Thromboxane A2 receptor contributes to the activation of rat pancreatic stellate cells induced by 8-epi-prostaglandin F2α

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Abstract

Background: Pancreatic stellate cells (PSCs) contribute to the development of pancreatic fibrosis. Identification of different molecules that may contribute to the activation of PSCs will provide a potential target for more effective antioxidant therapy for pancreatic fibrosis. The thromboxane A2 receptor (TxA2r) is a seven-transmembrane G-protein-coupled receptor. Our prior study confirmed that TxA2r was overexpressed in PSCs following pancreatic injuries in high-fat diet (HFD)-treated rats. Therefore, understanding the molecular mechanisms of over-activation of PSCs may pave the way for new treatments of CP. The thromboxane A2 receptor (TxA2r) is a seven-transmembrane G-protein-coupled receptor. [9,10] Hence, we used the isolated rat PSCs (iPSCs) in vitro to identify the potential target for more effective antioxidant therapy for pancreatic fibrosis.

Methods: iPSCs were isolated from the pancreas of 50 days old female SD rats treated with 0.15% (w/v) high-fat diet (HFD) for 4 weeks. The iPSCs were treated with different concentrations of 8-epi-PGF2α (10⁻⁴, 10⁻³, and 10⁻² mol/L) for 48 h, and compared with quiescent iPSCs (all P < 0.001). Real-time polymerase chain reaction was performed to detect the messenger RNA (mRNA) levels of α-smooth muscle actin (α-SMA) and collagen I. Comparisons between the groups were performed using Student’s t test.

Results: TxA2r was up-regulated in activated iPSCs compared with quiescent iPSCs (all P < 0.001). After being treated with SQ29548 (10⁻³ mol/L), the mRNA levels of TxA2r, α-SMA, and collagen I were significantly reduced in iPSCs (P = 0.008). However, different concentrations of SQ29548 all significantly reduced the expression of collagen I (10⁻³ mol/L: 0.35 ± 0.07 vs. 1.00 ± 0.07; t = 10.47, P < 0.001; 10⁻² mol/L: 0.56 ± 0.10 vs. 1.00 ± 0.07; t = 6.185, P < 0.001; 10⁻¹ mol/L: 0.27 ± 0.04 vs. 1.00 ± 0.07; t = 15.41, P < 0.001) and α-SMA (10⁻³ mol/L: 0.06 ± 0.01 vs. 1.00 ± 0.11; t = 15.17, P < 0.001; 10⁻² mol/L: 0.28 ± 0.03 vs. 1.00 ± 0.11; t = 11.29, P < 0.001; 10⁻¹ mol/L: 0.14 ± 0.04 vs. 1.00 ± 0.11; t = 12.86, P < 0.001). After being treated with SQ29548 (10⁻³ mol/L) and then 8-epi-PGF2α (10⁻³ mol/L), the mRNA levels of α-SMA (0.20 ± 0.08 vs. 1.00 ± 0.00; t = 17.46, P < 0.001) and collagen I (0.69 ± 0.13 vs. 1.00 ± 0.00; t = 4.20, P = 0.014) were significantly lower than those of the control group.

Conclusions: The results show that 8-epi-PGF2α promoted iPSC activation, while SQ29548 inhibited iPSC activation induced by 8-epi-PGF2α in vitro. This receptor may provide a potential target for more effective antioxidant therapy for pancreatic fibrosis.

Keywords: Pancreatic stellate cells; Thromboxane A2 receptor; 8-epi-prostaglandin F2α; SQ29548.
Moreover, the expression of the TXA2r is correlated with the expression of the fibrosis marker α-smooth muscle actin (α-SMA) in PSCs. Up-regulation of α-SMA expression in PSCs suggests that PSCs are activated, but it is unclear whether TXA2r is involved in the activation of PSCs. In addition, the upstream signal of up-regulation of the TXA2r is not fully understood.

There is increasing evidence that oxidative stress is involved in the activation of PSCs in CP.\[7,12-14\] In the oxidative stress response, a series of prostaglandin F-like compounds\[15\] called F2-isoprostanes are produced, which are currently considered to be the most reliable markers of oxidative stress.\[16-18\] 8-epi-prostaglandin F2α (8-epi-PGF2α) is a relatively abundant member of the F2-isoprostane family and is the most representative isomer.\[19-21\] 8-epi-PGF2α induced hepatic stellate cell (HSC) proliferation and collagen production mediated liver fibrosis.\[22-24\] In addition, a recent study showed that the effect of 8-epi-PGF2α on HSCs is dependent on the expression of the prostaglandin-related receptor TXA2r.\[25\] Therefore, we hypothesized that 8-epi-PGF2α might promote PSC activation mediated through TXA2r. The purpose of this study was to investigate the role of TXA2r in the activation of PSCs induced by 8-epi-PGF2α.

**Methods**

**Ethical approval**

This protocol was approved by the Institutional Animal Care and Use Committee of Capital Medical University and complied with the NIH Laboratory Animal Care and Use Guidelines (NIH Publication No. 80-23). Every effort was made to reduce the number of animals and minimize their suffering.

**Isolation and culture of PSCs**

Briefly, the pancreas of male Sprague-Dawley rats (180–200 g, n = 30) were digested with Gey balanced salt solution (GBSS) containing collagenase P (0.05%), pronase (0.02%), and DNase (0.1%). Then the resultant cell suspension was gently mixed with 28.7% (wt/vol) Nycodenz gradient and centrifuged at 1400 × g, 4°C for 20 min. After centrifugation, the stellate cells were enriched in the fuzzy band between the Nycodenz cushion and the GBSS with albumin from bovine serum. The PSCs were harvested from the band, and washed and resuspended in Iscove modified Dulbecco medium (IMDM) with 4 mmol/L glutamine, 10% fetal calf serum and antibiotics (streptomycin 100 μg/mL and penicillin 100 U/mL). Freshly isolated cells were seeded at (50–100) × 10^5 cells/well in uncoated plastic six-well plate in IMDM containing 10% fetal bovine serum and incubated for 24 h (quiescent cells) or 48 h (activated cells).

**TXA2r immunocytochemistry on PSCs**

Activated PSCs grown on glass coverslips were fixed with cold acetone for 5 min at 4°C. The cells were washed twice with phosphate-buffered saline (PBS) and blocked with 3% bovine serum albumin (BSA) for 1 h. The cells were then incubated with a polyclonal antibody (1:100 in 3% BSA) against the human thromboxane (TX) A2/prostaglandin (PG) H2 (TP) receptor\[26\] for 1 h. An EliVision™ Plus kit (Maixin Bio, Fuzhou, Fujian, China) was used to visualize the staining according to the manufacturer’s instructions. Sections were incubated with a post-blocking reagent for 20 min at room temperature. After the sections were washed, secondary antibodies were recognized by an EliVision Plus kit (Maixin Bio) were applied for 30 min at room temperature. The visualization signal was developed with diaminobenzidine. Cells were counterstained with Mayer hematoxylin for 5 min. The group of cells without primary antibody incubation were the negative controls.

**Co-localization of α-SMA and TXA2r in PSCs**

Activated PSC was plated on glass coverslips and incubated for 24 h. The fixation of the cells is described as above. For immunofluorescence localization of α-SMA, the fixed cells were first blocked with 3% BSA, then incubated with monoclonal mouse antibody anti-α-SMA (clone 1A4; Neomarkers, Lab Vision, Fremont, CA, USA) (1:10) for 1 h, and finally incubated for 1 h with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin (IgG) (Jackson Immunoresearch, Lancaster, PA, USA) (1:200). Subsequently, the immunofluorescence localization of TXA2r was performed in the same procedure as α-SMA, except that the primary antibody was replaced with a rabbit polyclonal antibody against human TP receptor (1:100), and the fluorescent secondary antibody was replaced by Alexa Fluor 594-conjugated anti-rabbit IgG (1:200). Each time the solution was changed, it was washed with PBS for 1 min, three times. The results of immunofluorescence were observed under a Nikon Eclipse TE300 microscope (Nikon Corporation, Tokyo, Japan).

**Immunoblot analysis of TXA2r in PSCs**

Freshly isolated PSCs were trypsinized at 4°C for 5 min and collected, and then lysed with pre-cooled radio-immuno-precipitation assay lysis buffer (600 μL; 1% sodium deoxycholate, 1 mmol/L disodium ethylenediaminetetraacetate, 0.1% sodium dodecyl sulfate, 150 mmol/L sodium chloride, 1% Triton X-100, 50 mmol/L Tris-base; Beyotime Institute of Biotechnology, Shanghai, China) for 30 min on ice. The lysate of cells was centrifuged at 4°C, 12,000 r/min for 10 min. The supernatant was transferred to a 1.5 mL tube. And protein concentration was determined using Pierce BCA Protein Assay Kit (RTP7102, Real-Times Biotechnology Co., Ltd, Beijing, China). The samples were mixed with loading buffer and then denatured in boiling water bath for 10 min. Next, samples (20 μg/sample) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 1 h. The proteins in gel after SDS-PAGE were transferred to polyvinylidene fluoride membranes using Western blotting transfer system (250 mA, 1 h) on ice. The blocking reaction used 5% skimmed milk for 1 h. Then, the membranes were incubated with a solution containing antibody of TXA2r (1:1000, BioRad, Hercules, CA, USA) or antibody of β-actin (1:1000; Abcam, Cambridge, UK) overnight at 4°C. After washing with PBS containing 1% Tween 20 for
5 min and for five times, the membranes were incubated with a solution including goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1000; Abcam) for 1 h. Then, immunodetected proteins were visualized using an electrochemiluminescence assay kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer’s recommended protocol[23]. Image lab v3.0 software (Bio-Rad, Hercules, California, USA) was used to acquire and analyze imaging signals. The relative contents of TxA2r in samples were normalized to β-actin.

Quantitation of messenger RNA (mRNA) with real-time polymerase chain reaction (RT-PCR)

The extraction of total RNA used TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of RNA was measured by Nanodrop ND2000 (Thermo Fisher Scientific). Then, reverse transcription was performed with 1 µg of total RNA to obtain complementary DNA using an Ipsogen RT kit (Qiagen, Hilden, Germany). The QuantiNova SYBR Green PCR Kit (Qiagen) was used for quantitative polymerase chain reaction (PCR). The sequences of primers were as follows: α-SMA (GGGATCCTGACCCCTGAAGTA; CACGCGAAGCTCGTGTTATAGA) and collagen I (GGTGGAACACTGTTATGCT; GGTTGGGACAGTCCAAGTCT). Expression levels were normalized to β-actin. The RT-PCR experimental procedure was repeated three times.

Treatment of 8-epi-PGF2α or SQ29548 for PSCs

PSCs isolated from four rats were seeded in six-well cell plates and cultured for 24 h. Cells were then treated with 8-epi-PGF2α (10^{-6}, 10^{-7}, 10^{-8} mol/L) or SQ29548, a TxA2r-specific antagonist (10^{-4}, 10^{-6}, and 10^{-7} mol/L) for 48 h, respectively to identify the drug concentration with the best biological effect and the least cytotoxicity. Then isolated PSCs were treated with SQ29548 (10^{-4} mol/L) for 2 h, followed by 10^{-5} mol/L 8-epi-PGF2α for 48 h. The mRNA expression levels of α-SMA and collagen I were quantified by RT-PCR. The RT-PCR experimental procedure is described above. The experiment was repeated three times.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 statistical software (IBM, Armonk, NY, USA). The continuous data were shown as the mean ± standard deviation, and the normality of the data was checked using the Kolmogorov-Smirnov test. Comparisons between the two groups were performed using Student’s t test. P < 0.05 indicates that the difference was statistically significant.

Results

TxA2r is up-regulated in cultured activated PSCs in vitro

First, the protein level of TxA2r in primary PSCs was measured by immunocytochemistry. The immunohistochemistry (IHC) staining showed that TxA2r was expressed in PSCs [Figure 1A]. The co-localization of α-SMA and TxA2r in PSCs staining showed that TxA2r was expressed in quiescent PSCs [Figure 1B-D]. After 48 h culture, the expression of TxA2r and α-SMA in PSCs was detected by immunofluorescence staining. As shown in Figure 2, TxA2r was also expressed in activated PSCs. Furthermore, compared to quiescent PSCs, the expression of TxA2r was increased in activated PSCs (all P < 0.001) [Figure 3]. Collectively, these data showed that TxA2r was expressed both in quiescent and activated PSCs and its increased level was associated with the activation of PSCs.

TxA2r contributes to PSC activation in vitro

To determine the role of TxA2r in PSC activation, SQ29548 was used to inhibit TxA2r in PSCs. Compared with the control, inhibiting TxA2r reduced the expression of collagen I at different concentrations of SQ29548 (10^{-4} mol/L: 0.55 ± 0.07 vs. 1.00 ± 0.07, t = 10.47, P < 0.001; 10^{-6} mol/L: 0.56 ± 0.10 vs. 1.00 ± 0.07, t = 6.185, P < 0.001; 10^{-7} mol/L: 0.27 ± 0.04 vs. 1.00 ± 0.07, t = 15.41, P < 0.001) and α-SMA mRNA (10^{-4} mol/L: 0.06 ± 0.01 vs. 1.00 ± 0.11, t = 15.17, P < 0.001; 10^{-6} mol/L: 0.28 ± 0.03 vs. 1.00 ± 0.11, t = 11.29, P < 0.001; 10^{-7} mol/L: 0.14 ± 0.04 vs. 1.00 ± 0.11, t = 12.86, P < 0.001) [Figure 4]. These data indirectly indicated that TxA2r contributed to PSC activation.
activation. mRNA level of α-SMA was the lowest after SQ29548 (10^{-4} mol/L) treatment. Thus, SQ29548 (10^{-4} mol/L) was used in subsequent experiments.

8-epi-PGF2α promoted PSCs activation via inducing TxA2r

Different concentrations of 8-epi-PGF2α were used to treat PSCs (10^{-6}, 10^{-7}, and 10^{-8} mol/L) for 48 h. mRNA levels of α-SMA and collagen I were quantitatively analyzed by RT-PCR [Figure 5]. Compared with the control group, 8-epi-PGF2α increased mRNA levels of α-SMA (10^{-6} mol/L: 2.23 ± 0.18 vs. 1.00 ± 0.07, t = 10.70, P < 0.001; 10^{-7} mol/L: 2.91 ± 0.29 vs. 1.01 ± 0.08, t = 10.83, P < 0.001; 10^{-8} mol/L: 1.67 ± 0.07 vs. 1.00 ± 0.08, t = 11.40, P < 0.001) and collagen I (10^{-6} mol/L: 2.68 ± 0.09 vs. 1.00 ± 0.07, t = 24.94, P < 0.001; 10^{-7} mol/L: 2.12 ± 0.29 vs. 1.01 ± 0.12, t = 6.08, P < 0.001; 10^{-8} mol/L: 1.46 ± 0.15 vs. 1.00 ± 0.05, t = 4.93, P = 0.008) in PSCs. Meanwhile, given that mRNA expression of α-SMA was the highest after 8-epi-PGF2α (10^{-7} mol/L) treatment, 8-epi-PGF2α (10^{-7} mol/L) was used in subsequent experiments. Then, the isolated PSCs were treated with SQ29548 (10^{-4} mol/L) for 2 h, followed by 10^{-7} mol/L 8-epi-PGF2α for 48 h. As shown in Figure 6, SQ29548 10^{-4} mol/L significantly reduced 8-epi-PGF2α-induced mRNA expression of α-SMA (0.20 ± 0.08 vs. 1.00 ± 0.00, t = 17.46, P < 0.001) and collagen I (0.69 ± 0.13 vs. 1.00 ± 0.00, t = 4.20, P = 0.014) in PSCs as compared with the control group. Together, these results suggested that 8-epi-PGF2α promoted PSCs activation via inducing TxA2r.
Discussion

There is growing recognition that persistent oxidative stress may play a role in the development and maintenance of CP. Oxidative stress triggers pancreatic inflammation and fibrogenesis via the nuclear factor-κB signaling pathway. Inhibiting oxidative stress by scoparone protects against pancreatic fibrosis via the transforming growth factor-β/Smad signaling pathway in rats. In our previous study, increased expression of malondialdehyde and decreased superoxide dismutase activity were observed in rat pancreatic tissues under HFD treatment. In our present study, we explore the underlying mechanism by which oxidative stress triggers CP, with a focus on TxA2r and PSCs.

PSC activation has been reported to play a critical role in the development of CP. However, the underlying mechanism remains unclear. The distribution and expression of TxA2r in pancreatic tissues were demonstrated by immunohistochemical staining in our previous study. TxA2r was up-regulated in rat pancreatic tissues under chronic pancreatic injuries induced by HFD, and TxA2r was associated with α-SMA expression in activated PSCs. Our present study showed that up-regulated TxA2r may contribute to the activation of PSCs. Immunocytochemistry showed that TxA2r was localized to the cell surface and perinuclear cytoplasm in PSCs. Co-localization of TxA2r and α-SMA in PSCs by immunofluorescence double staining demonstrated that TxA2r was expressed in activated PSCs. Inhibiting TxA2r by SQ29548 significantly reduced the mRNA levels of α-SMA and collagen I in PSCs. To our knowledge, we are the first to provide a description of the relationship between TxA2r and PSC activation in CP.

Recent in vitro and in vivo research has proven that oxidative stress plays an important role in PSC activation. When PSCs are stimulated by an oxidative stress insult, these quiescent fat-storing cells become...
activated and subsequently trigger a downstream signal transduction cascade involving a severe inflammatory process. In response to stimuli such as inflammatory cytokines, chemokines or growth factors, activated PSCs produce more extracellular matrix products as well as various chemokines. In our previous study, we conducted a double-immunofluorescence staining test and found that 4-hydroxynonenal was strongly localized within activated PSCs rather than quiescent PSCs. This result provided evidence that the activation of PSCs was directly related to lipid oxidation. In our present study, we confirmed that oxidative stress induced by 8-epi-PGF$_{2\alpha}$ promoted PSC activation in vitro.

Based on the oxidative stress hypothesis and experimental and clinical findings, antioxidant supplementation has been suggested as a potentially useful treatment for CP. Pre-clinical studies suggest that some antioxidants, including vitamin A, vitamin E, and epigallocatechin-3-gallate complex may be useful in relieving pain in CP. However, the development of clinical trials based on this novel concept is limited due to various reasons. In our present study, we found that 8-epi-PGF$_{2\alpha}$ promoted PSCs activation through the up-regulation of TxA$_2$R, which may provide a new target for the treatment of CP. Due to the easy activation of PSCs in vitro, erroneous activation of PSCs will inevitably occur in the experimental data. Therefore, animal studies should be used in future research.

In conclusion, PSCs activation has been recognized to play a critical role in the development of CP. Oxidative stress is one of the most important underlying mechanisms in PSCs activation. In this study, we provided evidence that 8-Epi-PGF$_{2\alpha}$, an abundant member of the F2-isoprostanes, can induce PSCs activation in vitro. To explore the molecular mechanism of the activation of PSCs induced by 8-epi-PGF$_{2\alpha}$, we found that the expression of TxA$_2$R was increased in activated PSCs. Further study showed that the TxA$_2$R inhibitor SQ29548 significantly reduced the PSC activation by 8-epi-PGF$_{2\alpha}$. These results show that TxA$_2$R may contribute to the activation of PSCs, and TxA$_2$R may be a potential target for the treatment of CP.

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**Conflicts of interest**

None.

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