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Original Paper

FTY720P Upregulates the Na^+/K^+ ATPase in HepG2 Cells by Activating S1PR3 and Inducing PGE2 Release

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Key Words
FTY720-P • Na^+/K^+ATPase • HepG2 • PKC • ERK • PGE2

Abstract
Background/Aims: Liver regeneration is induced by S1P and accompanied with an increase in hepatic Na^+/K^+ ATPase activity, suggesting a potential modulatory role of the sphingolipid on the ATPase activity. The ability of S1P to alter the ATPase activity was confirmed in a previous work which showed a time dependent effect, with an inhibition appearing at 15min and a stimulation at two hours. The aim of this work was to investigate if FTY720-P, an analogue of S1P used in the treatment of multiple sclerosis, exerts a similar effect at 2 hours. ***Methods:*** HepG2 cells were treated with FTY720-P for two hours and the activity of the Na^+/K^+ ATPase was assayed by measuring the amount of inorganic phosphate liberated in presence and absence of ouabain. The involvement of NF-κB in the pathway was investigated by determining changes in the protein expression of IκB. ***Results:*** FTY720-P induced a 2.5-fold increase in the activity of the Na^+/K^+ ATPase which was maintained in the presence of JTE-013, a specific blocker of S1PR2, but disappeared completely in presence of CAY 10444, a specific S1PR3 antagonist. The involvement of S1PR3 was supported by the stimulation observed with Cym5541, a S1PR3 agonist. FTY720-P increased the expression of COX2, and reduced that of IκB. Its effect was not manifested in presence of indomethacin, a COX inhibitor, or in presence of an NF-κB inhibitor. Exogenous PGE2 induced a significant stimulatory effect. Inhibiting PKC and ERK with respectively calphostin C and PD98059 abolished the effect of FTY720-P on the ATPase and on IκB, but not that of exogenous PGE2 indicating that the two kinases are upstream of NF-κB and PGE2.The PKC activator PMA increased the activity of the Na^+/K^+ ATPase as well as the expression of phopho-ERK, inferring that PKC is upstream of ERK. ***Conclusion:*** It was concluded that FTY720-P stimulates the Na^+/K^+ ATPase via PGE2 by activating sequentially S1PR3, PKC, ERK, NF-κB. The latter enhances COX-2 expression leading to PGE2 release.

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Introduction

The liver plays an important role in protein synthesis, bile production and carbohydrates' metabolism. In addition, it maintains hormonal balance and eliminates toxic substances [1]. Several of these liver functions are dependent on the transcellular sodium gradient established by the Na^+/K^+ ATPase. Amino acids and bile acids enter hepatocytes via a sodium dependent transport process and gluconeogenesis, which may use amino acids as precursors, can be also considered as a sodium dependent metabolic activity [2]. The Na^+/K^+ pump or Na^+/K^+ ATPase uses the energy derived from the breakdown of one ATP molecule to transport 2 K^+ ions to the inside and 3Na^+ ions to the outside of the cell, generating thus a sodium and potassium electro-chemical gradient across the hepatocyte membrane. The hepatic ATPase plays an important role in liver physiology and pathology. Its activity is enhanced by glucagon, insulin, norepinephrine and vasopressin [3, 4], and its alpha subunit has been implicated in ROS generation and the progression of nonalcoholic fatty liver disease (NAFLD) [5]. A higher activity of the pump was noted to accompany liver regeneration [6], which is induced by Sphingosine 1-phosphate (S1P) [7]. Whether S1P is behind the enhanced activity of the ATPase is a question that was addressed in a previous work [8] that revealed a dual time dependent effect of S1P with an inhibition appearing at 15min and a stimulation appearing at 2hrs. Recently, FTY720, a sphingosine analog and immune-modulator, has been approved by the US Food and Drug Association as an oral treatment for relapsing forms of multiple sclerosis [9, 10]. *In vivo* FTY720 is rapidly phosphorylated by sphingosine kinase to FTY720-P which acts as an agonist at S1P receptors and activates downstream signaling pathways [10]. The side effects of the drug on hepatic Na^+/K^+ ATPase and consequently on some liver functions have not been studied. If FTY720P has a similar activity profile to that of S1P, then we would expect it to exert a dual and opposite time dependent effect on the ATPase leading to time dependent alterations in liver activities. We demonstrated previously in Caco-2 cells [11] and in HepG2 cells [12, 13], a significant FTY720P-induced inhibition of the Na^+/K^+ ATPase at 15min whether at two hours FTY720P exerts in HepG2 cells, like S1P, a stimulatory effect is a question that we aim to address in this work. This work is thus a follow up and a continuation of the previous one and is undertaken to complete the time course study. Determining the action and the signaling pathway of the drug at different time intervals is crucial to ensure patients' safety especially when the response varies with time and changes unexpectedly from inhibition to stimulation. The results would thus determine the still unknown effect of FTY720P on the ATPase at two hours and reveal if the drug exerts a dual and opposite time-dependent effect, an effect that has not been demonstrated before. Unraveling the time course of FTY720P would help in circumventing any of its anticipated undesirable effects by blocking the S1P receptors involved or by inhibiting any mediator in its signaling pathway.

Materials and Methods

Materials

Prostaglandin E2 (PGE2), ouabain, indomethacin, Dulbecco's Minimal Essential Medium (DMEM) with 4500mg/L Glucose and pyridoxine HCL, Trypsin-EDTA, Penicillin/Streptomycin, Fetal Bovin Serum (FBS), 10x Phosphate Buffered Saline (PBS) without magnesium and calcium, and Adenosine 5'-triphosphate disodium salt (ATP) were procured from Sigma, Chemical Co, St Louis Missouri, USA. The Human hepatocellular carcinoma cell line, HepG2, was purchased from ATCC. FTY720-P and anti-COX2 antibody were purchased from Santa Cruz Biotechnology, CA, USA. Anti-ERK 1/2 antibody was from Promega, WI, USA, while anti-p-ERK 1/2 antibody was from Cell Signaling, MA, USA.

Phorbol-12-myrsitate-13-acetate (PMA), Calphostin C, and PD98059 were obtained from Calbiochem, San Diego, USA. Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim, Germany.

Biorad protein assay reagent, nitrocellulose membranes, and ECL substrate (Clarity TM Western ECL Substrate) were obtained from Biorad, California, USA.

All other chemicals were purchased from Sigma, Chemical Co, St Louis Missouri, USA.

Culture of HepG2 cells

HepG2 cells at passages 28-35 were grown in DMEM supplemented with 1% penicillin (100µg/ml), streptomycin (100µg/ml) and 10% FBS, in 100 mm culture plates at a density of 120, 000 cells/ml. The cells were kept in humidified incubator (95% O2, 5% CO2) at 37°C and treated at 85-90% confluence after an overnight starvation.

Treatment of HepG2 cells with FTY720-P

HepG2 cells were treated for 2 hours with 7.5 nM FTY720-P, a concentration that showed in a previous work [11] a significant inhibitory effect at 15 min. An equal volume of the vehicle (DMSO) was added to the control.

Protein Extraction and Determination

At the end of the treatment period, the cells were washed with PBS buffer (pH=7.4), scraped in lysis buffer (150mM histidine buffer pH=7.4) to which protease inhibitors were added, homogenized, and spun for 30 min at 20000g and 4°C. Proteins in the supernatant were quantified colorimetrically at a wavelength of 595 nm using the Bradford protein assay.

Na^+/K^+ ATPase Activity

Cell homogenates were diluted to a protein concentration 0.5 µg/µl with histidine buffer (pH 7.4, 150mM) and incubated for 15 min at room temperature with 1% saponin added at a ratio of 1:4, in presence of phosphatase inhibitors (2.7 mM pyrophosphate, 2.7 mM glycerophosphate). Aliquots were then taken from each sample and incubated in histidine buffer containing NaCl (121.5mM), KCl (19.6 mM,), MgCl2 (3.92 mM), adenosine tri-phosphate (2.94 mM), in presence or absence of ouabain (1.47 mM), a specific inhibitor of the ATPase. When ouabain was absent, it was replaced with water. The reaction was stopped by addition of 50% trichloroacetic acid at a ratio of 1:10 (v/v) and the samples were spun at 3000g for 5 min. The amount of inorganic phosphate liberated in the supernatant was measured colorimetrically at 750 nM according to the method of Taussky H, Shorr [14].

S1P receptors mediating FTY720-P's effect

The type of S1P receptors (S1PRs) involved in the effect of FTY720-P (7.5 nM;2hrs) was investigated by pretreating HepG2 cells for 15min with JTE-013 (1 µM, DMSO) or CAY10444 (17.4 µM, DMF), respective blockers of S1P receptors 2 (S1PR2) and 3 (S1PR3). The effect of specific S1PR2 and S1PR3 agonists (CYM5520; 2.5 µM, DMSO, S1PR2 agonist; CYM5541; 2 µM, DMSO, S1PR3 agonist) applied for two hours was also investigated. The vehicles were always added in the same amount to the control.

Involvement of PGE2

HepG2 cells were treated for 30 min prior to FTY720-P with indomethacin, a COX inhibitor (100 µM, DMSO). The effect of different concentrations of exogenous PGE2 (0.1, 1, 10, 100, 200, 300nM, 2hrs) was also studied. In addition, changes in the protein expression of COX-2, the enzyme that catalyzes PGE2 synthesis, were examined by western blot analysis.

Involvement of PKC, ERK and NF-κB

The involvement of PKC in the signaling pathway was tested by addition of a PKC inhibitor, Calphostin C (50 nM, DMSO) 20 min before FTY720-P or by treating the cells with a PKC activator, PMA (100nM, DMSO) for 2 hours. The vehicle was added in the same amount to the control.

The involvement of ERK was studied by treating HepG2 cells with FTY720-P in presence of an ERK inhibitor PD98059 (50 µM, DMSO added 30 min prior to FTY720P). Activation of ERK was tested by investigating changes in the protein expression of phospho- ERK by western blot analysis.

Synthesis of PGE2 is catalyzed by the enzyme COX-2 whose expression is controlled by the transcription factor NF-κB. Implication of NF-κB was investigated by studying the effect of FTY720-P on the ATPase

activity when the transcription factor was inactivated with a specific inhibitor (15 μM, added 30 min before FTY720P). NF-κB is sequestered in the cytosol associated with IκB which keeps it inactive. Its translocation to the nucleus is dependent on IκB degradation [15]. Thus a decrease in the protein expression of IκB is an indicator of NF-κB activation. To confirm any role of NF-κB in the effect of FTY720-P, changes in the protein expression of IκB were studied by western blot analysis.

Locating the mediators with respect to each other along the signaling pathway

The location of PKC with respect to PGE2 was determined by treating HepG2 cells with PMA (100nM, DMSO), a PKC activator in presence of indomethacin (100 μM, DMSO), and with calphostin C (50 nM, DMSO), a PKC inhibitor, in presence of PGE2 (100 nM).

The position of ERK relative to PGE2 was studied by treating cells with PGE2 in presence of an inhibitor of ERK (PD98059). Western blot analysis was used also to study the effect of ERK inhibition on the protein expression of COX-2.

The position of ERK relative to PKC was determined by studying the ATPase activity in cells treated with PMA in presence of ERK inhibitor (PD98059). Changes in ERK phosphorylation were also determined in cells treated with FTY720-P when PKC was inhibited.

To position NF-κB relative to ERK, the protein expression of IκB was determined in cells treated with FTY720-P in presence of an inhibitor of ERK, while the position of NF-κB relative to PGE2 was studied by determining changes in the protein expression of COX-2 when NF-κB was inhibited.

Western Blot Analysis

Equal amounts of proteins (40 μg) were loaded and resolved on 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane which was then blocked and incubated overnight at 4°C with a primary anti-ERK1/2, anti-p-ERK1/2, anti-IκB, anti-COX-2, or anti-GAPDH antibody followed by an incubation with a secondary HRP conjugated antibody. The signal was detected by chemiluminescence using Clarity ECL Substrate and the intensity of the signal was detected using a ChemiDoc™ imaging system. GAPDH expression was used to check for equal loading. The bands were normalized to GAPDH using Image lab software.

Statistical Analysis

The data are reported as mean ± SEM and tested for statistical significance using a one-way analysis of variance followed by a Tukey-Kramer multiple comparison test using GraphPad InStat 3.

Results

FTY720-P increases the activity of hepatic Na^+/K^+ ATPase

HepG2 cells treated with FTY720-P (7.5 nM) for two hours showed a very significant (2.5 folds) increase in Na^+/K^+ ATPase activity (Fig. 1A).

FTY720-P exerts its stimulatory effect via S1PR3

To determine the type of receptors involved in FTY720-P's action, HepG2 cells were treated with FTY720-P in presence of blockers to S1PR2 and S1PR3. The stimulatory effect of FTY720-P persisted in the presence of JTE-013 (S1PR2 antagonist) but disappeared totally in presence of CAY1104 (S1PR3 antagonist). The S1PR3 agonist, Cym5541 exerted a significant stimulatory effect on the ATPase, while Cym5520, a S1PR2 agonist, had no effect, confirming thus the involvement of S1PR3 (Fig. 1B & C).

Determination of the mediators involved

Involvement of PKC. S1PR3 is coupled to Gq protein which is known to activate PKC [16]. Treating the cells with PMA, a PKC activator, induced a significant increase in the activity of the ATPase (Fig. 2B) and in presence of calphostin C, a PKC inhibitor, the effect of FTY720-P was completely abolished (Fig. 2A).

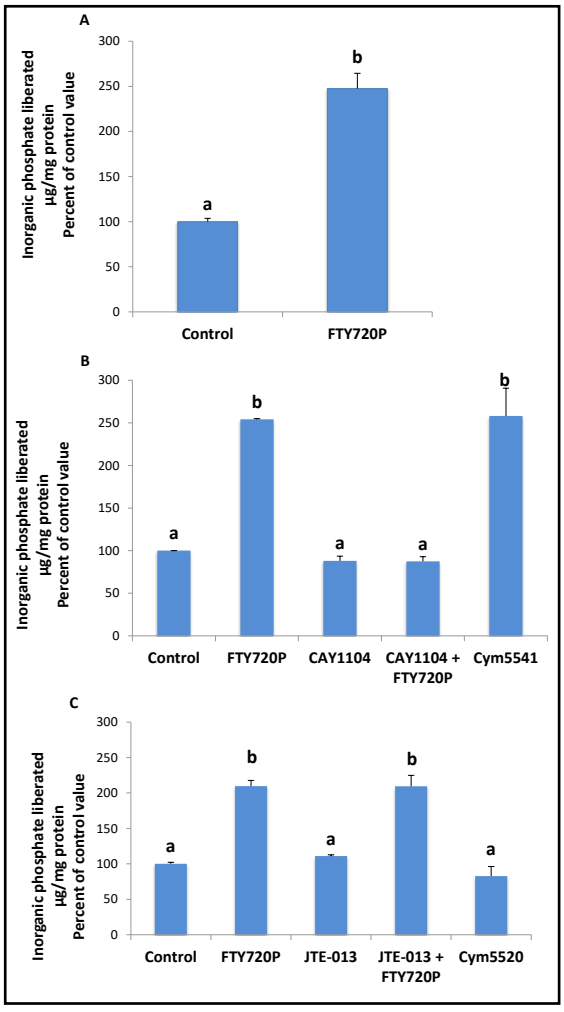

Fig. 1. FTY720-P increases the activity of the Na^+/K^+ ATPase via S1PR3. (A) Effect of FTY720-P (7.5nM. 2hours) on the activity of the hepatic Na^+/K^+ ATPase in presence of (B) a S1PR3 antagonist (CAY10444,17.4 µM, added 30min before FTY720-P) and agonist (CYM5541; 2 µM, 2hours), and (C) a S1PR2 antagonist (JTE-013; 1 µM, added 30min before FTY720-P) and agonist (CYM5520; 2.5 µM, 2hours) . Values are means ± SEM of 3 observations. Bars not sharing a common letter are significantly different from each other at p< 0.001.

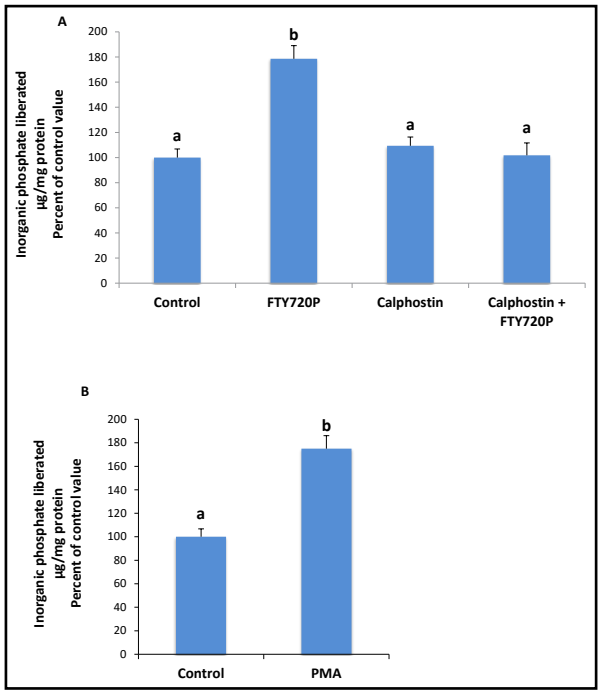

Fig. 2. FTY720-P activates PKC. (A) Cells were pre-treated 30 min before FTY720-P (7.5nM. 2hours) with the PKC inhibitor calphostin C (50 nM,). (B) Effect of PMA (100nM, 2hours), an activator of PKC on the activity of the Na^+/K^+ ATPase. Values are means ± SEM of at least 3 observations. Bars not sharing a common letter are considered significantly different from each other at p<0.01.

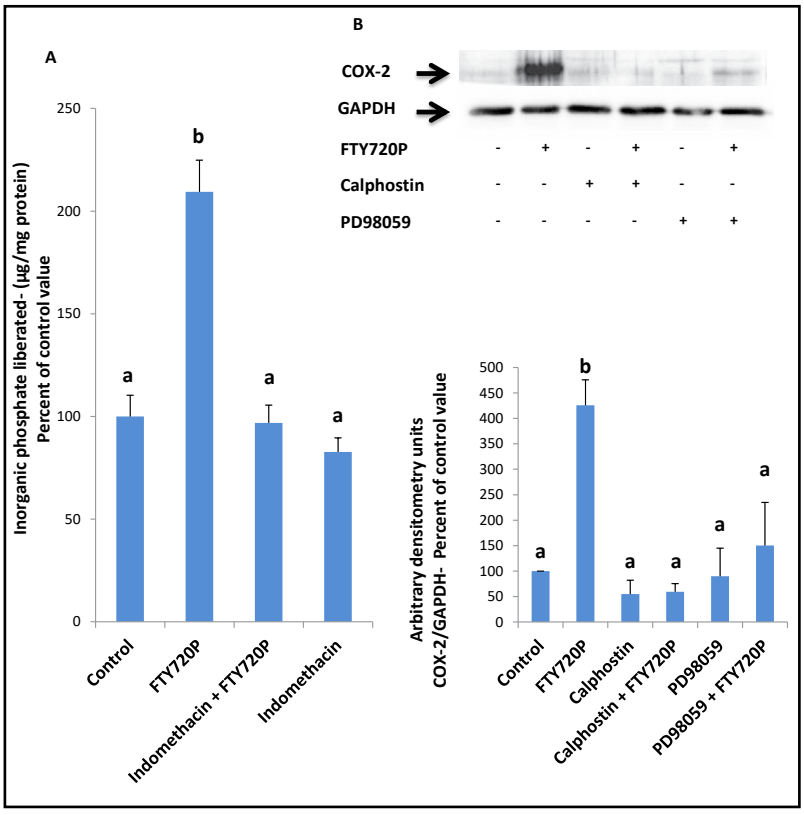

Fig. 3. FTY720-P activates COX-2. (A) HepG2 cells were treated, 30 min before FTY720-P (7.5nM. 2hours), with indomethacin, a COX inhibitor (100 µM). (B) Effect of FTY720-P (7.5nM. 2hours), on the protein expression of COX-2 in presence of a PKC inhibitor (calphostin C; 50 nM, 30min) and ERK inhibitor (PD98059; 50 µM, 30min) Values are normalized to GAPDH using Image lab software. The blot is representative of an experiment repeated 3 times. Values are means ± SEM of at least 3 observations. Bars not sharing a common letter are considered significantly different from each other at $p<0.01$.

FTY720-P acts via PGE2. FTY720-P increased the expression of COX-2 (Fig. 3B) and its stimulatory effect disappeared completely (Fig. 3A) in presence of indomethacin.

The effect of exogenous PGE2 was investigated to further confirm its role in FTY720-P's action. A dose response study (0.1 to 300 nM) revealed an inhibitory effect at low concentrations (0.1, 1 nM) and a stimulatory effect that started appearing at 10nM and reached a plateau at 100nM and beyond (Fig. 4A). Consequently, cells were treated with 100nM PGE2, a dose that stimulates the ATPase as FTY720-P did. At such a concentration a 2.5-fold increase in the activity of the ATPase was observed (Fig. 4B).

PKC acts upstream of PGE2. The stimulatory effect of PGE2 persisted in presence of Calphostin C (Fig. 5A), while that of PMA disappeared in presence of indomethacin (COX enzymes inhibitor) (Fig. 5B). Calphostin C and indomethacin alone had no effect on the ATPase. Calphostin C reduced the FTY720-P induced increase in the expression of COX-2 and brought it back to control levels (Fig. 3A). The results suggest that PKC is upstream PGE2.

FTY720-P acts via ERK which is downstream PKC. The effect of S1P was reported in many instances to be mediated through ERK [17-19]. On the other hand, ERK is a known modulator of the Na^+/K^+ ATPase [19, 20]. Hence ERK was suspected to be along the signaling pathway. Inhibiting ERK with PD98059, abolished completely the stimulatory effects of both FTY720-P (Fig. 6A), and PMA (Fig. 6B).

FTY720-P increased in addition the expression of phospho-ERK. This increase did not appear when PKC was inhibited with calphostin C (Fig. 6C).

ERK is upstream of PGE2. ERK inhibition did not alter the effect of PGE2 (Fig. 7), but abolished the FTY720P-induced increase in the expression of COX-2 (Fig. 3B), suggesting that ERK is upstream PGE2.

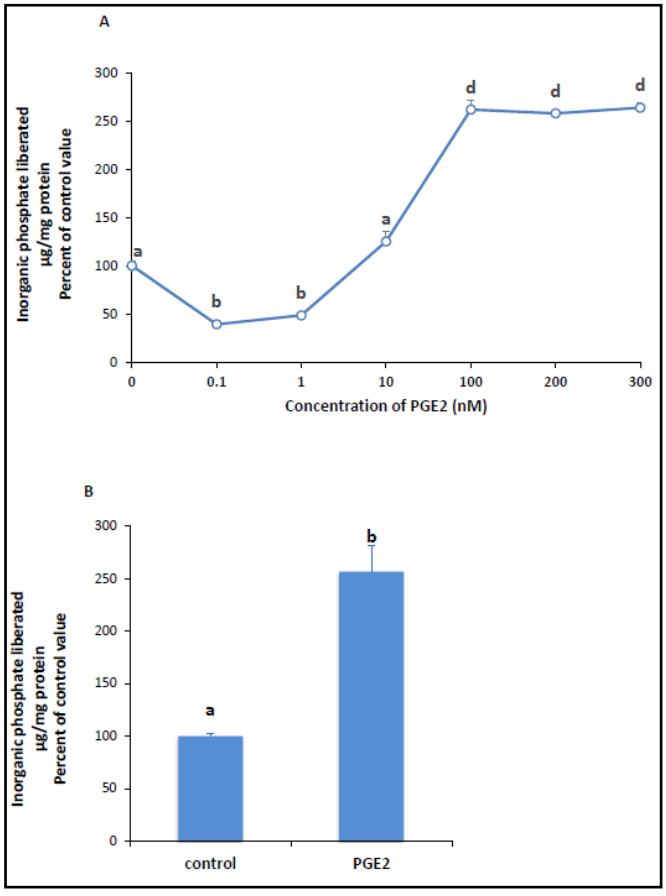

Fig. 4. PGE2 exerts a dose-dependent effect on the Na^+/K^+ ATPase activity. (A) Dose response study on the effect of exogenous PGE2 (0, 0.1, 1, 10,100,200, and 300 nM) added for 2 hours on the Na^+/K^+ ATPase activity. (B) Effect of exogenous PGE2 (100nM; 2hours) on the activity of the Na^+/K^+ ATPase. Values are means ± SEM of at least 3 observations. Bars not sharing a common letter are considered significantly different from each other at $p< 0.01$.

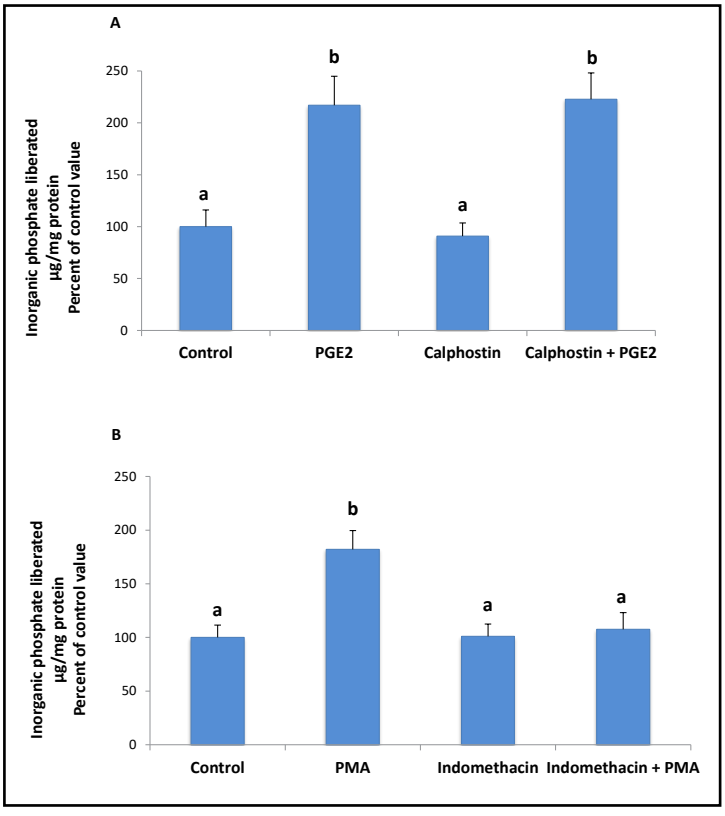

Fig. 5. PKC acts upstream COX-2. (A) Effect of PGE2 (100nM; 2hours) on the ATPase activity when cells were pre-treated for 30 min with calphostin C (50 nM). (B) Effect of PMA (100nM, 2hrs) on the activity of the Na^+/K^+ ATPase, in presence of Indomethacin (100 µM) added 30min before). Values are means ± SEM of at least 3 observations. Bars not sharing a common letter are considered significantly different from each other at: $p< 0.01$.

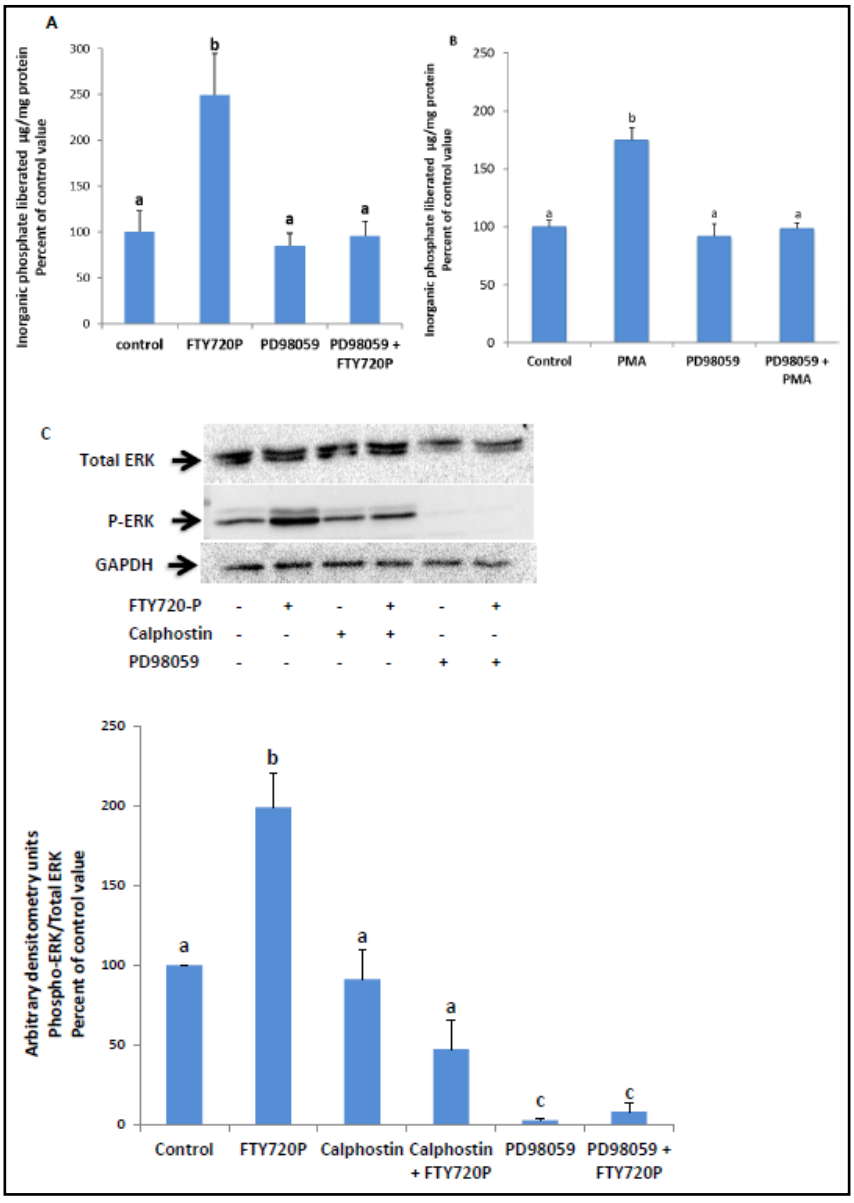

Fig. 6. FTY720-P and PKC activates ERK. Effect of (A) FTY720-P (7.5nM. 2hours) and (B) PMA (100nM, 2hours) on Na^+/K^+ ATPase activity in cells pre-treated for 30 min with the ERK inhibitor PD98059 (50 µM). (C) Effect of PKC and ERK inhibition on the Protein expression of p-ERK. Values are normalized to GAPDH using Image lab software. The blot is representative of an experiment repeated 3 times. Values are means ± SEM of at least 3 observations. Bars not sharing a common letter are considered significantly different from each other at $p<0.01$.

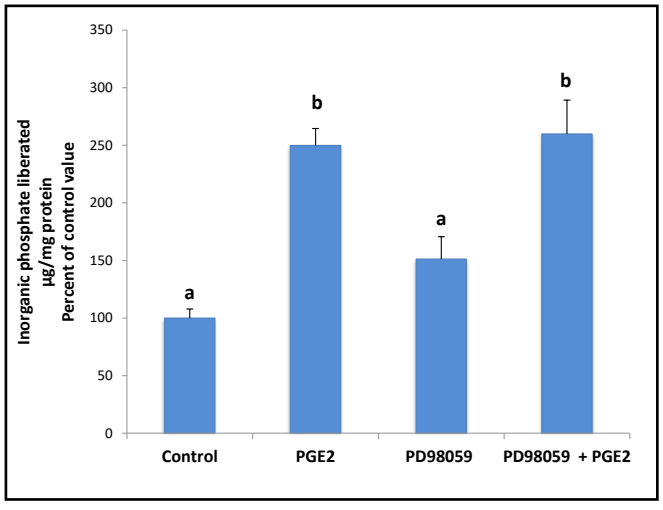

Fig. 7. ERK acts upstream PGE2. Effect of PGE2(100nM; 2hours) on the activity of the hepatic Na^+/K^+ ATPase in cells pre-treated for 30min with an ERK inhibitor (PD98059; 50 µM). Values are means ± SEM of at least 3 observations. Bars not sharing a common letter are considered significantly different from each other at $p< 0.01$.

NF-κB is involved and acts downstream PKC and ERK and upstream COX-2. In presence of an NF-κB inhibitor, the stimulatory effect of FTY720-P was not observed (Fig. 8A). Cells treated with FTY720-P had a lower expression of IκB (Fig. 8B) that was not manifested when PKC and ERK were inhibited respectively with PD98059 and calphostin C, (Fig. 9A), inferring that NF-κB acts downstream of PKC and ERK. Moreover, the FTY720P-induced increase in COX-2 expression was not observed any more when NF-κB was inhibited (Fig. 9B) indicating that NF-κB acts upstream COX-2.

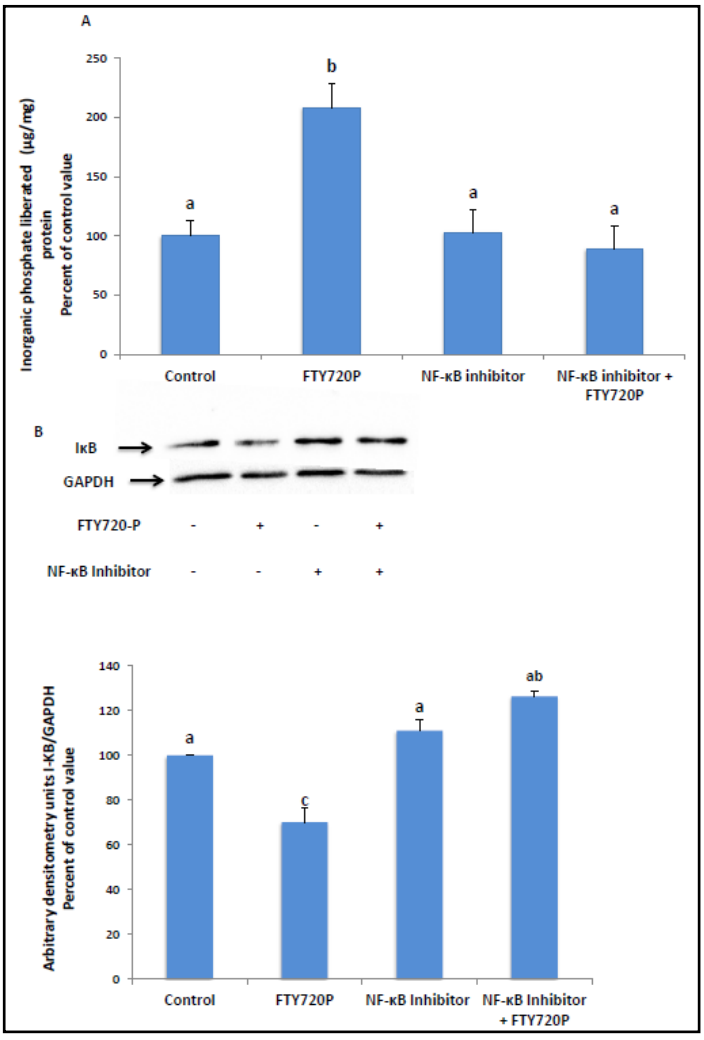

Fig. 8. FTY720-P acts through NF-κB. Effect of (A) NF-κB inhibition on the FTY720-P-induced stimulation of the ATPase. The inhibitor (15 µM) was added 30 min before FTY720-P (7.5nM. 2hours) (B) Effect of FTY720-P (7.5nM. 2hours) on the protein expression of IκB. Values are normalized to GAPDH using Image lab software. The blot is representative of an experiment repeated 3 times. Values are means ± SEM of at least 3 observations. Bars not sharing a common letter are considered significantly different from each other $p< 0.01$.

Discussion

Many hepatic cellular activities are dependent on the sodium gradient established by Na^+/K^+ ATPase. Accordingly, any alteration in the activity of the ATPase may result in impaired liver functions [21]. Previous studies in our lab demonstrated opposite and time-dependent effects of S1P on the Na^+/K^+ ATPase in HepG2 cells, with inhibition appearing at 15min and stimulation at 2hrs [8]. The S1P analogue, FTY720-P was shown to exert a similar effect on the pump at 15min at a concentration of 7.5nM [12]. Its effect at 2 hours however, was not investigated, and is the focus of the present study.

HepG2 cells treated with FTY720-P (7.5nM) for 2 hours showed a significant increase (2.5 folds) in Na^+/K^+ ATPase activity revealing a similar time dependent action profile to that of S1P. S1P acts through five different receptors that are all expressed in HepG2 cells [12]. Therefore, identifying the ones involved was our first concern. Blocking S1PR2s with JTE-013 did not alter the stimulatory effect of FTY720-P, and Cym5520, a S1PR2 specific agonist had no effect on the activity of the ATPase, implying that FTY720-P does not act through S1PR2s.

The involvement of S1PR3s was next investigated. The stimulatory effect of the drug was abolished completely in the presence of the antagonist CAY1104, and the S1PR3 specific agonist, Cym5541, exerted like FTY720P, a significant stimulatory effect. The results suggest

that FTY720-P acts mainly and solely via S1PR3s, since the activity of the pump went back to control values in presence of the antagonist. Had another type of receptor been activated, then a partial stimulation would have still been observed. Consequently, the involvement of other receptors was not pursued.

S1PR3s couple to Gq proteins which activate Protein Kinase C [16], a well-recognized modulator of Na^+/K^+ ATPase activity [22-26]. In this work, FTY720-P signaled via PKC since its effect disappeared when PKC was inhibited. Although S1PR3s may couple also to Gi and G13, the results suggest that Gq is the only G protein implicated in the action of FTY720-P since the activity was restored to control value in presence of a PKC inhibitor. The results are in line with the reported regulation of the ATPase by PKC which varied from stimulation to inhibition [12, 22, 25, 26].

The literature reports also a role for PGE_2 in the modulation of Na^+/K^+ ATPase activity [27-31]. Consequently, investigating the involvement of COX-2/PGE2 in the signaling cascade was deemed necessary. Exogenous PGE2 exerted a dose-dependent effect on the ATPase: an inhibitory effect appeared at low concentrations and a stimulatory one started at 10nM and reached a plateau at 100nM and beyond. PGE2 binds to four different types of EP receptors which have different affinities to the prostaglandin and activating each different signaling pathways [32].

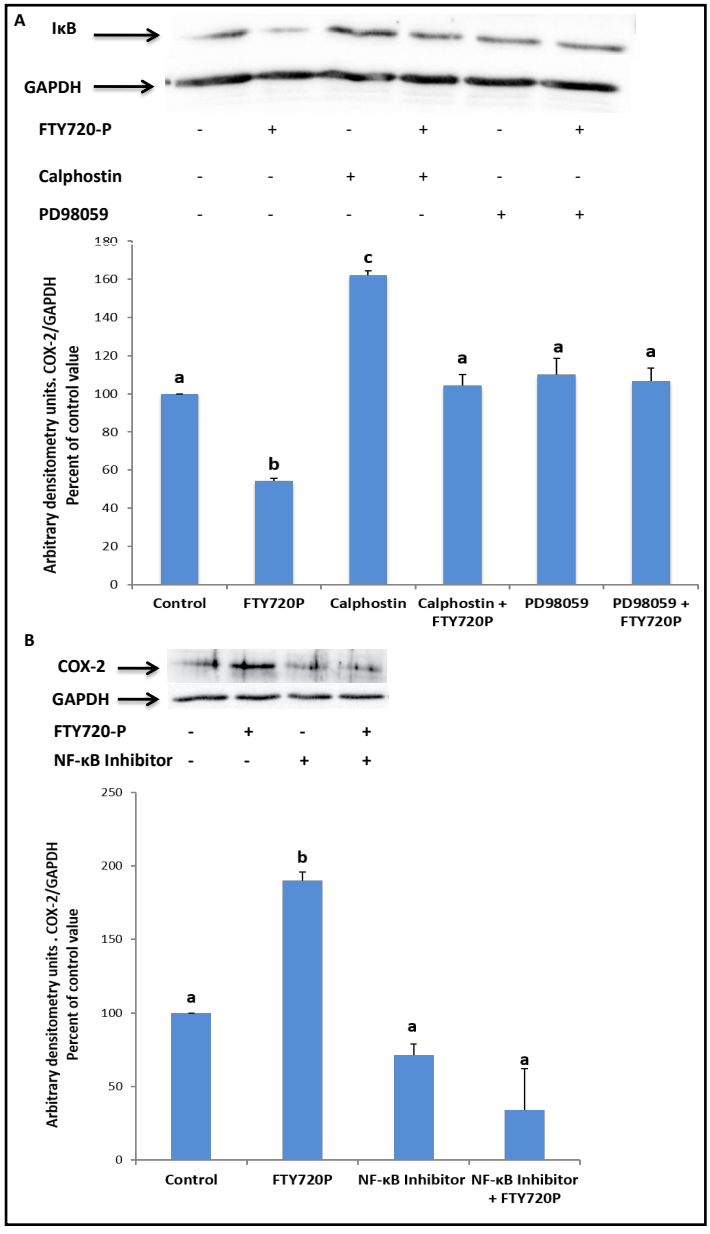

Fig. 9. NF-κB acts upstream COX-2 and downstream PKC & ERK. Effect of (A) FTY720-P (7.5nM. 2hours) on the protein expression of IκB in cells pre-treated for 30 min with either a PKC inhibitor (calphostin C; 50nM) or an ERK inhibitor (PD98059; 50 μM). (B) Effect FTY720-P (7.5nM. 2hours) on the protein expression of COX-2 enzyme in cells pre-treated for 30 min with an inhibitor of the NF-κB (15 μM). Values are normalized to GAPDH using Image lab software. The blot is representative of an experiment repeated 3 times. Values are means ± SEM of 3 observations. Bars not sharing a common letter are considered significantly different from each other at $p< 0.01$.

FTY720-P enhanced COX-2 expression and its effect was not manifested when PGE2 synthesis was blocked. The results infer that FTY720-P acts via PGE2 and induces the release of high levels of PGE2. Exogenous PGE2 at 100 nM exerted a significant stimulatory effect

that persisted in presence of calphostin C, while that of PMA disappeared in presence of indomethacin, indicating that PKC is upstream PGE2. In fact, FTY720-P did not increase the expression of COX-2 when PKC was inhibited, supporting thus the above conclusion. Activation of COX-2/PGE2 pathway by PKC has been reported in various tissues [33-35]. Although COX-2 possesses a PKC consensus sequence, the literature does not support its direct phosphorylation by the kinase [36] but rather an increase in its expression [37, 38].

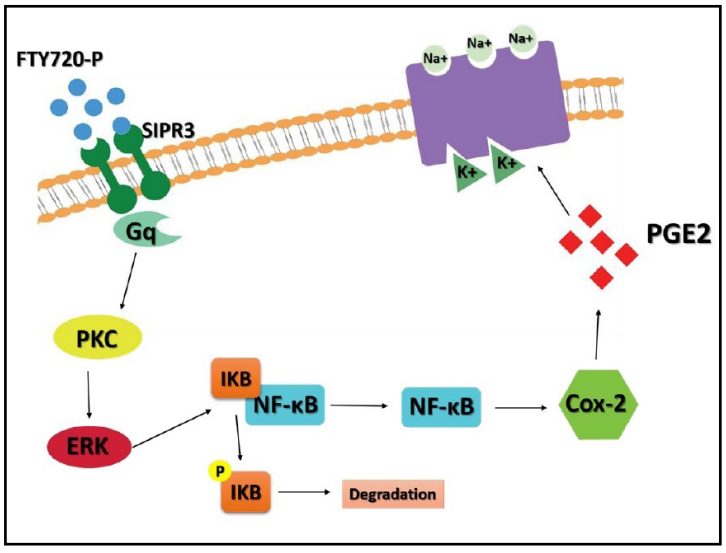

Fig. 10. Proposed signaling pathway.

The human COX-2 gene has two nuclear factor kappa B (NF-κB) sites [39], making NF-κB a potential transcriptional regulatory element that may be involved in the upregulation of COX-2 and PGE2 release. NF-κB is sequestered in the un-stimulated cell in the cytosol by IκB, an inhibitory protein to which it is complexed. In the stimulated cell, IκB is phosphorylated by IκB kinase leading to its ubiquination and degradation by the proteasome [15]. NF-κB then translocates to the nucleus and activates the transcription of specific genes. In fact, some studies reported phosphorylation and activation of IκB kinase by ERK [40]. On the other hand, PKC was reported to activate ERK via Raf [41] and to modulate Na^+/K^+ ATPase activity [42]. Whether in this work ERK is a direct or indirect regulator of the ATPase and is along the pathway activated by FTY720-P is a question that needed to be addressed. Inhibition of ERK with PD98059 abolished completely the stimulatory effect of FTY720-P and PMA, but not that of PGE2, implying that ERK is upstream of PGE2 and downstream of PKC. In further support of this implication, western blot analysis showed a higher FTY720-P induced expression of p-ERK that disappeared when PKC was inhibited.

Our results are in line with the reported involvement of ERK in PGE2 synthesis [43, 44], and as a mediator of PKC signaling [45-47]. It was concluded that FTY720-P binds to S1PR3, activates PKC which in turn activates ERK leading to PGE2 synthesis.

FTY720-P induced a decrease in the protein expression of IκB and consequently an activation of NF-κB. Inhibiting NF-κB abolished the stimulatory effect of FTY720-P on the ATPase confirming its position along the signaling pathway. FTY720-P did not reduce the expression of IκB when PKC and ERK were inhibited indicating that NFκB is downstream both kinases. Moreover, the FTY720-P-induced increase in COX-2 expression was not observed when NF-κB was inhibited revealing its involvement in COX-2 upregulation and PGE2 synthesis. Thus, the results indicate that NF-κB acts downstream of PKC and ERK and enhances COX-2 expression and PGE2 release.

Conclusion

It can be concluded that FTY720-P stimulates the Na^+/K^+ ATPase in HepG2 cells via S1PR3s which activate sequentially PKC, ERK, NF-κB, leading to an increase in COX-2 expression and PGE2 release.

The pathway involved is represented in Fig. 10.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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