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Phosphodiestrase-1 and 4 inhibitors ameliorate liver fibrosis in rats: Modulation of cAMP/CREB/TLR4 inflammatory and fibrogenic pathways

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ABSTRACT

Background: Phosphodiestrase (PDE) enzymes are suggested to play a leading role in fibrogenesis of liver where studies showed the possible implication of PDE 1 & 4 in liver injury proposing them as possible targets for treating liver fibrosis.

Aim: The present study was designed to investigate, for the first time, the possible therapeutic effects of selective inhibitors of PDE-1 (vinpocetine) and PDE-4 (roflumilast) in liver fibrosis induced by diethylnitrosamine (DEN) in rats.

Main methods: Rats were given DEN (100 mg/kg, i.p.) once weekly for 6 weeks to induce liver fibrosis. Vinpocetine (10 mg/kg/day) or roflumilast (0.5 mg/kg/day) was then orally administered for 2 weeks.

Key findings: Vinpocetine significantly suppressed the contents of hydroxyproline, transforming growth factor-beta 1 (TGF-β1), nuclear factor-kappa B (NF-κB) whereas roflumilast normalized them. Moreover, tumor necrosis factor-alpha (TNF-α) content and protein expressions of toll-like receptor 4 (TLR4) and tissue inhibitor of metalloproteinase-1 (TIMP-1) were markedly decreased whereas cAMP response element binding (CREB) protein expression was significantly elevated by both treatments. Additionally, vinpocetine and roflumilast up-regulated the gene expression of bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI) receptor where roflumilast showed better results. PDE1 and 4 activities were inhibited by vinpocetine and roflumilast, respectively. The superior results offered by roflumilast could be related to the higher cAMP level obtained relative to vinpocetine.

Significance: Our study manifested the up-regulation of PDE enzymes (1 & 4) in liver fibrosis and addressed the therapeutic role of vinpocetine and roflumilast as PDEIs through a cAMP-mediated TLR4 inflammatory and fibrogenic signaling pathways.

1. Introduction

Liver fibrosis is one of the most pressing health challenge worldwide especially in Egypt where it arises from the exposure to variable injuries such as viral hepatitis type B & C, metabolic, autoimmune and drug-induced liver diseases [1]. Hepatic fibrosis, as being a reversible process, should be treated as soon as possible before it ends with a serious complication such as liver cirrhosis, hepatocellular carcinoma or liver failure [2]. Owing to the growing steps in understanding its pathogenic mechanisms and the realization of the importance of approving an effective anti-fibrogenic therapy, a group of agents are now being tested for their potential benefit in hindering liver fibrosis [3].

Diethylnitrosamine (DEN) is one of the models of hepatic fibrosis that depends on generation of nitrosamine which, upon absorption, enters the liver and interferes with its detoxification system proceeding to cirrhosis and cancer [4]. It is an accepted and pragmatic model due to the common presence of nitrosamine in tobacco smoke, polluted water and wide variety of products that contain nitrites as a preservative [5,6]. DEN is reported to generate oxidative stress that could induce toll-like receptor 4 (TLR4) signaling which contributes to the development of hepatic inflammation as evaluated, for example by nuclear factor-kappa B (NF-κB) and tumor necrosis factor-alpha (TNF-α) that proceeds to hepatic fibrosis [7–9]. These stages of DEN-induced hepatic inflammation and fibrosis generates a positive loop that could finally end by hepatocellular carcinoma (HCC) [9]. Hence, this experimental model of liver fibrosis was chosen to conduct our investigation.

The implication of the second messenger, cyclic adenosine

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monophosphate (cAMP) in liver fibrosis has been previously identified [10,11]. The elevation of cAMP level was shown to decrease fibroblast proliferation and impede extracellular matrix (ECM) protein synthesis [12]. Moreover, the cellular levels of cAMP are much regulated by the hydrolyzing power of phosphodiestrases (PDEs) whose activities exceed the rate of synthesis by adenylyl cyclase (AC) [13]. Therefore, examining the effect of certain PDE inhibitors in liver fibrosis seemed appealing especially that limited data studying the role of PDEs in the pathogenesis of liver fibrosis are available.

Research conducted on PDE inhibitors proved their beneficial effect in experimental liver injuries [14–16]. Among these inhibitors are vinpocetine which is PDE1 inhibitor and roflumilast, a PDE4 inhibitor. Miller et al. [17] studied the effect of PDE1 inhibition in cardiac fibroblast where their results showed significant amelioration of transforming growth factor-beta 1 (TGF-β1)-induced myofibroblast activation and ECM synthesis in rat cardiac fibroblasts. Similarly, the investigation done by Gobejishvili et al. [18] assigned the regression of hepatic fibrosis to inhibition of PDE4 which prevented the elevation of the key fibrogenic marker, TGF-β1 and the expression of alpha-smooth muscle actin (α-SMA). Accordingly, exploring the potential antifibrogenic properties of vinpocetine and roflumilast in the DEN model of hepatic fibrosis would be of value due to the lack of clear evidence supporting the role of PDE1 and 4 in the pathogenesis of liver fibrosis. Furthermore, such studies would be of great benefit in the search for new antifibrogenic agents.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 200–250 g were obtained from the Modern Veterinary Office for Laboratory Animals, Giza, Egypt. The animals were housed in plastic cages and left to acclimatize for one week at the animal facility of Faculty of Pharmacy, Cairo University (Egypt). Rats were kept under constant temperature (23 ± 2 °C) and a 12-hour light/dark cycle as well as constant relative humidity throughout the experimental period. All animals were allowed free access to standard diet and water *ad libitum* during the investigation period. The experiment complies with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011) and was approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University [Permit Number: PT 1390].

2.2. Chemicals

DEN and roflumilast were purchased from Sigma-Aldrich (St. Louis, MO, USA) while vinpocetine was obtained from Amriya Pharm Industries (Alexandria, Egypt). DEN was prepared in saline and given as intraperitoneal (i.p.) injection using needle size of 23-gauge. However, vinpocetine and roflumilast were prepared in 1% carboxymethylcellulose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and orally administered using a stainless steel curved gavage-feeding needle with a round tip (16-gauge; tip diameter, 3 mm; length, 75 mm). Other chemicals unless specified were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Experimental design

Animals were allocated into six groups, 9 rats per group. The first group served as normal group receiving saline by i.p. injection, once/week for 6 weeks followed by daily oral administration of 1% carboxymethylcellulose for 2 weeks. The 2nd and 3rd groups served as vinpocetine and roflumilast controls, respectively where animals received i.p. saline injection once/week for 6 weeks followed by oral administration of drugs at a dose of 10 mg/kg/day [19] and 0.5 mg/kg/day [20], respectively at a dose volume of 10 ml/kg for 2 weeks. Animals of 4th group were given DEN (100 mg/kg, i.p.) [21] at a dose volume of 2.5 ml/kg once weekly for 6 weeks followed by daily oral administration of 1% carboxymethylcellulose for 2 weeks. Rats of 5th and 6th groups received DEN in same regimen as group 4 followed by vinpocetine and roflumilast, respectively starting from the 6th to 8th weeks as mentioned previously. At the end of experimental period, rats were anesthetized using thiopental (50 mg/kg, i.p.) [22] and the portal vein was exposed to monitor portal pressure (Power laboratory, AD Instruments, Australia) [23]. Blood samples were then collected in heparinized tubes from the retro-orbital sinus and animals were sacrificed by cervical dislocation under anesthesia. Livers were then rapidly excised, weighed and washed with saline. Parts of the left lobe of liver from each group were used for histological and immunohistochemical examinations whereas parts from the right lobe of liver were collected for biochemical analysis.

2.4. Histopathological examination & area of fibrosis

Liver samples were fixed in 10% formalin and processed for paraffin embedding. Sections of 5 μm were prepared and stained with H&E (magnification ×100) for examination of fibrogenic changes and scored according to Metavir scoring system [24]. Moreover, sections were stained with Masson's Trichrome (Sigma-Aldrich, St. Louis, MO, USA) (magnification ×100) to identify collagen fibers in liver tissues. The percentage of fibrosis was evaluated semiquantitatively using an image analyzer (Leica Qwin 550, Germany) and presented as mean of 10 randomly selected fields from each section (5 sections for each group) [25]. All the slides were examined under a light microscope.

2.5. α-SMA immunohistochemistry

Tissue sections of 5 μm thickness were deparaffinized with xylene then hydrated in graded ethanol solution and heated in citrate buffer (pH 6.0) for 5 min. Then, the sections were blocked using 5% bovine serum albumin (BSA) in tris buffered saline (TBS) for 2 h. Incubation of the slides was done overnight at 4 °C with anti-α-SMA (SC-32251, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody. After that, the slides were rinsed with TBS and incubated for 10 min in a solution of 0.02% diaminobenzidine containing 0.01% H_2O_2. The slides were then counterstained with hematoxylin, mounted and examined. The wall of blood vessels acts as internal positive control [26]. The positivity of α-SMA appeared as brown cytoplasmic staining and the immunoreactive area percentage in individual sections (magnification ×400) was measured using an image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA).

2.6. Biochemical measurements

2.6.1. Determination of plasma liver function tests

Plasma prepared was used for measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities using commercially available kit (Biodiagnostics, Giza, Egypt) according to manufacturer's instructions and results were expressed as U/l.

2.6.2. Enzyme-linked immunosorbent assays (ELISA) of hydroxyproline, cAMP, TGF-β1, NF-κB and TNF-α

Parts of liver samples were homogenized in ice cold saline to prepare 10% homogenates and were used to determine hydroxyproline, cAMP and TGF-β1 using the corresponding rat ELISA kits (LifeSpan BioSciences, Washington, USA; Cat. # LS-F25018-1; LS-F27893-1; LS-F24972-1, respectively). Likewise, NF-κB and TNF-α contents were quantified in liver homogenates with rat specific ELISA kits purchased from MyBioSource, San Diego, CA, USA (Cat. # MBS764450) and R&D Systems Inc., Minneapolis, USA (Cat. # RTA00), respectively. Procedures were performed according to manufacturer's instructions

and results were expressed as ng/mg protein for hydroxyproline and NF-κB and as pg/mg protein for the other mentioned parameters. Protein content was measured according to the method of Lowry et al. [27].

2.6.3. Western blot analysis of TLR-4, TIMP-1, CREB

Liver portions were homogenized in radioimmunoprecipitation assay (RIPA) buffer and protein contents were quantified using a protein assay kit (Thermo Fisher Scientific Inc., USA). The expressions of the mentioned parameters were assessed as previously described [28] using primary antibodies against TLR-4, TIMP-1 and CREB (Thermo Fisher Scientific Inc., USA) in addition to β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The amount of protein was quantified by densitometric analysis of the autoradiograms using a scanning laser densitometer (Biomed Instrument Inc., USA). Results were expressed as arbitrary units after normalization for β-actin protein expression.

2.6.4. Quantitative RT-PCR analysis of high mobility group box-1 (HMGB-1), PDE 1 & 4 isoforms and bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI) receptor

2.6.4.1. RNA extraction. Total RNA was purified from liver tissues using RNeasy Kit (Qiagen, Valencia, CA) according to manufacturer's instruction. RNA concentration and purity were determined spectrophotometrically at OD 260/280 nm and its integrity was assessed by gel electrophoresis on 1% agarose gel (Invitrogen Co. USA) stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA).

2.6.4.2. Quantitative real-time PCR technique. In brief, first-strand cDNA synthesis was performed with the SuperScript Choice System (Life Technologies, Breda, Netherlands) according to the manufacturer's protocol. For quantitative real-time PCR, 5 µl of first-strand cDNA was used in a total volume of 25 µl, containing 12.5 µl 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 ng of each primer. The sequence of the primers used was listed in Table 1. PCR reactions consist of 95 °C for 10 min (1 cycle), 94 °C for 15 s and 60 °C for 1 min (40 cycles). These reactions were performed on an ABI Prism 7900 HT Fast Real Time PCR system (Applied Biosystems). The mRNA levels were calculated based on the method of $2^{-\Delta\Delta CT}$ formula after normalization to glyceraldehyde 3-phosphate dehydrogenase (GADPH) [29].

Table 2
Effect of vinpocetine and roflumilast on DEN-induced changes of liver enzymes and liver index in rats.

Groups	ALT (U/l)	AST (U/l)	Liver index (%)
Normal	32.14 ± 3.76	79.92 ± 5.54	3.14 ± 0.19
Vin	33.03 ± 3.29	85.58 ± 3.77	3.23 ± 0.16
Rof	32.90 ± 3.53	83.86 ± 4.11	3.35 ± 0.17
DEN	75.10 ± 2.62*	159.30 ± 4.12*	4.42 ± 0.06*
Vin + DEN	55.01 ± 1.40*#	125.50 ± 2.76*#	3.67 ± 0.10#
Rof + DEN	53.46 ± 3.36*#	127.30 ± 3.72*#	3.41 ± 0.10#

Each value represents the mean of 5 experiments ± S.E.M. Statistical analysis was done using One way ANOVA followed by Tukey's post-hoc test. *$p < 0.05$ *vs.* normal, #$p < 0.05$ *vs.* DEN. Vin: vinpocetine, Rof: roflumilast, DEN: diethylnitrosamine, ALT: alanine aminotransferase, AST: aspartate aminotransferase.

2.6.5. Estimation of PDE1A and 4A activities

Based on the expression of different isoforms of PDE 1 and 4, PDE1A and 4A displayed the highest expression in DEN model when compared to the other isoforms. Hence, in the present work, PDEs specific enzymatic activities were determined using PDE1A and PDE4A assay kits (BPS Bioscience Inc., San Diego, USA, Cat. # 60310; 60340, respectively). Procedures were performed according to manufacturer's instructions and results were expressed as amount of cAMP hydrolyzed/min/mg protein.

2.7. Statistical analysis

Data were expressed as means ± standard error (SEM). Analysis of the results was done using one-way-analysis of variance test (ANOVA) followed by Tukey Kramer post-hoc multiple comparison's test except gene expression of different PDEs isoforms which was done using unpaired Student's *t*-test. For all statistical tests, the level of significance was set at $p < 0.05$ except gene expression of different PDEs isoforms which was estimated at different levels of significance. GraphPadPrism® software package, version 5 (GraphPad Software, Inc., USA) was used to carry out all statistical tests.

Table 1
The primer sequence of the HMGB-1, PDE1 (A, B, C) isoforms, PDE4 (A, B, C, D) isoforms, BAMBI receptor and GADPH genes.

Parameter	Primer sequence	Gene bank accession number
HMGB-1	Forward primer: 5′TCAATTCTGTCACACCATGGGA3′ Reverse primer: 5′AAGCTCACGCTTTTGGGGAT3′	NM_012963.2
PDE-1A	Forward primer: 5′ACCATGATTGGGTTCCATGTT3′ Reverse primer: 5′CAGCCAACTCTTTCCACCT3′	NM_030871
PDE-1B	Forward primer: 5′TCCACATCCAGACCAAGTCA3′ Reverse primer: 5′GCAGGACATGTCTGTGGCT3′	NM_022710
PDE-1C	Forward primer: 5′CAGCCTACCGTCTTCTCCA3′ Reverse primer: 5′TTCAATTGCTTCTGGTTGCTG3′	NM_031078
PDE-4A	Forward primer: 5′GAAGACAACCGGGACTGGT3′ Reverse primer: 5′CCTCAGTGGTAGGCAATCC3′	NM_013101
PDE-4B	Forward primer: 5′CCTCCGACACCTTCGTAAC3′ Reverse primer: 5′CCAGGTCTGTGAAGACAGC3′	NM_017031
PDE-4C	Forward primer: 5′GAAGGGCACTACCACTCCA3′ Reverse primer: 5′GTGTATAGCGCACGCAAAGA3′	XM_001070301
PDE-4D	Forward primer: 5′TGGGCAGACCTCGTACATC3′ Reverse primer: 5′CAGTGTCTGACTCGCCATC3′	NM_017032
BAMBI receptor	Forward primer: 5′CCATGCCCACTTTGGAATGC3′ Reverse primer: 5′TTCTGCTGCTGTCATGCTGG3′	NM_139082.3
GADPH	Forward primer:5′-TCCCTCAAGATTGTCAGCAATG-3′ Reverse primer:5′-AGATCCACAACGGATACATTGG-3′	NM_017008.3

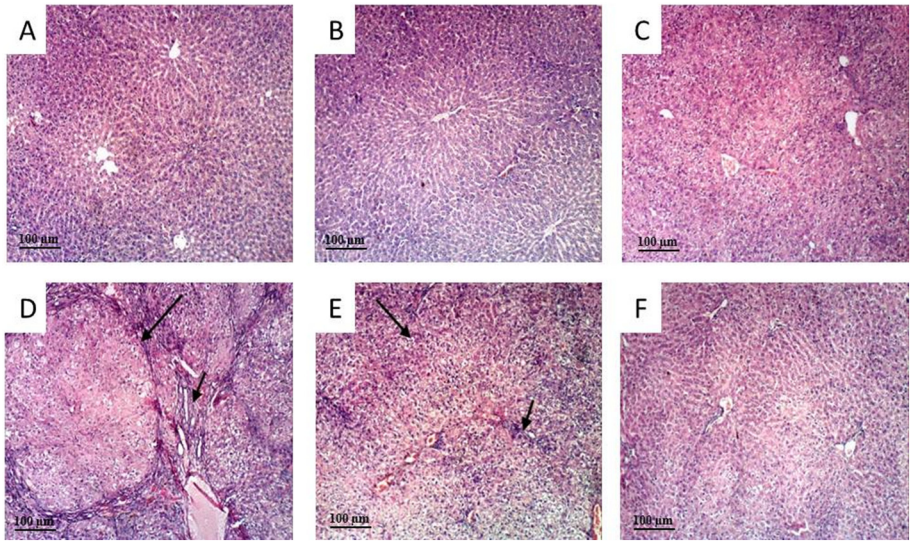

Fig. 1. Effect of vinpocetine and roflumilast on DEN-induced histological changes in liver tissues in rats (magnification ×100). (A–G) H & E staining. (A) Normal group (B) Vin group (C) Rof group (D) DEN group; short arrow: portal fibrosis, long arrow: nodules formation (E) Vin + DEN group; short arrow: fine fibroblasts in the portal triad, long arrow: degeneration of hepatocytes (F) Rof + DEN group. Vin: vinpocetine, Rof: roflumilast, DEN: diethylnitrosamine.

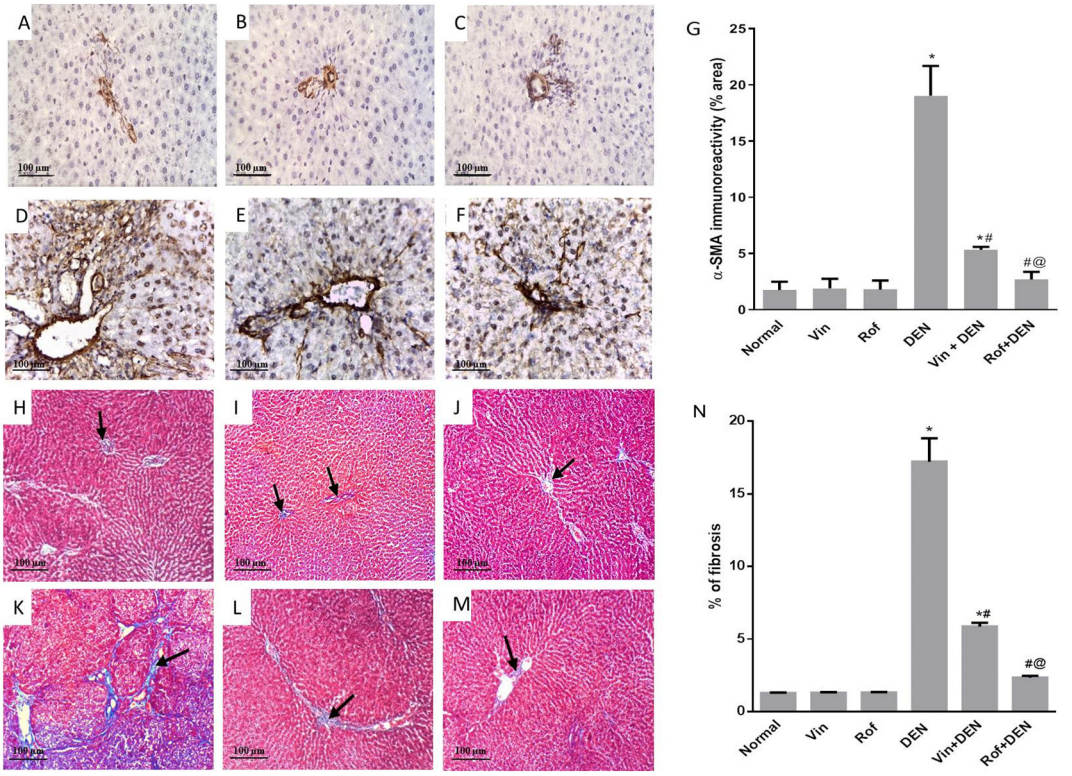

Fig. 2. Effect of vinpocetine and roflumilast on DEN-induced histological changes in liver tissues in rats. (A–G) α-SMA immunostaining (magnification ×400) as a marker for hepatic stellate cells (HSCs) activation (brown color). (A) Normal group (B) Vin group (C) Rof group (D) DEN group (E) Vin + DEN group (F) Rof + DEN group (G) Image analysis of α-SMA immunoreactivity (% area). (H–M) Specimens stained with Masson's trichrome (magnification x 100) for estimation of liver fibrosis (blue color). (H) Normal group (I) Vin group (J) Rof group (K) DEN group (L) Vin + DEN group (M) Rof + DEN group (N) Image analysis of % of fibrosis; Arrows represent collagen deposition in portal triad. Each value represents the mean of 5 experiments ± SEM. Statistical analysis was done using One way ANOVA followed by Tukey's post-hoc test. $p < 0.05$ *vs.* normal, $^{\#}p < 0.05$ *vs.* DEN, $^{@}p < 0.05$ *vs.* Vin + DEN. Vin: vinpocetine, Rof: roflumilast, DEN: diethylnitrosamine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Effect of vinpocetine and roflumilast on DEN-induced changes of liver enzymes and liver index in rats

DEN induced liver fibrosis as manifested by significant increase in liver index (liver weight/body weight %) indicating liver enlargement and confirmed by the elevation of the levels of ALT and AST by 2.3 and 1.9 times, respectively compared to the normal level (Table 2). Moreover, exposure to DEN showed strong deposition of collagen fibers in the portal triad and around the hepatic lobules (Metavir score F3) (Fig. 1) leading to a significant boosting in α-SMA immunoreactivity and % of fibrosis (Fig. 2). Treatment with vinpocetine and roflumilast succeeded to normalize liver index as well as to significantly suppress the enzymatic levels of ALT and AST compared to DEN group (Table 2). Furthermore, both drugs showed significant improvement in

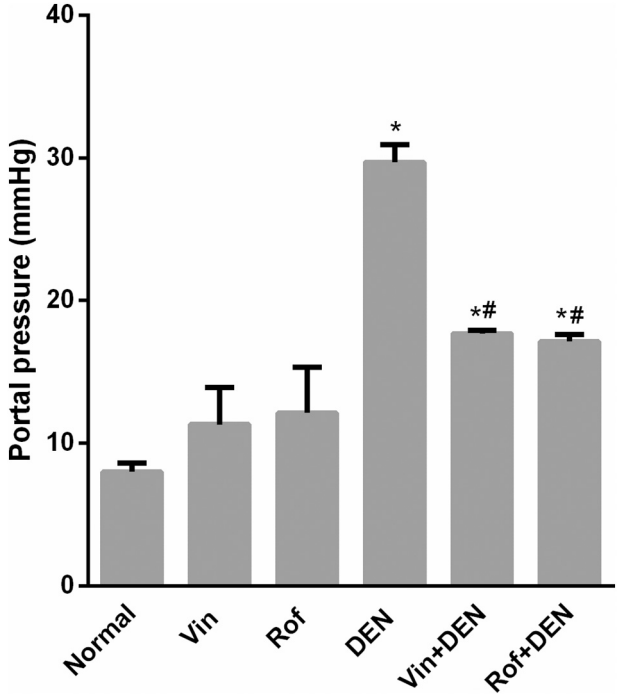

Fig. 3. Effect of vinpocetine and roflumilast on DEN-induced portal hypertension in rats. Each value represents the mean of 4 experiments ± SEM. Statistical analysis was done using One way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$ *vs.* normal, $^{\#}p < 0.05$ *vs.* DEN. Vin: vinpocetine, Rof: roflumilast, DEN: diethylnitrosamine.

histological picture ranging from complete restoration of liver parenchyma to fine strands of fibroblasts between the hepatocytes (Metavir score F0–F1) (Fig. 1) thus ameliorating fibrosis as revealed by α-SMA expression and % of fibrosis (Fig. 2).

3.2. Effect of vinpocetine and roflumilast on DEN-induced portal hypertension in rats

Rats treated with DEN showed a marked rise in the mean portal pressure when compared to normal group while this elevation was significantly ameliorated in vinpocetine- and roflumilast-treated groups (Fig. 3).

3.3. Effect of vinpocetine and roflumilast on DEN-induced changes of hepatic HMGB-1 mRNA expression and TLR-4 protein expression in rats

As an evidence of liver injury, treatment with DEN boosted the gene expression of HMGB-1 ten times the normal value which in turn led to sevenfold increase in TLR-4 protein expression. Treatment with vinpocetine and roflumilast significantly decreased gene expression of HMGB-1 compared to DEN-treated group where roflumilast significantly reduced TLR-4 protein expression to a greater extent than that observed with vinpocetine (Fig. 4).

3.4. Effect of vinpocetine and roflumilast on DEN-induced changes of hepatic inflammatory mediators in rats

Liver contents of NF-κB and TNF-α were elevated by 1.5 fold whereas the protein expression of TIMP-1 showed 10 fold increment upon the administration of DEN. Vinpocetine treatment significantly reduced the rise in the aforementioned parameters whereas treatment with roflumilast normalized NF-κB and TNF-α contents and mitigated TIMP-1 protein expression when compared to vinpocetine group (Fig. 5).

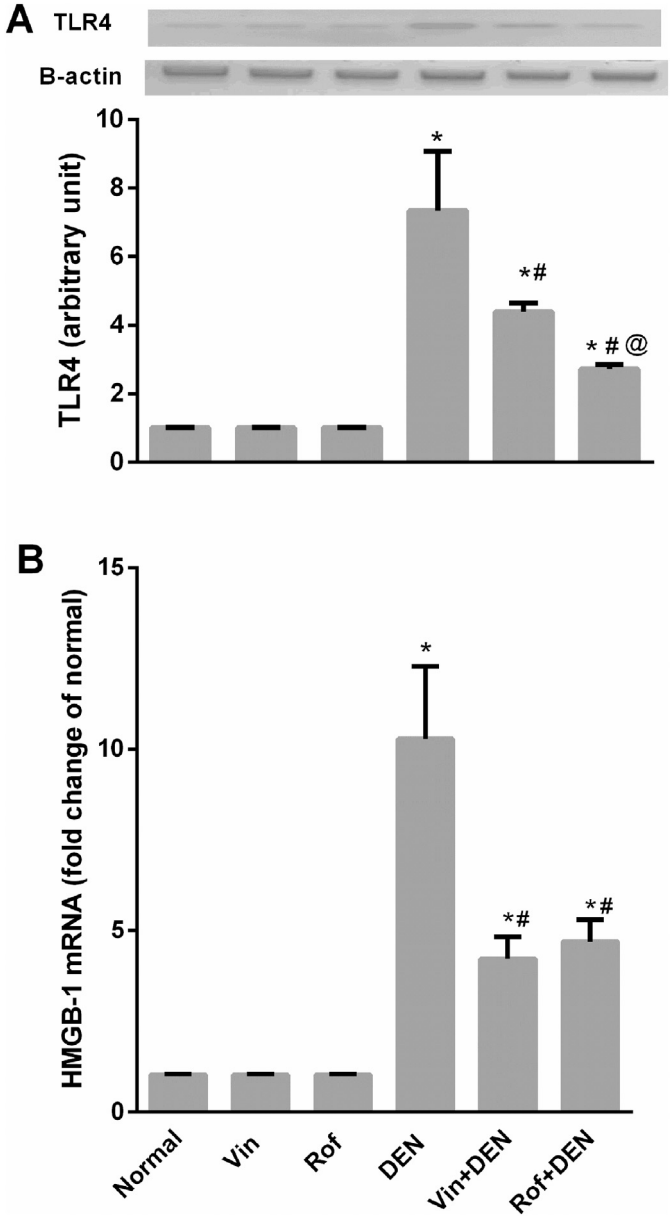

Fig. 4. Effect of vinpocetine and roflumilast on DEN-induced changes of hepatic protein expression of (A) TLR4 and gene expression of (B) HMGB-1 in rats. Each value represents the mean of 5 experiments ± SEM. Statistical analysis was done using One way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$ *vs.* normal, $^{\#}p < 0.05$ *vs.* DEN, $^{@}p < 0.05$ *vs.* Vin + DEN. Vin: vinpocetine, Rof: roflumilast, DEN: diethylnitrosamine.

3.5. Effect of vinpocetine and roflumilast on DEN-induced changes of hepatic fibrogenic markers in rats

DEN-treated group demonstrated significant liver fibrosis as evidenced by markedly elevated liver contents of TGF-β1 and hydroxyproline in addition to significantly downregulated BAMBI receptor gene expression. Administration of either vinpocetine or roflumilast ameliorated these changes where vinpocetine significantly diminished the increments in TGF-β1 and hydroxyproline contents and significantly up-regulated the expression of BAMBI receptor. On the other hand, roflumilast succeeded to normalize TGF-β1 and hydroxyproline hepatic contents and displayed a greater restoration of the expression of the BAMBI receptor compared to vinpocetine group (Fig. 6).

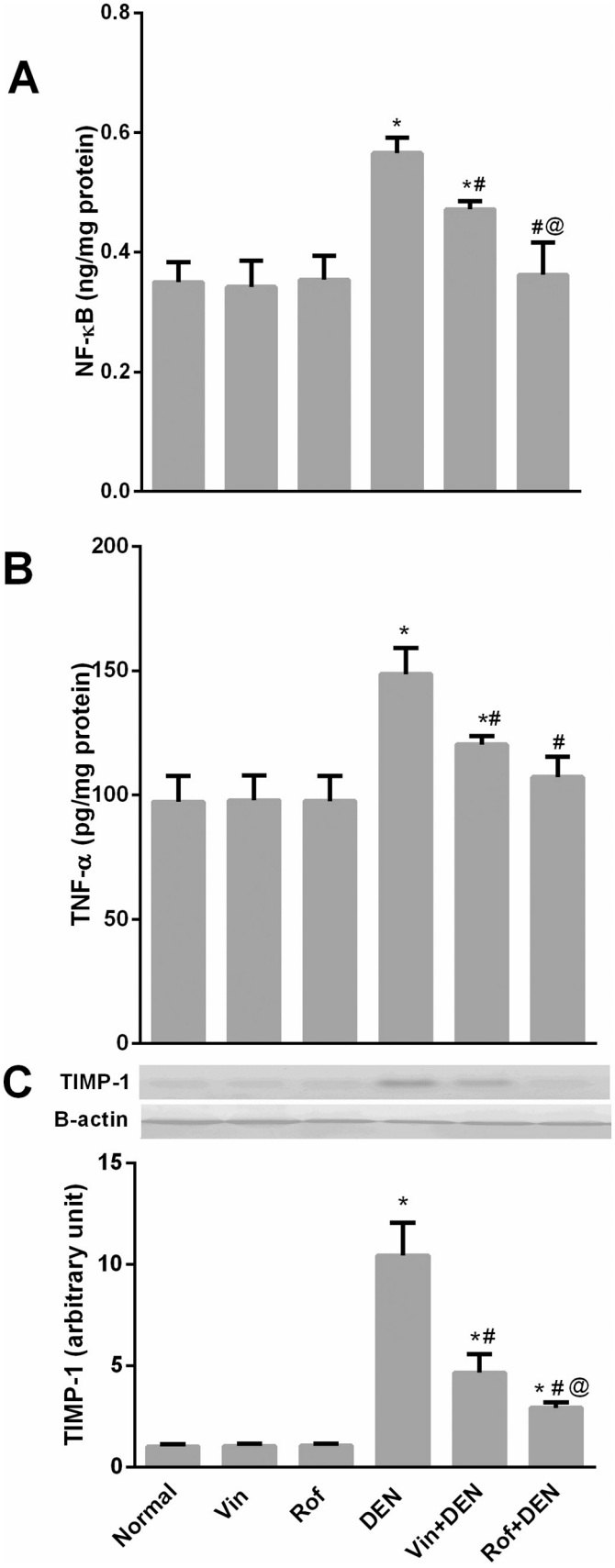

Fig. 5. Effect of vinpocetine and roflumilast on DEN-induced changes in hepatic contents of (A) NF-κB and (B) TNF-α as well as the protein expression of (C) TIMP-1 in rats. Each value represents the mean of 5 experiments ± SEM. Statistical analysis was done using One way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$ *vs.* normal, $^{\#}p < 0.05$ *vs.* DEN, $^{@}p < 0.05$ *vs.* Vin + DEN. Vin: vinpocetine, Rof: roflumilast, DEN: diethylnitrosamine.

3.6. Effect of vinpocetine and roflumilast on DEN-induced changes of hepatic PDE1 and PDE4 enzymes in rats

Based on the estimation of expression of different isoforms of PDE 1 (A, B, C) and PDE 4 (A, B, C, D), PDE1A and 4A displayed the highest expression in DEN model when compared to the other isoforms indicating their significant role in hepatic fibrosis. Interestingly, their enzymatic activities were also increased to 3.5 (PDE1A) and 4.5 (PDE4A) times the normal values, respectively in DEN group. Treatment with vinpocetine significantly inhibited the activity of PDE1A without affecting that of PDE4A confirming the specificity of vinpocetine as a PDE1 inhibitor. While upon the administration of roflumilast, it solely inhibited PDE4A activity as being a selective PDE4 inhibitor (Fig. 7).

3.7. Effect of vinpocetine and roflumilast on DEN-induced changes of hepatic cAMP content and CREB protein expression in rats

As a consequence of the elevation in PDEs expressions and activities, hepatic cAMP content as well as CREB protein expression were reduced upon the administration of DEN. Accordingly, the administration of vinpocetine showed a significant rise in cAMP content and CREB protein expression due to the inhibition of PDE1 activity. The same effect was demonstrated, but in a superior manner, using roflumilast based on its inhibitory effect on PDE4 activity (Fig. 8).

4. Discussion

Liver fibrosis is a major health concern that could threaten or even end the patient's life. Efforts done to discover a treatment for hepatic fibrosis would decrease liver-related mortality rate. In the current study, DEN was used to induce liver fibrosis. DEN is an experimental model of chemically-induced hepatic fibrosis that depends on the generation of a chemical substance named nitrosamine. This model resembles, to a certain extent, the way humans can experience liver fibrosis due to the common presence of nitrosamine in tobacco smoke, polluted water, processed meat, soybean, cheese and a wide variety of products that contain nitrites as a preservative [5,6]. DEN is known to induce oxidative stress [30,31] which produces liver injury and damages the liver matrix leading to the generation of the danger signals, damage associated molecular patterns (DAMPs), one of which is HMGB-1 as demonstrated in our study. HMGB-1 has the ability to induce HSC proliferation and subsequently α-SMA expression [32] which matches our results. HSCs, when activated, express receptors for recognizing and signaling TLR4 thus linking DAMPs and TLR4 signaling [33].

The expression of TLR4 plays a crucial role in HSCs activation, inflammation and fibrogenesis in the context of chronic liver injury [34,35]. In the present study, DEN-induced hepatic fibrosis was evidenced by the elevated levels of plasma ALT and AST [6,7,21,36] which was further confirmed by the histological findings revealing several features deviating from the normal picture. These changes included portal fibrosis with bridging of fibroblasts between the hepatic lobules and nodules formation. These results are in agreement with previous studies [37,38]. The histological changes were reflected by the measured portal pressure which could be related to TLR4 mediated fibronectin production from HSCs which prompts liver sinusoidal endothelial cells to induce portal hypertension [39,40]. Furthermore, the activation of HSCs, the chief executor of fibrosis, was accompanied by a

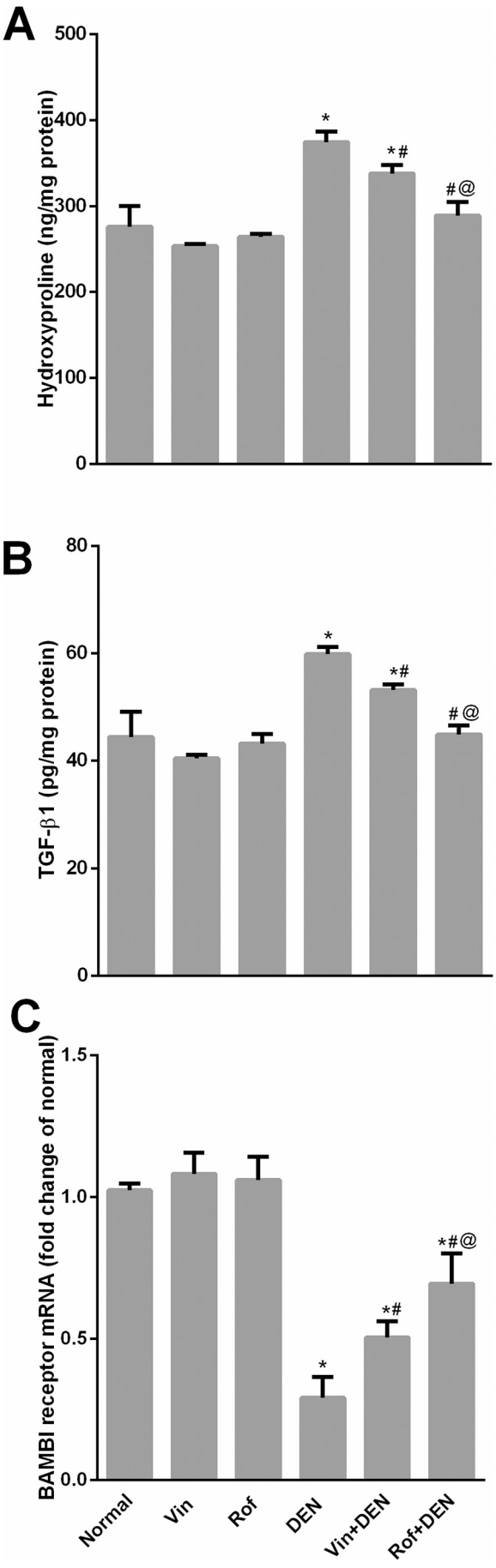

Fig. 6. Effect of vinpocetine and roflumilast on DEN-induced changes in hepatic contents of (A) hydroxyproline and (B) TGF-β1 besides the gene expression of (C) BAMBI receptor in rats. Each value represents the mean of 5 experiments ± SEM. Statistical analysis was done using One way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$ *vs.* normal, $^{\#}p < 0.05$ *vs.* DEN, $^{@}p < 0.05$ *vs.* Vin + DEN. Vin: vinpocetine, Rof: roflumilast, DEN: diethylnitrosamine.

rise in hydroxyproline content, increased expression of α-SMA immunohistochemically and marked appearance of collagen fibers in the histological examination. These results were in accordance with the work done in a previous model of hepatic and renal toxicities in rats [41]. In addition, DEN-induced TLR4 activation was associated with an increase in the inflammatory markers (NF-κB and TNF-α) as well as the protein expression of TIMP-1 which comply with the previous reports [42–44]. The inflammatory cascade has been reported to be mediated through the dissociation of NF-κB from IκB through the phosphorylation of the latter by the TLR4-mediated activation of the IκB kinase (IKK) complex [45–47]. By turn, NF-κB promotes the transcription of pro-inflammatory cytokines such as TNF-α [48,49].

TNF-α has pleiotropic effects through itself and its receptor TNFR1. The production of TNF-α induces HSCs matrix metalloproteinases and TIMP-1 expression [50]. Additionally, Pradere et al. [51] suggested the contribution of TNF-α in the survival of activated HSC through NF-κB dependent mechanism. Studies done on different fibrogenic models also showed the chief involvement of TNFR1 in the progression of liver fibrosis [52,53]. Completing the inflammatory-fibrogenic loop initiated by TLR4 activation, reduction in the expression of BAMBI receptors and elevation of TGF-β1 content were shown in the present study. TLR4 exerts a pro-fibrogenic activity through intensifying the effect of TGF-β by down-regulating TGF-β pseudoreceptor, BAMBI [54,55]. Moreover, Seki et al. [55] reported the ability of NF-κB to aid in TGF-β-activation of HSCs.

In the current study, the expressions PDE1 and 4 enzymes were up-regulated in DEN group although PDE4 expression surpassed PDE1 isoform supporting the evidence of PDE4 critical involvement in hepatic fibrosis [56]. As a consequence, the hepatic cAMP content and CREB protein expression were significantly reduced matching former studies documented in different experimental models [17,57]. Interestingly, our study focused on these two enzymes namely; PDE1 and PDE4 which are known to be upregulated in various fibrogenic models [17,58,59]. Their inhibition showed promising results through elevating cAMP levels which suppress collagen synthesis and fibroblast activation [17,58,59]. Treatment with vinpocetine (PDE1 inhibitor) or roflumilast (PDE4 inhibitor) demonstrated a significant reduction in PDE1 or PDE4 activity, respectively thus preventing the degradation of cAMP and elevating CREB protein expression through activation of PKA and phosphorylation of CREB protein turning it to the active form [57]. Hence, we conducted the current experiment to study their roles in treating liver fibrosis and to address their therapeutic mechanisms by virtue of their capabilities of elevating the cAMP level and connecting this elevation to TLR4 inflammatory and fibrogenic pathways.

Concerning the inflammatory aspect, vinpocetine and roflumilast showed a reduction in protein expression of TLR4 and its downstream inflammatory signals; NF-κB and TNF-α. Previous studies documented that elevation of cAMP-PKA axis inhibited TLR4 inflammatory response by suppressing TNF-α level and boosting IL-10 one [60,61]. This effect could be attributed to modulation of NF-κB transcriptional activity through PKA direct and indirect mechanisms [61]. PKA directly suppressed NF-κB through inhibiting phosphorylation of p65 subunit or stabilizing IκB [62] and indirectly by increasing CREB phosphorylation, as demonstrated herein, which would decrease NF-κB activation [63] thus damping off cytokines transcription such as TNF-α. On the prevention of TNF-α production, TIMP-1 expression was suppressed [50] as shown by our treatments. However, in particular, vinpocetine

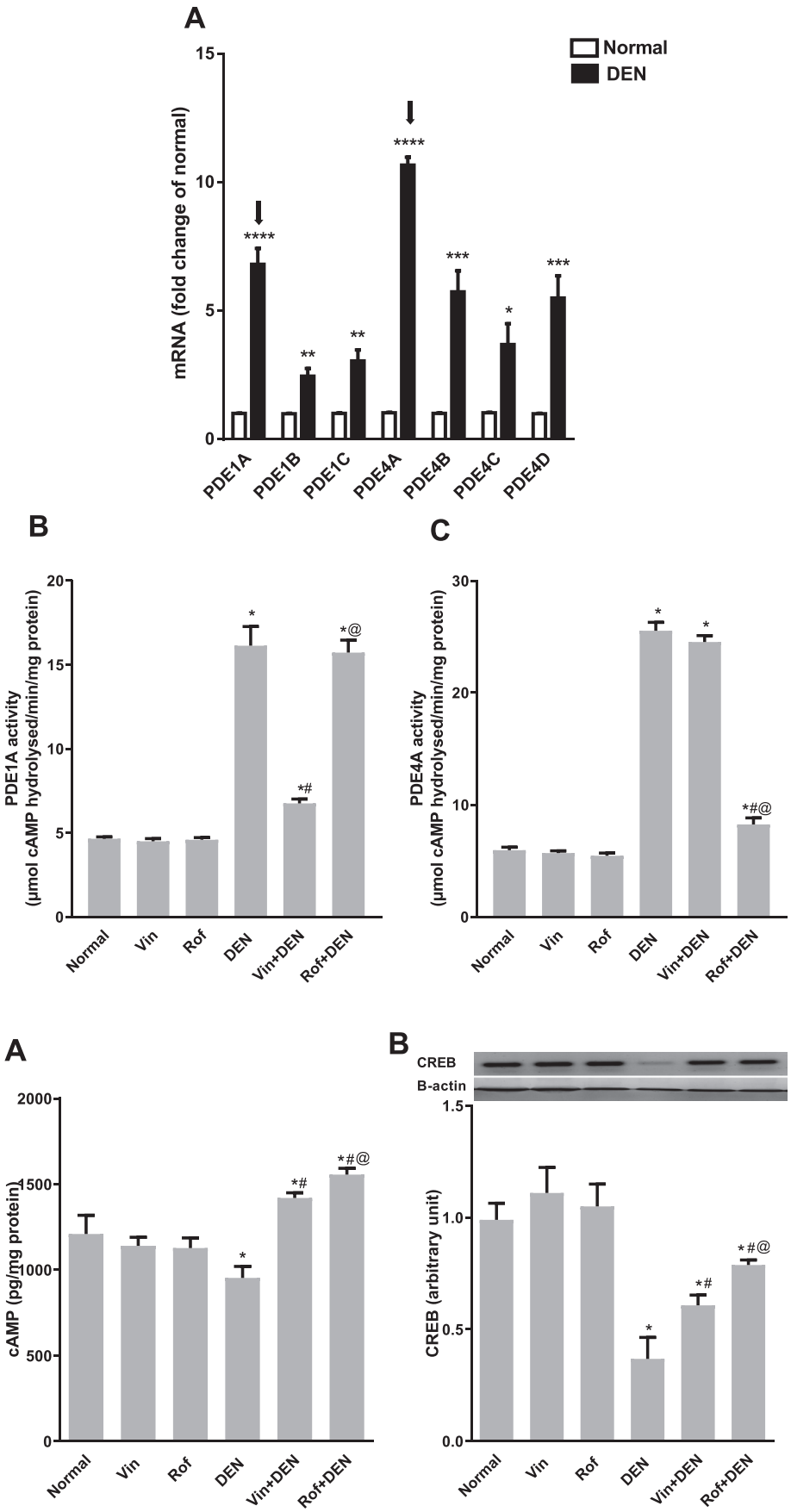

Fig. 7. Effect of DEN on hepatic gene expression of (A) PDE1 and PDE4 isoforms as well as the effect of vinpocetine and roflumilast on DEN-induced changes in hepatic enzymatic activities of (B) PDE1A and (C) PDE4A in rats. Each value represents the mean of 5 experiments ± SEM. For gene expression of different isoforms of PDEs, statistical analysis was done using unpaired Student's *t*-test. $^{*}p < 0.05$ *vs.* normal, $^{**}p < 0.01$ *vs.* normal, $^{***}p < 0.001$ *vs.* normal, $^{****}p < 0.0001$ *vs.* normal. For the other parameters, statistical analysis was done using One way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$ *vs.* normal, $^{\#}p < 0.05$ *vs.* DEN, $^{@}p < 0.05$ *vs.* Vin + DEN. Vin: vinpocetine, Rof: roflumilast, DEN: diethylnitrosamine.

Fig. 8. Effect of vinpocetine and roflumilast on DEN-induced changes in hepatic content of (A) cAMP and protein expression of (B) CREB in rats. Each value represents the mean of 5 experiments ± SEM. Statistical analysis was done using One way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$ *vs.* normal, $^{\#}p < 0.05$ *vs.* DEN, $^{@}p < 0.05$ *vs.* Vin + DEN. Vin: vinpocetine, Rof: roflumilast, DEN: diethylnitrosamine.

presented its inhibitory effect on NF-kB-induced inflammation not only through affecting cAMP level but also *via* IKK-dependent mechanism [64].

Connecting the inflammatory side to the fibrogenic one, vinpocetine and roflumilast treatments showed elevated hepatic expression of BAMBI receptor, decreased content of TGF-β1 and subsequently HSCs activation as indicated by reduced α-SMA expression and hydroxyproline content. TLR4 inhibition has previously shown to prevent the down-regulation of BAMBI receptor thus abating the elevation of TGF-β1 and subsequently TGF-β1-mediated HSCs activation [55,65]. Moreover, the elevated cAMP level was shown to reduce TGF-β1-stimulated α-SMA expression in rat cardiac fibroblast [66]. In the present study, the amelioration of the inflammatory and fibrogenic parameters by PDE inhibitors was reflected by the histopathological restoration of liver parenchyma and decrease in collagen deposition as well as the reduction in portal pressure.

Interestingly, PDE4 inhibition displayed better results in the current study that could be clarified based on two reasons. First, PDE4 expression exceeded PDE1 in DEN group which was reflected by a further increase in cAMP level upon use of roflumilast rather than vinpocetine giving the former an advantage. Second, PDE1 hydrolyzes not only cAMP but also cGMP and with a higher affinity [67]. The increased cGMP level was previously revealed to induce TGF-β1 production and collagen synthesis [68]. Accordingly, the elevation of cGMP could counteract in part the beneficial effect of cAMP stimulated by vinpocetine administration.

5. Conclusion

Our present work indicates the significance of PDE1 and PDE4 overexpression as a crucial pathogenic factor in the context of liver fibrosis. Thus, the administration of vinpocetine (PDE1 inhibitor) or roflumilast (PDE4 inhibitor) offered an efficient treatment in liver fibrosis induced by DEN as evidenced by the amelioration of histological and biochemical parameters. The therapeutic mechanisms of our drugs could be related to enhancing cAMP and CREB levels thus affecting the induced fibrosis through modulating TLR4 inflammatory and fibrogenic pathways. Therefore, PDE inhibitors may propose a new therapeutic perspective in the management of liver fibrosis.

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Conflict of interest

None declared.

References

[1] S.L. Friedman, Mechanisms of hepatic fibrogenesis, Gastroenterology 134 (2008) 1655–1669.
[2] K. Miyazawa, M. Moriyama, M. Mikuni, H. Matsumura, H. Aoki, T. Shimizu, H. Yamagami, M. Kaneko, A. Shioda, N. Tanaka, Y. Arakawa, Analysis of background factors and evaluation of a population at high risk of hepatocellular carcinoma, Intervirology 46 (2003) 150–156.
[3] A. Dhiman, A. Nanda, S. Ahmad, A recent update in research on the antihepatotoxic potential of medicinal plants, Zhong Xi Yi Jie He Xue Bao 10 (2012) 117–127.
[4] C. Thirunavukkarasu, D. Sakthisekaran, Influence of sodium selenite on glycoprotein contents in normal and N-nitrosodiethylamine initiated and phenobarbital promoted rat liver tumors, Pharmacol. Res. 48 (2003) 167–173.
[5] L. Verna, J. Whysner, G.M. Williams, N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation, Pharmacol. Ther. 71 (1996) 57–81.
[6] H. Sun, L. Yu, H. Wei, G. Liu, A novel antihepatitis drug, bicyclol, prevents liver carcinogenesis in diethylnitrosamine-initiated and phenobarbital-promoted mice tumor model, J Biomed Biotechnol 2012 (2012) 584728.
[7] K. Pradeep, C.V.R. Mohan, K. Gobianand, S. Karthikeyan, Silymarin modulates the oxidant-antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats, Eur. J. Pharmacol. 560 (2007) 110–116.
[8] E.O. Farombi, S. Shrotriya, Y.-J. Surh, Kolaviron inhibits dimethyl nitrosamine-induced liver injury by suppressing COX-2 and iNOS expression via NF-kappaB and AP-1, Life Sci. 84 (2009) 149–155.
[9] D.H. Dapito, A. Mencin, G.-Y. Gwak, J.-P. Pradere, M.-K. Jang, I. Mederacke, J.M. Caviglia, H. Khiabanian, A. Adeyemi, R. Bataller, J.H. Lefkowitch, M. Bower, R. Friedman, R.B. Sartor, R. Rabadan, R.F. Schwabe, Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4, Cancer Cell 21 (2012) 504–516.
[10] K. Houglum, K.S. Lee, M. Chojkier, Proliferation of hepatic stellate cells is inhibited by phosphorylation of CREB on serine 133, J. Clin. Invest. 99 (1997) 1322–1328.
[11] E. Shimizu, Y. Kobayashi, Y. Oki, T. Kawasaki, T. Yoshimi, H. Nakamura, OPC-13013, a cyclic nucleotide phosphodiesterase type III, inhibitor, inhibits cell proliferation and transdifferentiation of cultured rat hepatic stellate cells, Life Sci. 64 (1999) 2081–2088.
[12] P.A. Insel, F. Murray, U. Yokoyama, S. Romano, H. Yun, L. Brown, A. Snead, D. Lu, N. Aroonsakool, CAMP and Epac in the regulation of tissue fibrosis, Br. J. Pharmacol. 166 (2012) 447–456.
[13] X. Wang, C.J. Ward, P.C. Harris, V.E. Torres, Cyclic nucleotide signaling in polycystic kidney disease, Kidney Int. 77 (2010) 129–140.
[14] C. Windmeier, A.M. Gressner, Pharmacological aspects of pentoxifylline with emphasis on its inhibitory actions on hepatic fibrogenesis, Gen. Pharmacol. 29 (1997) 181–196.
[15] I. Taguchi, K. Oka, K. Kitamura, M. Sugiura, A. Oku, M. Matsumoto, Protection by a cyclic AMP-specific phosphodiesterase inhibitor, rolipram, and dibutyryl cyclic AMP against Propionibacterium acnes and lipopolysaccharide-induced mouse hepatitis, Inflamm. Res. 48 (1999) 380–385.
[16] T. Matsuhashi, M. Otaka, M. Odashima, M. Jin, K. Komatsu, N. Konishi, I. Wada, T. Sato, Y. Horikawa, R. Ohba, J. Oyake, N. Hatakeyama, S. Watanbe, Specific type IV phosphodiesterase inhibitor ameliorates thioacetamide-induced liver injury in rats, J. Gastroenterol. Hepatol. 20 (2005) 135–140.
[17] C.L. Miller, Y. Cai, M. Oikawa, T. Thomas, W.R. Dostmann, M. Zaccolo, K. Fujiwara, C. Yan, Cyclic nucleotide phosphodiesterase 1A: a key regulator of cardiac fibroblast activation and extracellular matrix remodeling in the heart, Basic Res. Cardiol. 106 (2011) 1023–1039.
[18] L. Gobejishvili, S. Barve, K. Breitkopf-heinlein, Y. Li, J. Zhang, D.V. Avila, S. Dooley, C.J. Mcclain, Rolipram attenuates bile duct ligation – induced liver injury in rats: a potential pathogenic role of PDE4, J. Pharmacol. Exp. Ther. 347 (2013) 80–90.
[19] H.F. Zaki, R.M. Abdelsalam, Vinpocetine protects liver against ischemia-reperfusion injury, Can. J. Physiol. Pharmacol. 91 (2013) 1064–1070.
[20] M. Izikki, B. Raffestin, J. Klar, A. Hatzelmann, D. Marx, H. Tenor, P. Zadigue, S. Adnot, S. Eddahibi, Effects of roflumilast, a phosphodiesterase-4 inhibitor, on hypoxia- and monocrotaline-induced pulmonary hypertension in rats, J. Pharmacol. Exp. Ther. 330 (2009) 54–62.
[21] P. Madankumar, P. Naveenkumar, S. Manikandan, H. Devaraj, S. Niranjalidevaraj, Morin ameliorates chemically induced liver fibrosis in vivo and inhibits stellate cell proliferation in vitro by suppressing Wnt/β-catenin signaling, Toxicol. Appl. Pharmacol. 277 (2014) 210–220.
[22] Y.S. Mohamed, L.A. Ahmed, H.A. Salem, A.M. Agha, Role of nitric oxide and KATP channel in the protective effect mediated by nicorandil in bile duct ligation-induced liver fibrosis in rats, Biochem. Pharmacol. 151 (2018) 135–142.
[23] D.M. El-Tanbouly, W. Wadie, R.H. Sayed, Modulation of TGF-β/Smad and ERK signaling pathways mediates the anti-fibrotic effect of mirtazapine in mice, Toxicol. Appl. Pharmacol. 329 (2017) 224–230.
[24] A.C. Larson, Carbogen Gas – Challenge BOLD MR Imaging in a Rat Model of Purpose: Methods: Results, vol. 254, (2010).
[25] X. Tian, C. Zhao, J. Guo, S. Xie, F. Yin, X. Huo, X. Zhang, Carvedilol attenuates the progression of hepatic fibrosis induced by bile duct ligation, Biomed. Res. Int. 2017 (2017) 1–10.
[26] K.R. Bridle, C. Popa, M.L. Morgan, A.L. Sobbe, A.D. Clouston, L.M. Fletcher, D.H.G. Crawford, Rapamycin inhibits hepatic fibrosis in rats by attenuating multiple profibrogenic pathways, Liver Transpl. 15 (2009) 1315–1324.
[27] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[28] L.A. Ahmed, N.I. Shehata, N.F. Abdelkader, M.M. Khattab, Tempol, a superoxide dismutase mimetic agent, ameliorates cisplatin-induced nephrotoxicity through alleviation of mitochondrial dysfunction in mice, PLoS One 9 (2014) e108889.
[29] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[30] K.L. Kolaja, J.E. Klaunig, Vitamin E modulation of hepatic focal lesion growth in mice, Toxicol. Appl. Pharmacol. 143 (1997) 380–387.
[31] Y. Qi, X. Chen, C. Chan, D. Li, C. Yuan, F. Yu, M.C. Lin, D.T. Yew, H.-F. Kung, L. Lai, Two-dimensional differential gel electrophoresis/analysis of diethylnitrosamine induced rat hepatocellular carcinoma, Int. J. Cancer 122 (2008) 2682–2688.

[32] Y.-H. Kao, B. Jawan, S. Goto, C.-T. Hung, Y.-C. Lin, T. Nakano, L.-W. Hsu, C.-Y. Lai, M.-H. Tai, C.-L. Chen, High-mobility group box 1 protein activates hepatic stellate cells in vitro, Transplant. Proc. 40 (2008) 2704–2705.
[33] J. Guo, S.L. Friedman, Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis, Fibrogenesis Tissue Repair 3 (2010) 21.
[34] Y. Paik, R.F. Schwabe, R. Bataller, M.P. Russo, C. Jobin, D.A. Brenner, Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells, Hepatology 37 (2003) 1043–1055.
[35] M. Darnaud, J. Faivre, N. Moniaux, Targeting gut flora to prevent progression of hepatocellular carcinoma, J. Hepatol. 58 (2013) 385–387.
[36] M. El-Shahat, S. El-Abd, M. Alkafafy, G. El-Khatib, Potential chemoprevention of diethylnitrosamine-induced hepatocarcinogenesis in rats: myrrh (Commiphora molmol) vs. turmeric (Curcuma longa), Acta Histochem. 114 (2012) 421–428.
[37] K. Pradeep, C.V. Raj Mohan, K. Gobianand, S. Karthikeyan, Protective effect of Cassia fistula Linn. on diethylnitrosamine induced hepatocellular damage and oxidative stress in ethanol pretreated rats, Biol. Res. 43 (2010) 113–125.
[38] A. Behfar, Z. Nazari, M.H. Rabiee, G. Raeesi, M.R. Oveisi, N. Sadeghi, B. Jannat, Pharmaceutical Products, vol. 8, (2013), pp. 0–5.
[39] K. Jagavelu, C. Routray, U. Shergill, S.P. O'Hara, W. Faubion, V.H. Shah, Endothelial cell toll-like receptor 4 regulates fibrosis-associated angiogenesis in the liver, Hepatology. 52 (2010) 590–601.
[40] Q. Zhu, L. Zou, K. Jagavelu, D.A. Simonetto, R.C. Huebert, Z.-D. Jiang, H.L. DuPont, V.H. Shah, Intestinal decontamination inhibits TLR4 dependent fibronectin-mediated cross-talk between stellate cells and endothelial cells in liver fibrosis in mice, J. Hepatol. 56 (2012) 893–899.
[41] A. Rezaie, A. Fazlara, M. Haghi Karamolah, A. Shahriari, H. Najaf Zadeh, M. Pashmforosh, Effects of Echinacea purpurea on hepatic and renal toxicity induced by diethylnitrosamine in rats, Jundishapur J. Nat. Pharm. Prod. 8 (2013) 60–64.
[42] M. Liu, Y. Xu, X. Han, L. Yin, L. Xu, Y. Qi, Y. Zhao, K. Liu, Dioscin alleviates alcoholic liver fibrosis by attenuating hepatic stellate cell activation via the TLR4/MyD88/NF-κ B signaling pathway, Nat. Publ. Group (2015) 1–13.
[43] L. Liang, X. Yang, Y. Yu, X. Li, Y. Wu, R. Shi, Babao Dan Attenuates Hepatic Fibrosis by Inhibiting Hepatic Stellate Cells Activation and Proliferation Via TLR4 Signaling Pathway, vol. 7, (2016), pp. 82554–82566.
[44] Y. Yuan, Q. Han, S. Li, Z. Tian, J. Zhang, Wnt2b attenuates HSCs activation and liver fibrosis through negative regulating TLR4 signaling, Sci. Rep. 7 (2017) 3952.
[45] T. Van der Bruggen, S. Nijenhuis, E. van Raaij, J. Verhoef, B.S. van Asbeck, Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the raf-1/MEK1-MEK2/ERK1-ERK2 pathway, Infect. Immun. 67 (1999) 3824–3829.
[46] K. Andersson, R. Sundler, Signalling to translational activation of tumour necrosis factor-alpha expression in human THP-1 cells, Cytokine 12 (2000) 1784–1787.
[47] H.-S. Choi, D. Cho, H.-K. Choi, S.Y. Im, S.-Y. Ryu, K.-M. Kim, Molecular Mechanisms of Inhibitory Activities of Tanshinones on Lipopolysaccharide-Induced Nitric Oxide Generation in RAW 264.7 Cells (Undefined), (2004).
[48] B. Beutler, Tlr4: central component of the sole mammalian LPS sensor, Curr. Opin. Immunol. 12 (2000) 20–26.
[49] S.M. Kerfoot, P. Kubes, Local coordination verses systemic disregulation: complexities in leukocyte recruitment revealed by local and systemic activation of TLR4 in vivo, J. Leukoc. Biol. 77 (2005) 862–867.
[50] T. Knittel, M. Mehde, D. Kobold, B. Saile, C. Dinter, G. Ramadori, Expression patterns of matrix metalloproteinases and their inhibitors in parenchymal and non-parenchymal cells of rat liver: regulation by TNF-alpha and TGF-beta1, J. Hepatol. 30 (1999) 48–60.
[51] J.-P. Pradere, J. Kluwe, S. De Minicis, J.-J. Jiao, G.-Y. Gwak, D.H. Dapito, M.-K. Jang, N.D. Guenther, I. Mederacke, R. Friedman, A.-C. Dragomir, C. Aloman, R.F. Schwabe, Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice, Hepatology 58 (2013) 1461–1473.
[52] K. Kitamura, Y. Nakamoto, M. Akiyama, C. Fujii, T. Kondo, K. Kobayashi, S. Kaneko, N. Mukaida, Pathogenic roles of tumor necrosis factor receptor p55-mediated signals in dimethylnitrosamine-induced murine liver fibrosis, Lab. Investig. 82 (2002) 571–583.
[53] N. Tarrats, A. Moles, A. Morales, C. García-Ruiz, J.C. Fernández-Checa, M. Marí, Critical role of tumor necrosis factor receptor 1, but not 2, in hepatic stellate cell proliferation, extracellular matrix remodeling, and liver fibrogenesis, Hepatology 54 (2011) 319–327.
[54] S.L. Friedman, A deer in the headlights: BAMBI meets liver fibrosis, Nat. Med. 13 (2007) 1281–1282.
[55] E. Seki, S. De Minicis, C.H. Österreicher, J. Kluwe, Y. Osawa, D.A. Brenner, R.F. Schwabe, TLR4 enhances TGF-β signaling and hepatic fibrosis, Nat. Med. 13 (2007) 1324–1332.
[56] L. Gobejishvili, K. Breitkopf-Heinlein, J. Zhang, D. Avila, S. Dooley, S. Barve, C.J. McClain, 386 development of liver inflammation and fibrosis is critically regulated by phosphodiesterase 4 sub-family, Gastroenterology 142 (2012) S–914.
[57] H. Feng, J. Chen, H. Wang, Y. Cheng, Z. Zou, Q. Zhong, J. Xu, Roflumilast Reverses Polymicrobial Sepsis-induced Liver Damage by Inhibiting Inflammation in Mice, vol. 97, (2017), pp. 1008–1019.
[58] B.D. Sachs, G.S. Baillie, J.R. McCall, M.A. Passino, C. Schachtrup, D.A. Wallace, A.J. Dunlop, K.F. MacKenzie, E. Klussmann, M.J. Lynch, S.L. Sikorski, T. Nuriel, I. Tsigelny, J. Zhang, M.D. Houslay, M.V. Chao, K. Akassoglou, p75 neurotrophin receptor regulates tissue fibrosis through inhibition of plasminogen activation via a PDE4/cAMP/PKA pathway, J. Cell Biol. 177 (2007) 1119–1132.
[59] E. Kolosionek, R. Savai, H.A. Ghofrani, N. Weissmann, A. Guenther, F. Grimminger, W. Seeger, G.A. Banat, R.T. Schermuly, S.S. Pullamsetti, Expression and Activity of Phosphodiesterase Isoforms During Epithelial Mesenchymal Transition: The Role of Phosphodiesterase 4, vol. 20, (2009), pp. 4751–4765.
[60] E.A. Wall, J.R. Zavzavadjian, M.S. Chang, B. Randhawa, X. Zhu, R.C. Hsueh, J. Liu, A. Driver, X.R. Bao, P.C. Sternweis, M.I. Simon, I.D.C. Fraser, Suppression of LPS-induced TNF-alpha production in macrophages by cAMP is mediated by PKA-AKAP95-p105, Sci. Signal. 2 (2009) ra28.
[61] H. Ji, X. Shen, Y. Zhang, F. Gao, C.Y. Huang, W.W. Chang, C. Lee, B. Ke, R.W. Busuttil, J.W. Kupiec-Weglinski, Activation of cyclic adenosine monophosphate-dependent protein kinase a signaling prevents liver ischemia/reperfusion injury in mice, Liver Transpl. 18 (2012) 659–670.
[62] S.B. Mustafa, M.S. Olson, Expression of nitric-oxide synthase in rat Kupffer cells is regulated by cAMP, J. Biol. Chem. 273 (1998) 5073–5080.
[63] M. Delgado, D. Ganea, Inhibition of endotoxin-induced macrophage chemokine production by vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide in vitro and in vivo, J. Immunol. 167 (2001) 966–975.
[64] K.-I. Jeon, X. Xu, T. Aizawa, J.H. Lim, H. Jono, D.-S. Kwon, J.-I. Abe, B.C. Berk, J.-D. Li, C. Yan, Vinpocetine inhibits NF-kappaB-dependent inflammation via an IKK-dependent but PDE-independent mechanism, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 9795–9800.
[65] C. Liu, X. Chen, L. Yang, T. Kisseleva, D.A. Brenner, E. Seki, Transcriptional repression of the transforming growth factor β (TGF-β) Pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI) by nuclear factor κB (NF-κB) p50 enhances TGF-β signaling in hepatic stellate cells, J. Biol. Chem. 289 (2014) 7082–7091.
[66] J.S. Swaney, D.M. Roth, E.R. Olson, J.E. Naugle, J.G. Meszaros, P.A. Insel, Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 437–442.
[67] W.E. Knight, S. Chen, Y. Zhang, M. Oikawa, M. Wu, Q. Zhou, C.L. Miller, Y. Cai, D.M. Mickelsen, C. Moravec, E.M. Small, J. Abe, C. Yan, PDE1C deficiency antagonizes pathological cardiac remodeling and dysfunction, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) E7116–E7125.
[68] Y.-C. Hsu, M. Hsiao, Y.W. Chien, W.-R. Lee, Exogenous nitric oxide stimulated collagen type I expression and TGF-beta1 production in keloid fibroblasts by a cGMP-dependent manner, Nitric Oxide Biol. Chem. 16 (2007) 258–265.