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ORIGINAL ARTICLE

The adenosine transporter, ENT1, in cardiomyocytes is sensitive to inhibition by ethanol in a kinase-dependent manner: implications for ethanol-dependent cardioprotection and nucleoside analog drug cytotoxicity

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Abstract The adenosine transporter 1 (ENT1) transports nucleosides, such as adenosine, and cytotoxic nucleoside analog drugs. ENT1 is well established to play a role in adenosinergic signaling in the cardiovascular system by modulating adenosine levels. Moderate ethanol consumption is cardioprotective and underlying mechanisms of action are not clear although adenosinergic signaling has been implicated. Here, we show that ethanol (5–200 mM) significantly reduces ENT1-dependent [^{3}H] 2-chloroadenosine uptake (by up to 27 %) in the cardiomyocyte cell line, HL-1. Inhibition or absence of ENT1 is known to be cardioprotective, suggesting that the interaction of ethanol with ENT1 may promote adenosinergic cardioprotective pathways in the cardiovasculature.

Ethanol sensitivity of adenosine uptake is altered by pharmacological activation of PKA and PKC. Primary cardiomyocytes from PKCε-null mice have significantly greater sensitivity to inhibition (by approximately 37 %) of adenosine uptake by ethanol than controls. These data suggest that the presence of ethanol may compromise ENT1-dependent nucleoside analog drug cytotoxicity, and indeed, ethanol (5 mM) reduces the cytotoxic effects of gemcitabine (2 nM), an anti-cancer drug, in the human cancer cell line, HTB2. Thus, the pharmacological inhibition of ENT1 by ethanol may contribute to ethanol-dependent cardioprotection but compromise gemcitabine cytotoxicity.

Keywords ENT1 · Ethanol · Gemcitabine · Regulation · Adenosine · Transporter

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Introduction

The adenosine transporter, ENT1, is a member of the family of equilibrative nucleoside transporters (ENTs) that are integral membrane proteins responsible for the bidirectional flux of nucleosides, nucleobases, and nucleoside analog drugs, across the cell membrane [1]. ENTs are important clinically as the route of entry of cytotoxic nucleoside analog drugs [2]. ENTs also modulate extracellular levels of adenosine and thus play significant roles in purinergic signaling, particularly in the central nervous system and the cardiovasculature [3, 4].

Adenosine is a critically important purine nucleoside in the heart, and flux of adenosine across the cardiomyocyte cell membrane is mediated primarily via ENT1 [5], which plays a central role in regulating adenosinergic cardioprotection [3, 4, 6, 7]. ENT1-null mice have elevated plasma adenosine levels [4] and are inherently cardioprotected [3].

Previous studies have identified ethanol as a pharmacological inhibitor of adenosine uptake, resulting in increased extracellular adenosine in human lymphocytes [8], primary cultures of rat hepatocytes [9], human placental cells [10], human bronchial cells [11], S49 mouse lymphoma cells, and rodent neuronal–glial cells NG108-15 cells [12–15].

Moderate ethanol consumption, defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as an

average consumption of one to two drinks per day (equivalent to peak blood alcohol levels of ~10 mM ethanol), has long been known to be cardioprotective in a variety of animal models [16–18], and a contribution of purinergic signaling has been implicated in many of these studies.

Despite the proposed putative central role of ENT1 in mediating the cardioprotective effects of ethanol, there are no data demonstrating that adenosine uptake is inhibited by ethanol in cardiomyocytes. Thus, we hypothesized that adenosine uptake via ENT1 is inhibited by ethanol in cardiomyocytes, which would lead to enhanced adenosinergic signaling and cardioprotection. Moreover, previous studies demonstrated that ethanol sensitivity of ENT1 in neuronal cells is regulated by kinase-dependent pathways [12, 13, 19]. PKA activity is required to maintain or restore ethanol sensitivity of ENT1 [12], while PKC activation is correlated with ethanol insensitivity of ENT1-dependent uptake [13]. Thus, we hypothesized that PKC and PKA would also modulate the ethanol sensitivity of ENT1 in cardiomyocytes. Finally, while partial ethanol inhibition of adenosine uptake would enhance cytoprotective effects of adenosine in the cardiovasculature (and therefore be beneficial to health), we hypothesized that cytotoxic effects of nucleoside analog drugs, used in the treatment of solid tumors, might be compromised in the presence of ethanol (and thus be contraindicated).

Materials and methods

Reagents

Claycomb medium, nitrobenzylthioinosine (NBTI), dipyridamole, chloroadenosine, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). McCoy's 5A medium was purchased from Wisent Inc. (St-Bruno, QC, Canada). Radiolabeled [^{3}H]-2-chloroadenosine and [^{3}H] NBTI were purchased from Moravek Biochemicals (CA, USA). Chemicals including Sp-cAMPs (cAMP analog) and OAG (2-acetyl-1-oleoyl-sn-glycerol) were from Tocris Bioscience (MO, USA) and of cell culture grade.

HL-1 cell culture

HL-1 cells are an adult immortalized cardiac cell line and were maintained as previously described [5]. For uptake assays, cells were used between passages 30 and 50, were seeded in six-well plates, and allowed to grow until 75–80 % confluency. The medium was exchanged for serum-free media [supplemented Claycomb media lacking 10 % (v/v) fetal bovine serum] 24 h prior to conducting uptake assays.

HTB2 cell culture

HTB2 cells, derived from a papilloma (superficial transitional carcinoma), are a human bladder cancer cell line. They were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in McCoy's 5A medium supplemented with 10 % (v/v) FBS in humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C. For gemcitabine toxicity assays, cells were plated in 96-well plates and left to attach for 24 h before treatment.

Protein kinase C epsilon (PCKε) animals and genotyping

All animal handling procedures were approved by the Animal Care Committee of York University in accordance with the Canadian Council on Animal Care guidelines. The mice were housed in standard cages with chow and water available ad libitum. The animal room was maintained on a 12-h light/dark cycle.

Heterozygous PKCε (+/−) mice were generated as previously described [20]. F2 mice of inter-crossed heterozygotes were genotyped for PKCε gene detection using standard PCR analysis, using two sense primers F1 5′-CTT TAT CCT GGG TAG CCT AGT G-3′ and Neo 5′-ATA TTG CTG AAG AGC TTG GCG GC-3′, and anti-sense primer R1 5′-CCT AAC TGA ATG CTG CTC CTA C-3′ prepared by Sigma-Aldrich Canada (Oakville, ON, Canada). PCR cycling conditions were as follows: 4 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 45 s at 58 °C, and 2 min at 72 °C with final extension of 7 min at 72 °C. Experiments in this study were performed with homozygous wild-type (WT) (PKCε +/+) and homozygous PKCε-null (PKCε −/−) mice between 2 and 4 months of age.

PKCε primary cardiomyocyte isolation

Primary cardiomyocytes isolation was conducted as previously described [3].

Ethanol exposure conditions

Cells grown in six-well plates were incubated in the presence or absence of 50–200 mM ethanol (100 %) for 10 min, while others were treated with 10–50 mM for 24 h. In addition, primary cardiomyocytes isolated from PKCε-null (−/−) mice were treated with varying concentrations of ethanol (50–200 mM) for 10 min. Concentrations and time points were based on previous studies [8–15]. [^{3}H] 2-Chloroadenosine uptake assays were conducted following ethanol treatments. The cells were not washed after the ethanol treatments.

[^{3}H] 2-Chloroadenosine uptake assay

[^{3}H] 2-Chloroadenosine uptake assays were conducted to examine the effect of ethanol on adenosine nucleoside uptake. [^{3}H] 2-Chloroadenosine is a more stable analog of adenosine. The uptake assay was conducted as previously described [5]. The concentration of unlabeled 2-chloroadenosine was 10 μM and the specific activity of radiolabeled [^{3}H] 2-chloroadenosine was 20.7 Ci/mmol. Transport values were expressed as picomoles of [^{3}H] 2-chloroadenosine per milligram of protein.

Trypan blue exclusion assay

To determine the effects of ethanol on cardiomyocyte viability, trypan blue exclusion assays were conducted on HL-1 cells exposed to ethanol (200 mM; 10 min or 50 mM; 24 h).

NBTI treatment

NBTI is a high affinity (nM), non-transportable, specific inhibitor of ENT1 [5]. HL-1 cells were treated with NBTI (100 nM; 15 min) prior to incubation in the presence or absence of ethanol (200 mM; 10 min), followed by [^{3}H] 2-chloroadenosine uptake assays. This concentration of NBTI will inhibit ENT1 but not ENT2.

PKA and PKC treatment

The specific PKA activator, Sp-cAMPs, or the general PKC activator, OAG, was used to determine the effect of PKA/PKC activation on ethanol sensitivity of ENT1-dependent transport in cardiomyocytes. Sp-cAMPs (200 μM; 10 min) or OAG (50 μM; 30 min) was added to HL-1 cells prior to incubation in the presence or absence of ethanol (200 mM; 10 min), and transport assays were then conducted.

[^{3}H] NBTI binding assay

NBTI binding assays were conducted on HL-1 cells and PKCε primary cardiomyocytes as previously described [5]. [^{3}H]NBTI binding constants (K_d and B_{max}) were obtained using non-linear regression analysis using GraphPad Prism v4.00 (GraphPad, San Diego, CA, USA).

Cytotoxicity assay

HTB2 cells were seeded in 96-well plates at a density of approximately 8,000 cells per well. Cells were allowed to attach for 24 h and then increasing concentrations of gemcitabine were added and cells were incubated for 48 h. Cells were treated with 5 nM gemcitabine (IC_{50}) plus 2 mM ethanol for 48 h to determine the effect of ethanol. An MTT assay was then performed [21]. IC_{50} values (the concentration of gemcitabine that blocks the HTB2 cells growth by 50 % compared to untreated, control cells) were calculated by non-linear regression analysis of values plotted as percentages of control values against the logarithm of gemcitabine concentration.

Statistical analysis

Data were compared using ANOVA with Newman–Keuls multiple comparison test. Data are expressed as mean±SEM or SD with a $p < 0.05$ considered statistically significant. Statistical analyses were conducted using GraphPad Prism v4.00 (GraphPad Software).

Results

Short-term exposure to high ethanol concentrations significantly inhibited adenosine uptake in HL-1 cells

Ethanol interacts with a number of membrane proteins, such as receptors and ion channels, by binding at a specific site [22]. Ethanol likely interacts with ENT1 in a similar manner although the target site is unknown. Using similar approaches to previous studies [9, 11, 14], we found that ethanol consistently and significantly inhibited [^{3}H] 2-chloroadenosine uptake in HL-1 cells (50 mM; 10.18±0.41 pmol/mg, $p < 0.01$, 100 mM; 9.76±0.3 pmol/mg, $p < 0.001$, 200 mM; 8.91±0.24 pmol/mg, $p < 0.001$; Fig. 1a) compared to control untreated cells (11.65±0.28 pmol/mg; Fig. 1a). These data show an increasing level of inhibition with increasing concentrations (50 mM, 12.6 %; 100 mM, 16.2 %; 200 mM, 23.5 %) compared to the control. These results suggest that adenosine uptake in HL-1 cells is sensitive to inhibition by short-term exposure to high concentrations of ethanol.

These concentrations of ethanol may have potential cytotoxic effects. However, data from trypan blue exclusion assays demonstrated that the percentage of cell death under our conditions was not significantly different from levels in untreated cells (data not shown).

Previous in vitro studies have demonstrated that ENT1, but not ENT2, is specifically inhibited by acute exposure to alcohol [8–11]. Similarly, HL-1 cells pre-treated with NBTI showed a significant decrease in [^{3}H] 2-chloroadenosine uptake compared to control cells (4.34±0.19 pmol/mg protein vs. 10.4±0.24 pmol/mg protein, $p < 0.001$; Fig. 1b), confirming that NBTI inhibits ENT1, but there was no further inhibition of uptake in the presence of ethanol (200 mM; 10 min) (NBTI+ethanol 4.35±0.22 pmol/mg; Fig. 1b). The inhibition of uptake in the ethanol treated versus control is

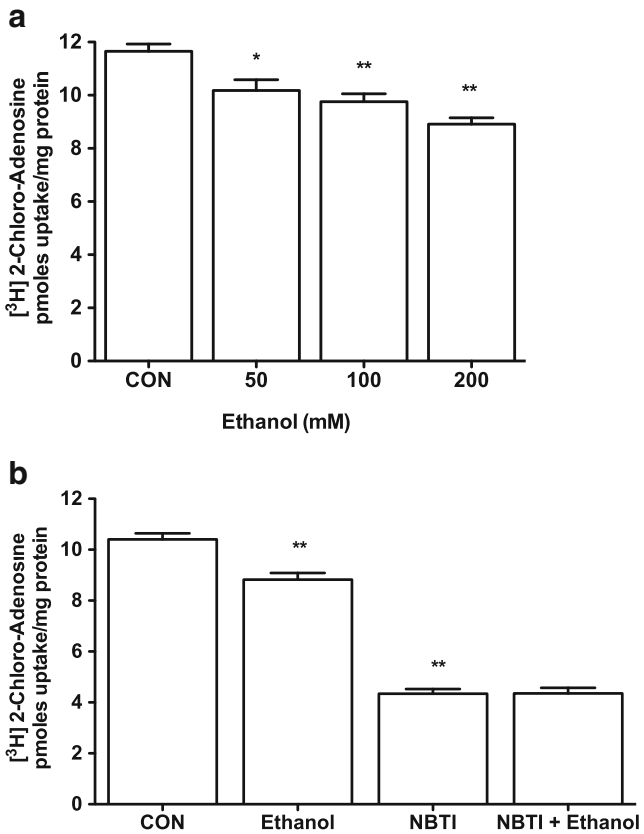

Fig. 1 [^{3}H]-2-Chloroadenosine uptake in HL-1 cardiomyocytes is inhibited by short-term exposure to ethanol. **a** HL-1 cells were incubated in serum-free media for 24 h prior to incubation in presence or absence of ethanol (50, 100, and 200 mM; 10 min). [^{3}H]-2-Chloroadenosine uptake was significantly inhibited in the presence of ethanol (50–200 mM) compared to the untreated control cells [$^*P<0.01$ ethanol (50 mM) vs. control; $^{**}P<0.001$ ethanol (100 and 200 mM) vs. control]. Pooled data are presented as mean±SEM where control $n=7$, ethanol (50 mM) $n=3$, ethanol (100 mM) $n=4$, and ethanol (200 mM) $n=5$; each experiment was conducted in sextuplicate. **b** [3H]-2-Chloroadenosine uptake via ENT2 in HL-1 cells is not inhibited by ethanol. HL-1 cells were treated with NBTI (100 nM, 15 min) prior to incubation in the presence or absence of ethanol (200 mM, 10 min). $^{**}P<0.001$ for NBTI vs. control and ethanol, and for ethanol vs. control and NBTI+ethanol. Ethanol does not increase in the inhibitory effect of NBTI, confirming that ENT1 is the main target

approximately 15 %, but subtraction of the NBTI insensitive component increases this to approximately 27 %. These data demonstrate that ethanol primarily and significantly inhibits uptake via ENT1 in HL-1 cells.

PKA activity regulates sensitivity of ENT1 to ethanol inhibition of adenosine uptake

Ethanol sensitivity of ENT1 has been previously correlated with PKA activation [12, 19], so we hypothesized that activation of PKA might increase ethanol sensitivity of ENT1 and lead to greater levels of inhibition by ethanol. Indeed, ethanol inhibition of 2-chloroadenosine uptake increased to 37 % ($p<0.001$, Fig. 2) in Sp-cAMPs-treated cells compared to untreated cells (24 %, $p<0.001$; Fig. 2). HL-1 cells treated with Sp-cAMPs alone displayed no significant difference in [^{3}H] 2-chloroadenosine uptake (11.27±0.62 pmol/mg protein; Fig. 2) relative to control (12.27±0.31 pmol/mg protein; Fig. 2). These findings suggest that ethanol sensitivity of [^{3}H] 2-chloroadenosine uptake via ENT1 is regulated by PKA activity.

PKC activity regulates sensitivity of ENT1 to ethanol inhibition of [^{3}H] 2-chloroadenosine uptake

PKC has been implicated in regulating cellular responses to ethanol [13]. Inhibition of [^{3}H] 2-chloroadenosine uptake by ethanol was significantly reduced in cells pre-incubated with OAG compared to cells treated with ethanol only (EtOH—6.96±0.38 pmol/mg protein, EtOH+OAG—8.47±0.45 pmol/mg protein, $p<0.05$; Fig. 3). HL-1 cells treated with OAG alone showed similar [^{3}H] 2-chloroadenosine uptake levels (11.1±0.43 pmol/mg protein; Fig. 3) compared to control cells (10.55±0.34 pmol/mg protein; Fig. 3), suggesting that the presence and activation of PKC can result in the loss of ethanol sensitivity of ENT1.

The PKC isoforms epsilon (ε) has been implicated in cellular responses to ethanol [23]. Uptake of [^{3}H] 2-chloroadenosine in WT cardiomyocytes isolated from PKCε (+/+) mice showed a small (~20 %) but significant inhibition of [^{3}H] 2-chloroadenosine uptake in the presence of ethanol (Fig. 4a). In contrast, PKCε-null (−/−) primary cardiomyocytes showed striking and significant (~50 %) inhibition of [^{3}H] 2-chloroadenosine uptake ($p<0.001$) in the presence of ethanol (Fig. 4b). These data indicate that loss of PKCε results in a

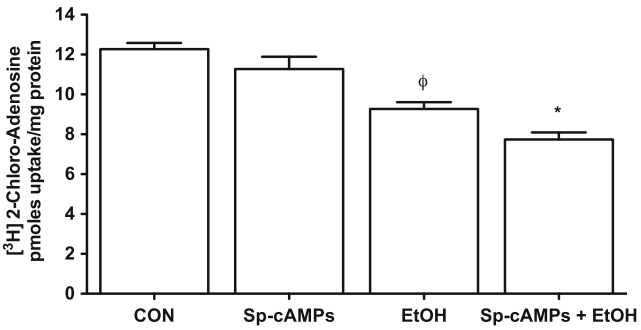

Fig. 2 PKA activation enhances ethanol sensitivity of [^{3}H]-2-chloroadenosine uptake in HL-1 cells. HL-1 cells incubated in serum-free media were treated in the presence or absence of ethanol (200 mM, 10 min) with pre-treatment in the presence or absence of a specific PKA activator, Sp-cAMPs (200 μM, 10 min). As expected, cells treated with ethanol showed a significant decrease in [^{3}H]-2-chloroadenosine uptake compared to control untreated cells ($\Phi P<0.001$). A further inhibition of [^{3}H]-2-chloroadenosine uptake was obtained in cells incubated with Sp-cAMPs prior to ethanol treatment compared to ethanol-only treated cells ($^*P<0.05$). Pooled data are presented as mean±SEM where $n=6$ for control and $n=3$ for other treatments; each experiment was conducted in sextuplicate

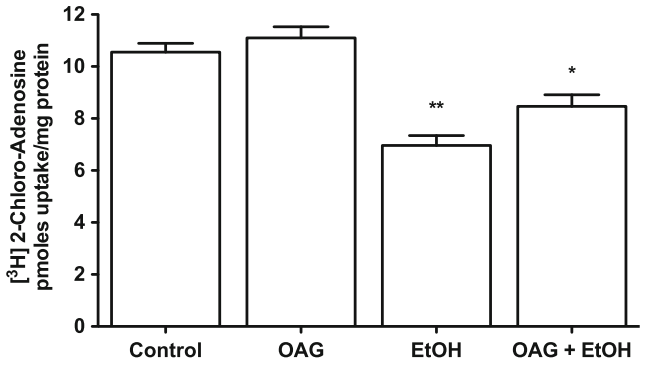

Fig. 3 PKC activation is correlated with decreased ethanol sensitivity of [^{3}H]-2-chloroadenosine uptake in HL-1 cells incubated with OAG (50 μM, 30 min), a general PKC activator, prior to ethanol treatment (200 mM; 10 min), and ethanol-only treated cells showed significant inhibition of [^{3}H]-2-chloroadenosine uptake relative to control untreated cells ($^{**}P<0.001$ EtOH and OAG+EtOH vs. CON). However, HL-1 cells incubated with OAG prior to ethanol treatment show a reduced inhibition of [^{3}H]-2-chloroadenosine uptake (i.e., less sensitivity to ethanol) compared to ethanol-treated cells ($^{*}P<0.05$ OAG+EtOH vs. EtOH). Data are presented as mean±SEM where $n=6$ for control and $n=3$ for other treatments

highly ethanol-sensitive mENT1 protein and thus enhanced inhibition of adenosine uptake.

Overall, the data suggest that PKC, and in particular PKCε, is a key element in determining ethanol sensitivity of ENT1.

Long-term exposure to low ethanol concentrations significantly inhibits adenosine uptake in HL-1 cells

Pharmacologically high ethanol concentrations partially inhibit [^{3}H] 2-chloroadenosine uptake in HL-1 cells (Fig. 1), but these levels are not physiologically significant when considering long-term moderate consumption of alcohol in humans. An ethanol concentration of 10 mM in the blood is a peak level that would be achieved by light to moderate alcohol intake, while a 25 mM blood ethanol concentration, which is just over the legal limit (17 mM), is routinely achieved in social settings.

Long-term exposure of HL-1 cells to low ethanol concentrations (10–50 mM; 24 h) resulted in a significant reduction in [^{3}H] 2-chloroadenosine uptake ($p<0.001$; Fig. 5) relative to control cells. Inhibition of [^{3}H] 2-chloroadenosine uptake in ethanol-treated cells was between 21 and 27 % ($p<0.001$; Fig. 5), suggesting that low concentrations of ethanol, which more closely mimic human intake, inhibit [^{3}H] 2-chloroadenosine uptake in HL-1 cells.

Long-term ethanol treatment leads to a decrease in NBTI binding in HL-1 cells

ENT1 can be regulated transcriptionally, for instance, by exposure to hypoxia such that there is less transporter at the membrane [24]. Since our data showed that longer incubation of HL-1 cells with low concentrations of ethanol (10–50 mM; 24 h) led to a decreased uptake of [^{3}H] 2-chloroadenosine (Fig. 5),

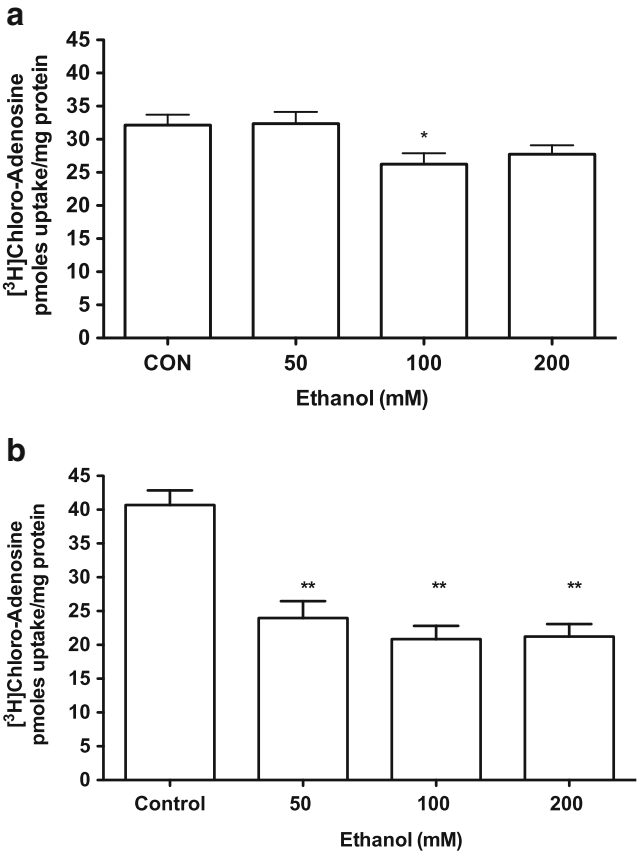

Fig. 4 Absence of PKCε is correlated with a highly ethanol-sensitive ENT1 in primary murine cardiomyocytes. **a** Adult primary PKCε WT cardiomyocytes were incubated with a range of ethanol concentrations (50–200 mM; 10 min). Cells treated with 100 mM ethanol showed inhibition of [3H]-2-chloroadenosine uptake compared to untreated cells ($^{*}P<0.05$, 100 mM ethanol vs. control and 50 mM ethanol). Pooled data are presented as mean±SEM, $n=3$. Each experiment was conducted in ≥quadruplicate. **b** Adult primary PKCε-null cardiomyocytes were incubated with a range of ethanol concentrations (50–200 mM; 10 min). PKCε-null cardiomyocytes showed significant inhibition of uptake compared to untreated control PKCε-null cells at various concentrations of ethanol, suggesting that [^{3}H]-2-chloroadenosine uptake is highly sensitive to inhibition by ethanol in PKCε-null cardiomyocytes [$^{**}P<0.001$ ethanol (50, 100, and 200 mM) vs. control]. Pooled data are presented as mean±SEM where $n=4$ for control and 200 mM ethanol-treated PKCε-null cardiomyocytes; $n=3$ for 50 and 100 mM ethanol-treated PKCε-null cardiomyocytes. Each experiment was conducted in ≥quadruplicate

we hypothesized that this might be due to the down-regulation of ENT1 at the membrane. NBTI binding assays confirmed that ethanol exposure (10 mM) led to a significant decrease in NBTI binding (B_{max} 0.32±0.01 pmol/mg protein; Fig. 6) relative to control cells (B_{max} 0.85±0.04 pmol/mg protein; Fig. 5). Ethanol-treated cells also had a higher affinity (K_d 0.14±0.02 nM) than control cells (K_d 1.3±0.19 nM) for NBTI. These data suggest that reduced ENT1 presence at the cell membrane is responsible for reduced [^{3}H] 2-chloroadenosine uptake and not the reduced affinity of ENT1. The reasons for the different affinity for NBTI binding following ethanol exposure are not clear. Previous studies

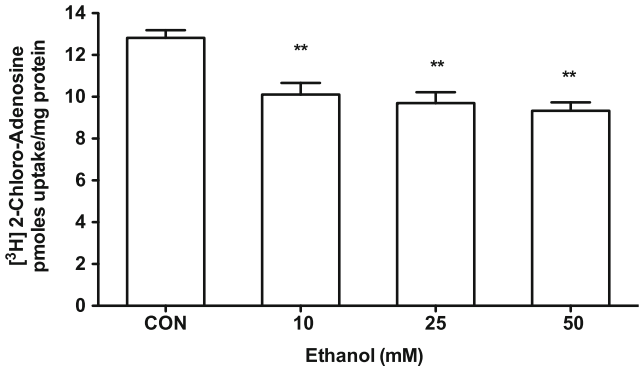

Fig. 5 Long-term exposure to low ethanol concentrations is correlated with reduced [^{3}H]-2-chloroadenosine uptake in HL-1 cells. HL-1 cells were incubated in serum-free media for 5 h prior to incubation in presence or absence of ethanol (10, 25, and 50 mM; 24 h). Increasing ethanol concentrations resulted in increasing inhibition of [^{3}H]-2-chloroadenosine uptake compared to untreated control cells, with maximal inhibition occurring at 50 mM [$^{**}P<0.001$ ethanol (10, 25, and 50 mM) vs. control]. Pooled data are presented as mean±SEM where $n=3$; each experiment was conducted in sextuplicate

[32, 33] have suggested the existence of two populations, low affinity and high affinity, of NBTI binding sites on cells, and exposure to ethanol may result in a shift from one population to another although more studies are required to identify the nature of these different populations.

Gemcitabine cytotoxicity is reduced in the presence of ethanol

We hypothesized that ethanol inhibition of ENT1-dependent nucleoside analog drug would compromise the cytotoxic effects of these drugs, which are routinely used to treat cancers. To test this, we conducted cytotoxicity assays using a human bladder cancer cell line HBT2 and a nucleoside analog drug, gemcitabine, which is a pyrimidine analog, in the presence of low, persistent levels of ethanol. Cells were treated with increasing concentrations of gemcitabine alone for 48 h (Fig. 7a) in order to calculate the EC_{50} value of gemcitabine for these cells. Using a concentration of gemcitabine (5 nM) equivalent to the EC_{50} for these cells, we found that cytotoxicity was indeed significantly reduced in the presence of even low levels of ethanol (Fig. 7b). Ethanol (2 mM) increases the viability of HTB2 cells treated with 5 nM gemcitabine.

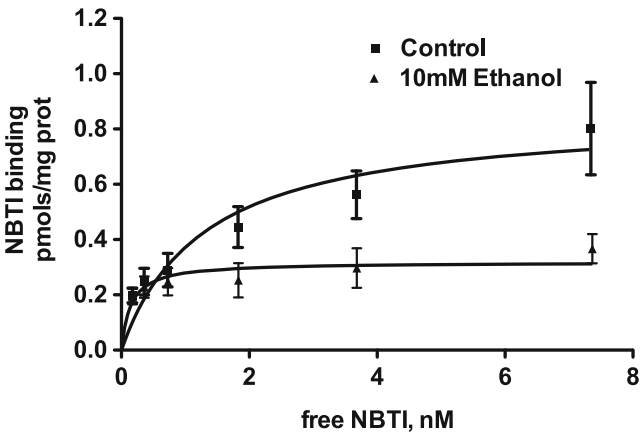

Fig. 6 Ethanol exposure is correlated with decreased NBTI binding in HL-1 cells. NBTI assay was conducted on HL-1 cells treated in presence or absence of ethanol (10 mM; 24 h). Pooled data are presented as mean bound [^{3}H] NBTI±SEM where $n=3$

Discussion

The cardiovascular purinome is a complex network of receptors, metabolic enzymes, channels, and transporters,

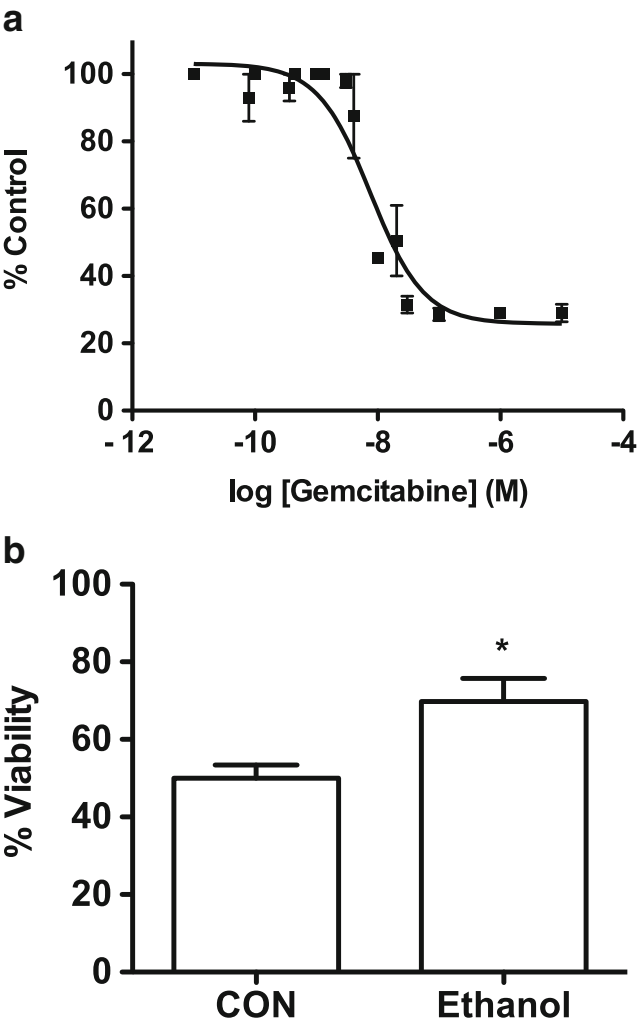

Fig. 7 Ethanol affects gemcitabine toxicity in HTB2 cells. **a** Gemcitabine toxicity in HTB2 cells. Cells were treated with increasing concentrations of gemcitabine for 48 h. Viability is plotted as percent control (untreated cells) against the logarithm of gemcitabine concentration. Pooled data are presented as mean±SEM, $n=3$. **b** HTB2 cells treated with 5 nM gemcitabine and ethanol (2 mM) show increased viability. Control—cells treated with 5 nM gemcitabine for 48 h; 2 mM ethanol—cells treated with 5 nM gemcitabine+2 mM ethanol for 48 h. The data are presented as mean±SEM, $n=16$ control, $n=7$ 2 mM ethanol. ($^*P<0.05$ 2 mM ethanol vs. control)

which coordinate the signaling and processing of a large class of purine-based compounds [25]. ENT1 is a critical member of the purinome and contributes to purinergic cardioprotection [1, 6, 7, 24, 26, 27]. Ethanol also has cardioprotective effects when consumed regularly at moderate levels (e.g., [28]), and there is clearly a link between ENT1 and the effects of ethanol in the nervous system [29]. In contrast, there have been no studies exploring the interaction of ethanol and ENT1 in the cardiovasculature. Here, we show that ENT1 can be partially inhibited by ethanol in cardiomyocytes and that this ethanol sensitivity is regulated by PKA and PKC. Moreover, long-term exposure to low levels of ethanol results in a decreased expression of ENT1 in cardiomyocytes. We propose that there is a direct partial inhibition of adenosine uptake via ENT1 and a long-term down-regulation of ENT1 expression resulting in less ENT1 protein at the plasma membrane both of which will contribute to purinergic cardioprotection since inhibition or absence of ENT1 has been demonstrated to result in a cardioprotective phenotype [4].

ENT1-dependent adenosine uptake is partially inhibited by ethanol in mouse lymphoma cells [14], human bronchial cells [11], rodent neural cells [15], and primary cultures of rat hepatocytes [9]. These data suggest that a similar mechanism of ethanol-mediated inhibition of ENT1 exists in multiple cell types. Ethanol-specific inhibition of ENT1 uptake (rather than ENT2) in cardiomyocytes is also consistent with previous studies showing that ENT2, which is expressed in much lower amounts than ENT1 in HL-1 cells [5], mouse, and human heart tissue [3, 30], is insensitive to ethanol [8]. Ethanol is thought to interact with ENT1 at a specific site in the protein as has been described for other ethanol-sensitive membrane proteins such as the *N*-methyl-D-aspartate receptors, gamma-amino butyric acid receptors, serotonin receptors, calcium- and voltage-activated potassium channels (BK_{Ca}), and ligand-gated ion channels [22, 31, 32]. Since very little is known about the structure of ENT1, we cannot identify the location of a putative ethanol binding pocket although the large intracellular loop between transmembrane domains 6 and 7 is the region of ENT1 with the least similarity to ENT2, and also the location of known phosphorylation targets [31], suggesting that this region of the protein may be the region with which ethanol interacts. Previous data [19] suggested that the adenosine transport system in neuronal cells is "primed" by phosphorylation of ENT1 before ethanol exposure, and this "priming" is critical in regulating the accessibility of the putative hydrophobic sites of ENT1 to ethanol by causing a conformational change in protein. Here, we show that the sensitivity of ENT1-dependent adenosine transport to inhibition by ethanol in cardiomyocytes is regulated by both PKA and PKC in an opposing manner, as previously described for neuronal cells [12, 13]. Additionally, our data support a role for a specific PKC isoform, PKCε, in regulation of ethanol sensitivity of ENT1 in cardiomyocytes. PKCε signaling is an important mediator of ethanol-dependent cardioprotection [23]. Downstream targets of PKCε include mitochondrial ATP-sensitive K^+ channels and, intriguingly, ENT1 [7].

Overall, acute ethanol exposure decreases ENT1-dependent adenosine uptake in cardiomyocytes most likely as a consequence of the binding of ethanol directly to pocket of ENT1 protein, an effect that can be modulated by PKA and PKC, and particularly PKCε. Long-term ethanol exposure decreases overall adenosine uptake in cardiomyocytes due to down-regulation of ENT1 protein at the cell membrane. Both mechanisms will lead to elevated extracellular adenosine levels, which will activate adenosinergic cytoprotective pathways. We believe this is therefore a previously undescribed molecular/cellular pathway, which contributes to the well-established cardioprotective effects of moderate ethanol consumption.

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