RESEARCH ARTICLE

Signaling pathway involved in the inhibitory effect of FTY720P on the Na^+/K^+ ATPase in HepG2 cells

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Received: 3 September 2016 / Accepted: 28 November 2016 / Published online: 14 February 2017
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Abstract The Na^+/K^+ ATPase modulates the activity of many transporters in the liver, and maintains the ionic constancy of the intracellular milieu, preserving thus normal functioning of hepatocytes. Previous work showed that FTY720P, a sphingosine one phosphate receptor agonist used in the treatment of multiple sclerosis, exerts in HepG2 cells, an inhibitory effect on the activity of the ATPase, mediated via PGE2. This study is an attempt to identify the signaling molecules involved downstream of the prostaglandin. The activity of the ATPase was assayed by measuring the amount of inorganic phosphate liberated in presence and absence of ouabain, a specific inhibitor of the enzyme. The effect of FTY720P and PGE2 disappeared completely in presence of PF-04418948, a blocker of EP2 receptors, RpcAMP, an inhibitor of PKA, PD98059, an inhibitor of ERK, as well as in presence of PTIO, a nitric oxide synthase inhibitor, but was mimicked by butaprost, an EP2 agonist, dbcAMP, a cell permeable cAMP analogue, and SNAP1, a nitric oxide generator. PGE2 and dbcAMP increased the expression of phosphorylated ERK but not total ERK. This increase did not appear however in presence of PTIO, indicating that PKA is upstream of NO. It was concluded that FTY 720P induces PGE2 release which activates NOS leading to NO production and ERK activation. ERK then inhibits directly or indirectly the Na^+/K^+ ATPase.

Keywords EP2 · ERK · HepG2 · Na^+/K^+ ATPase · NO · PKA

Introduction

The liver plays a major role in the body's metabolism. It is involved in the processing of absorbed nutrients, as well as in bile production and secretion, storage of glycogen, vitamins and minerals, synthesis of plasma proteins, removal of toxic substances, and many others.... The hepatocytes' functions are fulfilled and accomplished through the concerted action of various transporters like the Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchangers and $\text{Na}^+/\text{K}^+ - 2 \text{Cl}^-$ cotransport (Graf and Häussinger 1996), which rely directly or indirectly on the sodium gradient established by the Na^+/K^+ ATPase, known also as the Na^+/K^+ pump. The ATPase is located in the basolateral membrane (Sellinger et al. 1990) and maintains the ionic constancy of the intracellular milieu which is needed for proper cellular activities.

Sphingosine 1-phosphate (S1P) was shown previously to reduce the activity of the Na^+/K^+ ATPase in HepG2 cells (Dakroub and Kreydiyyeh 2012), a human liver cancer cell line, and a similar inhibitory effect was demonstrated for its analogue, FTY720P (Al Alam and Kreydiyyeh 2016), which has been approved lately for the treatment of multiple sclerosis because of its immunomodulatory properties. The drug is also gaining further attention as a potential anti-cancer agent (Zhang et al. 2013). With such a possible future expanded therapeutic use, elucidating its mode of action on hepatic Na^+/K^+ ATPase was deemed necessary since any alteration in the ATPase activity may result in impairment in the transport processes across hepatocytes, and consequently impairment in liver functions. Part of the mechanism of action of FTY720P on the Na^+/K^+ ATPase was revealed in a previous work (Al Alam and Kreydiyyeh 2016) which demonstrated that the drug exerts its effect via activation of S1PR2s which activate in turn Gq, PKC, NF-KB, leading to PGE2 release. The aim of this work is to elucidate further the signaling pathway by

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investigating, specifically, the downstream effectors of PGE₂. Knowing the different mediators involved in the action of FTY720P on the pump would help in circumventing any of its side effects that may intervene with proper functioning of hepatocytes.

Materials and methods

FTY720P, Glyco-SNAP1, Carboxy-PTIO, GAPDH antibody and secondary antibodies conjugated to horse radish peroxidase (HRP) were from Santa Cruz Biotechnology, CA, USA. Anti-ERK 1/2 rabbit polyclonal antibody and Anti-p-ERK 1/2 rabbit monoclonal antibody were respectively from Promega, WI, USA, and from Cell Signaling, MA, USA. Adenosine-3', 5'-cyclic Monophosphorothioate Rp-Isomer Triethylammonium salt (RpcAMP), and PD98059 were purchased from Calbiochem, San Diego, USA. Protease inhibitor cocktail tablets were from Boehringer Mannheim, Germany. Protein assay reagent and nitrocellulose membranes were from Biorad, California, USA. PF-04418948 was from Abcam, Cambridge, MA, USA. The human liver carcinoma cell line, HepG2, was

purchased from American Type Culture Collection (ATCC). All other chemicals and culture media used were from Sigma, Chemical Co, St Louis Missouri, USA.

Methods

Culture of HepG2 cells

HepG2 cells at passages 25–45 were grown in Dulbecco's Minimal Essential Medium (DMEM) with 4500 mg/L Glucose and pyridoxine HCl, supplemented with 1% penicillin (100 µg/ml) and streptomycin (100 µg/ml) and 20% FBS at a density of 2×10^6 , on 100 mm culture plates. The cells were kept in a humidified incubator (95% O₂, 5% CO₂) at 37 °C and treated at 85–90% confluence after an overnight serum starvation.

The concentration of the different inhibitors, activators, agonists and antagonist added to FTY 720P-treated cells, was selected based on values reported in the literature or on previous work conducted by the authors (Kreydiyyeh et al. 2007 for butaprost and RpcAMP; El-Zein et al. 2014 for PD98059).

Fig. 1 Determination of the type of EP receptors involved. At a confluence of 85–90% and after an overnight serum starvation, HepG2 cells were treated, 30 min before FTY 720P (7.5 nM, 15 min) or PGE₂ (1 nM, 15 min), with PF-04418948 (1 µM in DMSO), a specific EP₂ antagonist, or with butaprost (4 µM in DMSO; 15 min), an EP₂ agonist. The effect of FTY720P **a** and PGE₂ **b** on the Na⁺/K⁺ ATPase disappeared in cells pre-

treated with PF-04418948, and mimicked by butaprost **c**. Values are means $\pm$ SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p < 0.01$ as determined by a one-way Analysis of Variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test

Determination of the EP receptor involved

We demonstrated recently that in HepG2 cells, FTY720P acts on the Na^+/K^+ ATPase via PGE2 (Al Alam and Kreydiyyeh 2016) and in a previous work that exogenous PGE2 inhibits the ATPase by activating EP2 receptors (Dakroub and Kreydiyyeh 2012). Hence EP2 receptors were suspected to be the ones mediating the effect of FTY720P on the Na^+/K^+ ATPase. To test this hypothesis, HepG2 cells were treated, 30 min before FTY720P (7.5 nM, 15 min) or PGE2 (1 nM, 15 min), with PF-04418948 (1 μM in DMSO), a specific EP2 antagonist, or with butaprost (4 μM in DMSO; 15 min), an EP2 agonist, to see if they would block or mimic respectively, the effect of FTY720P or the prostaglandin.

Involvement of PKA

EP2 receptors couple to Gs proteins and increase the production of cAMP (Coleman et al. 1994) and consequently PKA activity. To investigate the involvement of PKA, HepG2 cells were treated 15 min prior to FTY720P or PGE2, with RpcAMP (30 μM) a PKA inhibitor, or with dbcAMP (10 μM), a PKA activator.

Involvement of ERK

Another known mediator of S1P signaling is ERK (Sato et al. 2012).

The involvement of ERK in FTY720P action was studied by assaying the activity of the Na^+/K^+ ATPase in HepG2 cells treated 15 min prior to FTY720P or PGE2, with PD98059 (50 μM) a MEK/ERK inhibitor. Changes in the protein expression of phospho-ERK were also studied by western blot analysis.

Involvement of NO

Cross talks between nitric oxide and COX enzymes, and nitric oxide and ERK have been reported in many works. To determine if NO is a mediator, the ATPase activity was assayed in cells treated with SNAP1 (2 μM , 15 min) a NOS activator, and in cells pre-treated (15 min prior to FTY720P or PGE2), with PTIO (30 μM), a NOS inhibitor.

The Na^+/K^+ ATPase activity assay

The activity of the Na^+/K^+ ATPase was assayed as described by Esmann (1988). Cell homogenates were diluted to a

Fig. 2 cAMP is involved in the effect of FTY720P and PGE2 on the activity of Na^+/K^+ ATPase. At a confluence of 85–90% and after an overnight serum starvation, HepG2 cells were treated 15 min prior to FTY720P (7.5 nM, 15 min) or PGE2 (1 nM, 15 min), with RpcAMP (30 μM , 15 min) a PKA inhibitor, or with dbcAMP (10 μM , 15 min), a PKA activator. The effect of FTY720P **a** and PGE2 **b** on the activity of

the Na^+/K^+ ATPase disappeared in cells pre-treated with RpcAMP and a similar inhibitory effect appeared in cells treated with dbcAMP. Values are means $\pm$ SEM of 4 observations. Bars not sharing the same letter are considered significantly different from each other at $p < 0.01$ as determined by a one-way Analysis of Variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test

concentration of 0.5 $\mu\text{g}/\mu\text{l}$ in histidine buffer (150 mM, pH 7.4) and incubated for 30 min at room temperature with saponin (1%) added at a ratio of 1:4(v/v). Aliquots were then drawn and incubated at 37 °C for an additional 30 min in histidine buffer containing NaCl (121.5 mM), KCl (19.6 mM), MgCl_2 (3.92 mM), adenosine tri-phosphate (2.94 mM), in presence or absence of ouabain (1.47 mM), a specific inhibitor of the ATPase. At the end of the incubation period, the reaction was stopped by addition of 50% trichloroacetic acid added at a ratio of 1:10 (v/v) and the samples were spun at 3000 g for 5 min. The amount of inorganic phosphate liberated in the supernatant was measured colorimetrically at 750 nm according to the method of Taussky and Shorr (1953).

Western blot analysis

Equal amounts of protein (40 μg) were resolved on 10% SDS polyacrylamide gels, and then transferred to nitrocellulose membranes which after blocking were incubated overnight at 4 °C with anti-p-ERK1/2, anti-ERK1/2, or anti-

GAPDH and then with secondary antibodies conjugated with horseradish peroxidase. The signal was detected by chemiluminescence.

Statistical analysis

Results are reported as means $\pm$ SEM and tested for statistical significance by a one-way Analysis of Variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test using Instat and Excel Analysis softwares.

Results

FTY720P acts via EP2 receptors

The inhibitory effect of FTY720P and PGE2 on the Na^+/K^+ ATPase disappeared completely in the presence of PF-04418948, a blocker of EP2 receptors, but was mimicked by butaprost, an EP2 agonist, indicating an involvement of EP2 receptors (Fig. 1).

Fig. 3 Nitric oxide mediates the inhibitory effect of FTY720P and PGE2 on the Na^+/K^+ ATPase activity. HepG2 cells were treated at a confluence of 85–90%, after an overnight serum starvation. The effect of FTY720P (7.5 nM, 15 min) **a** and PGE2 (1 nM, 15 min) on the Na^+/K^+ ATPase activity did not appear in cells pre-treated with PTIO (30 μM , 15 min), a NOS inhibitor. SNAP1 (2 μM , 15 min), a NOS activator, exerted a similar

inhibitor effect on the ATPase **c**. Values are means $\pm$ SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p < 0.01$ as determined by a one-way Analysis of Variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test

FTY720P activates PKA

FTY720P and PGE₂ did not exert any effect on the activity of the Na⁺/K⁺ ATPase in cells pre-treated with RpcAMP (Fig. 2a, b), a PKA inhibitor. An inhibitory effect was however observed when the cells were treated with dbcAMP, a cell permeable cAMP analogue (Fig. 2c), suggesting a role of cAMP as a second messenger and consequently an involvement of PKA.

NO is a mediator of FTY720P and PGE₂

In presence of PTIO, a nitric oxide synthase inhibitor, the effect of FTY720P (Fig. 3a) and PGE₂ (Fig. 3b) on the Na⁺/K⁺ ATPase disappeared completely, but was mimicked by SNAP1 (Fig. 3c), a nitric oxide generator, implying an involvement of NO, downstream of PGE₂, in the effect of FTY720P on the pump.

FTY720P activates ERK

Pre-treating the cells with PD98059, a MEK1/2 inhibitor, abolished the effect of FTY720P (Fig. 4a) and PGE₂ (Fig. 4b) on the Na⁺/K⁺ ATPase, suggesting that ERK is an intermediate signaling molecule that acts downstream of PGE₂. Since PGE₂ acts via EP2 receptors which activate PKA and lead to cAMP production, then ERK is expected to be activated by PGE₂ and dbcAMP. This hypothesis was confirmed by western blot analysis which showed an increase in phosphorylated ERK but not in total ERK (Fig. 5a, b). However this increase was not observed when the production of NO was blocked by PTIO (Fig. 5b), suggesting that NO acts upstream of ERK and downstream of PKA.

Discussion

PGE₂ acts in an autocrine/paracrine way and modulates cellular activities via its four G-protein coupled EP receptors namely EP1, EP2, EP3, and EP4 (Narumiya et al. 1999). We demonstrated previously an EP2 mediated PGE₂ inhibitory effect on the Na⁺/K⁺ ATPase in HepG2 cells (Kreydiyyeh et al. 2007). Since here the effect of FTY720P on the Na⁺/K⁺ pump was shown to be mediated via PGE₂, then EP2 receptors were suspected to be the ones involved in its action. As expected, butaprost, an EP2 receptor agonist, mimicked the effect FTY720P, while PF-04418948, an EP2 blocker abrogated it completely, confirming thus the involvement of EP2 receptors only. Had another type of receptors been involved, a partial effect of the drug would have still persisted in presence of the blocker PF-04418948. EP2 receptors are linked to Gs and activate adenylyl cyclase inducing thus an increase in cAMP which in turn activates protein kinase A (PKA). When PKA was inhibited with RpcAMP, the effect of FTY720P on the

ATPase as well as that of exogenous PGE₂ disappeared completely confirming further the involvement of EP2 receptors and PKA in the effect of FTY720P.

PTIO, a NOS inhibitor, abrogated the effect of FTY720P and PGE₂ on the Na⁺/K⁺ pump indicating that NO is an intermediate signaling molecule of PGE₂ that can alter the ATPase activity. Such an effect of NO has been observed before. Ellis et al. (2001) reported that the carbachol induced inhibition of the Na⁺/K⁺ pump in bovine ciliary processes was mimicked by SNAP and abolished by the NOS inhibitor, L-NAME. On the other hand, PGE₂ was shown to up-regulate the NOS enzyme through Gs coupled receptors. In microglial cells, COX inhibitors that blocked the production of endogenous prostanoids lead to a drop in iNOS expression (Minghetti et al. 1997) while in breast cancer cells, the COX-2 enzyme induced an up-regulation of iNOS, that was reduced by COX-2 inhibitors (Timoshenko et al. 2004).

Nitric oxide has been recognized also, as a mediator of S1P action in various cells. For instance, eNOS activation by TNF- α

Fig. 4 ERK is involved in the inhibitory effect of FTY720P and PGE₂ on the activity of the Na⁺/K⁺ ATPase. The effect of FTY720P (7.5 nM, 15 min) **a** and PGE₂ (1 nM, 15 min) on the activity of the Na⁺/K⁺ ATPase did not appear in HepG2 cells pre-treated with PD98059 (50 μ M, 15 min). Values are means $\pm$ SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $p < 0.01$ as determined by a one-way Analysis of Variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test

Fig. 5 PGE₂ (1 nM, 15 min) (a, c) and dbcAMP (10 μ M, 15 min) (b, d) increased the expression of phosphorylated ERK but not total ERK. The effect of dbcAMP did not appear in presence of PTIO (30 μ M, 15 min) (A, C). The blots are representative of an experiment repeated three times. The bands were quantified using imageJ software. Expression level of

phosphorylated ERK was normalized to GAPDH. Differences in the protein expression levels were tested for significant differences using one-way Analysis of Variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test. Bars not sharing a common letter are significantly different from each other at $P < 0.025$

in human endothelial cells was blocked by inhibitors of sphingomyelinase, sphingosine kinase, and blockers of S1P receptors (Mulders et al. 2006).

This work showed that ERK is another mediator of FTY720P's action on the pump. ERK modulation of the

ATPase activity has already been reported in the literature. In opossum kidney cells, the parathyroid hormone (PTH) was shown to inhibit the Na⁺/K⁺ ATPase through an activation of PKC that was mediated by ERK (Khundmiri et al. 2005). ERK was also shown to be a mediator of S1P signaling and

Fig. 6 The signaling pathway involved in the effect of FTY720P on the activity of the Na⁺/K⁺ ATPase

was reported to be involved in the SIP regulated aldosterone secretion by the zona glomerulosa cells of the adrenal glands (Brizuela et al. 2007).

Our findings indicate also that PGE2 and dbcAMP induce an up-regulation of Phospho-ERK, implicating a role of PKA in the activation of ERK. Since the effect of dbcAMP did not appear in presence of PTIO, it can be concluded that NO acts upstream of PKA. The results are in line with the reported direct phosphorylation of PKA by nNOS and eNOS through calcium-independent as well as calcium dependent pathways leading to an increase in their sensitivity to resting calcium/calmodulin levels (Butt et al. 2000; Hurt et al. 2012; Adak et al. 2001). PKA can also activate the iNOS isoform indirectly by a mechanism involving the activation of NF- κ B. (Shirakawa and Mizel 1989; Muroi and Suzuki 1993). Along the same line, Canals et al. (2003) demonstrated that nitric oxide donors activate ERK2 in primary midbrain cells, and cerebral Purkinje cells (Endo and Launey 2003). Lander et al. (1993) suggested that this could be achieved, through a nitric oxide activation of ras, a known upstream activator of ERK1/2 (Raman et al. 2007).

ERK is therefore the most downstream mediator in the studied signaling pathway. The kinase can phosphorylate directly the alpha subunit of the ATPase leading to a decrease in its activity and/or its endocytosis and subsequent degradation (Khundmiri et al. 2004). Figure 6 represents the signaling pathway activated by FTY720P and through which it inhibits the activity of the Na⁺/K⁺ ATPase.

Acknowledgements This work was supported by a grant from the University Research Board.

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