
Nucleotide signaling in hepatic stellate cells.
Dranoff, et al.

FIGURE 4

A

Day 1 HSC

Day 7 HSC

B

Relative Fluorescence (f/f₀)

ATP
n = 6

ATP
n = 11

ADP
n = 5

ADP
n = 3

UDP
n = 3

UDP
n = 15

UTP
n = 9

UTP
n = 6

Day 1 HSC

Day 7 HSC
Nucleotide signaling in hepatic stellate cells.
Dranoff, et al.

FIGURE 5

A

B

C

Ecto-nucleotidase activity (pmol Pi/10^5 cells)

Day 1    Day 4    Day 7

58 kD

ATP
n=6

ADP
n=6

AMP
n=5

ATP
n=6

ADP
n=5

AMP
n=5

Day 1    Day 7

Ecto-nucleotidase activity (pmol Pi/10^5 cells)

ATP
n=6

ADP
n=6

AMP
n=5

ATP
n=6

ADP
n=5

AMP
n=5

Day 1

Day 7
FIGURE 6
FIGURE 7

![Graph showing the effect of suramin and UDP on α(1)-procollagen mRNA expression.](image-url)