F₂-isoprostane receptors on hepatic stellate cells

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F₂-isoprostanes are considered as the most reliable markers of oxidative stress and can be used to evaluate the oxidative status in a number of human pathologies. Besides being markers of oxidative stress, F₂-isoprostanes proved to be mediators of important biological effects and would act through the activation of receptors analogous to those for thromboxane A₂. In a previous work, we provided evidence that F₂-isoprostanes, generated during carbon tetrachloride-induced hepatic fibrosis, mediate hepatic stellate cell (HSC) proliferation and collagen hyperproduction. To investigate whether TxA₂ receptor (TxA₂r or TPr) is involved in the effects of F₂-isoprostanes on HSC, experiments on DNA synthesis were carried out in the presence of 8-epi-prostaglandin F_{2α} (8-epi-PGF_{2α}) or the TxA₂r-specific agonist I-BOP ([1S-[1α,2α(Z),3β(1E,3S*), 4α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid). Both agonists significantly stimulated DNA synthesis, which was almost completely inhibited by the TxA₂r-specific antagonist SQ29548 ([1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl] hydrazino] methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptanoic acid), suggesting that much of the effect of 8-epi-PGF_{2α} is mediated by the TxA₂r. Further studies showed that increasing concentrations of SQ29548 progressively inhibit DNA synthesis, suggesting a possible competitive antagonism between the two molecules. In addition, we demonstrated that the stimulatory effect of 8-epi-PGF_{2α} on collagen synthesis could be mediated by TxA₂r. The occurrence of TxA₂r on HSC was also investigated using western blotting analysis and immunocytochemistry, which reveals that TP is distributed both on plasma membranes and within the cells. Moreover, binding studies indicated the presence of a specific binding site for ³H-SQ29548 on HSC. Competition binding studies indicated that 8-epi-PGF_{2α} and I-BOP were both able to displace ³H-SQ29548 binding with a very different affinity ($K_i = 4.14 \pm 1.9 \times 10^{-6}$ M and $K_i = 1.15 \pm 0.3 \times 10^{-9}$ M, respectively), suggesting the involvement of a modified form of isoprostane receptor, homologous to the classic thromboxane A₂-binding site in F₂-isoprostanes-evoked responses on HSC.

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Fibrotic progression of liver disease of different etiology to the end stage of cirrhosis is currently seen as a dynamic and highly integrated cellular response to chronic liver injury.

Increased deposition of collagen and other extracellular matrix proteins is a feature of many chronic diseases affecting the liver, lung, arteries and nervous system. In experimentally induced carbon tetrachloride hepatotoxicity, besides the well-known steatonecrosis, fibrosis also develops and evolves into cirrhosis in the chronic intoxication. Carbon tetrachloride hepatotoxicity is now considered as a model of oxidative stress *in vivo*.

The relation between oxidative stress and collagen hyperproduction was first proposed by Chojker *et al*¹ who observed that the addition of ascorbic acid and iron to cultured fibroblasts strongly stimulates lipid peroxidation and, at the same time, the production of collagen and procollagen α1(I) mRNA; the effects are reproduced by the addition to the same fibroblasts of malonaldehyde, one of the end products of lipid peroxidation.

Among liver nonparenchymal cells, hepatic stellate cells (HSC) represent a very important source of production of collagen and other matrix proteins. The activation of HSC, which quickly occurs even in culture, is accompanied by

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increased production of matrix proteins, by cell proliferation and by the typical change from the resting to the myofibroblast-like phenotype.

It has been reported that lipid peroxidation induced *in vitro* in human HSC or the treatment of the latter with 4-hydroxynonenal (the most cytotoxic aldehyde from lipid peroxidation)² stimulates expression and synthesis of procollagen $\alpha 1(I)$.^{3,4}

After the work of Morrow *et al*⁵ and Roberts *et al*,⁶ oxidative stress and lipid peroxidation are currently monitored in blood and urine by the detection of F_2 -isoprostanes, a series of prostaglandin F_2 -like compounds, formed by free radical-catalyzed peroxidation of phospholipid-bound arachidonic acid, a pathway which is independent of the cyclooxygenase pathway. Therefore, F_2 -isoprostanes can nowadays be considered the most reliable and reproducible markers of oxidative stress and can be used to evaluate the oxidative status in a number of human pathologies.

Besides being the markers of oxidative stress, F_2 -isoprostanes appear to be mediators of important biological effects, such as the vasoconstriction of renal glomerular arterioles (possible explanation of the Hepato-Renal Syndrome)⁷ or the effects on vascular muscle cells and on endothelial cells,^{8,9} in which cellular proliferation is stimulated. The effects of F_2 -isoprostanes are also observed on kidney mesangial and glomerular cells exposed to high glucose concentration, in which increased production of transforming growth factor- β (TGF- β) is seen,¹⁰ and similar effects are reported on retinal vessels.¹¹ Most of these effects appear to be mediated through receptors analogous to those for thromboxane A_2 (Tx A_2 r or TP receptors).^{12–14}

In a previous study,¹⁵ we have shown that, after chronic treatment of rats with carbon tetrachloride, the F_2 -isoprostanes levels, which are dramatically increased in the early stages, remain elevated for the entire experimental period and correlated to the increased hepatic collagen content. Furthermore, the addition of 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-PGF $_{2\alpha}$, the most represented isomer of the isoprostane series) to the cultured activated HSC in the range of concentrations (10^{-8} – 10^{-9} M) found in blood in the *in vivo* studies greatly stimulates cell proliferation and collagen synthesis. Moreover, TGF- β production by a monocyte macrophage cell line is also increased by the addition of 8-epi-PGF $_{2\alpha}$.¹⁵

The effect on DNA synthesis is inhibited by the Tx A_2 r-specific antagonist SQ29548 ([1S-[1 α ,2 α (Z),3 α ,4 α]-7-[3-[[2-[(phenylamino)carbonyl] hydrazino] methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptanoic acid), suggesting that much of the effect of 8-epi-PGF $_{2\alpha}$ is mediated by activation of Tx A_2 r.

The present study was aimed at investigating the possible expression of Tx A_2 r on HSC and its characterization by means of binding studies, using 3H -SQ29548 as a ligand. Moreover, competition studies were performed to assess the binding of 8-epi-PGF $_{2\alpha}$ to Tx A_2 r and the likely correlation between occupancy of these receptors and its biological effect.

MATERIALS AND METHODS

HSC Isolation and Culture

HSC were prepared using sequential pronase/collagenase digestion method as reported previously¹⁶ and purified by density-gradient centrifugation, using Nycodenz 18% (Nycomed, Oslo, Norway). HSC were harvested from the top of gradient and seeded (10^6 cells/ml) in 25 cm² flasks in Dulbecco's Modified Eagle's Medium (DMEM) plus 20% fetal bovine serum and antibiotics. The viability and purity of preparation was tested by trypan blue exclusion and auto-fluorescence of Vitamin A, respectively. Cells were incubated in 95% air–5% CO₂ at 37°C and allowed to adhere. Experiments were performed at the second serial passage, when the cells are fully activated. In fact, at this stage, all the cells exhibited the alpha-smooth muscle actin (α -SMA), detected using Smooth Muscle-alpha Actin Immunohistology Kit (IMMH-2, Sigma, St Louis, USA).

Immunoblot Analysis of Tx A_2 r on HSC

Immunoblot analysis of Tx A_2 r expression was performed in both quiescent (freshly isolated) and activated HSC, using a polyclonal antibody raised against human TP receptor C-terminal 323–343 amino acids (Cayman Chemical, Ann Arbor, USA).

An aliquot of freshly isolated HSC were resuspended in the lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 10 mM EGTA, 10 mM iodoacetamide, 0.02% sodium azide and 0.01% PMSF) and placed on ice for 30 min. Cells were homogenized, sonicated and centrifuged for 45 min at 40 000 g at 4°C. In parallel, activated HSC were seeded onto 25 cm² flasks at a density of 7×10^4 /ml of DMEM and allowed to reach confluence. Cells were washed with phosphate-buffered saline (PBS), collected from the culture plates using a cell scraper and centrifuged at 600 g $\times$ 10 min. The pellet was resuspended in the lysis buffer and treated as described above for quiescent cells.

A 30 μ g portion of membrane protein was loaded in each lane of a 10% SDS-polyacrylamide gel. Proteins were resolved electrophoretically and then transferred onto a nitrocellulose membrane. The nitrocellulose membranes were blocked for 1 h with 3% BSA in TBS-T (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5), incubated with primary antibody (1:1000) overnight at 4°C, followed by the secondary goat anti-rabbit IgG antibody (BioRad, Hercules, CA, USA) (1:15 000). Protein extracts of solubilized human platelet membranes¹⁷ were electrophoresed in parallel with HSC membrane lysates and used as positive control for TP receptor protein expression. Immunodetected proteins were visualized using ECL assay kit (Amersham Biosciences), following the manufacturer's recommended protocol.

Immunocytochemistry of Tx A_2 r on HSC

Activated HSC were grown on cover glasses and fixed with cold acetone at 4°C for 5 min. After washing the cells twice with PBS, nonspecific immune-binding sites were blocked by

incubation with 3% BSA in PBS for 1 h. Cells were then incubated for 1 h with primary polyclonal antibody raised against human TP receptor C-terminal 323–343 amino acids (1:100 in 3% BSA/PBS). Following a PBS wash (3×5 min), cells were incubated with FITC-conjugated anti-rabbit IgG (Sigma, St Louis, MO, USA) (1:100 in 3% BSA/PBS) for 1 h. The cells were washed again with PBS as above, and the fluorescence was analyzed by a confocal microscope Zeiss LSM 510 META.

Colocalization of α -SMA and TP Receptor on HSC

Activated HSC were grown on cover glasses and fixed as reported above. The colocalization of α -SMA and TP receptor on HSC was performed by a first block of nonspecific binding with 3% BSA, followed by incubation with primary monoclonal mouse antibody anti- α -SMA (Sigma) (1:100) for 1 h. Then, the cells were incubated with FITC-conjugated anti-mouse IgG (1:200) for 1 h. Nonspecific immune-binding sites were again blocked with 3% BSA and the cells were incubated with rabbit polyclonal antibody raised against human TP receptor (1:100) for 1 h. After incubation of the cells with Alexa Fluor 594-conjugated anti-rabbit IgG (1:200) for 1 h, the immunofluorescence was analyzed by a Nikon Eclipse TE300 microscope.

Study of DNA Synthesis

DNA synthesis was estimated as ^3H -thymidine uptake by HSC, according to Boscoboinik *et al.*¹⁸ In brief, cells were seeded on 24-well cell culture plates as 5×10^4 cells/ml and at the subconfluence made quiescent by culture in serum-free DMEM medium. After 2 h, the medium was changed to DMEM and the preincubation was carried out in the presence of 8-epi-PGF_{2 α} (10^{-9} M), I-BOP ([1S-[1 α ,2 α (Z),3 β (1E,3S*),4 α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid) (10^{-9} M), SQ29548 (10^{-8} M), 8-epi-PGF_{2 α} plus SQ29548, or I-BOP plus SQ29548 (Cayman Chemical, Ann Arbor, USA) in a 1:10 molar ratio. In parallel studies, cells were preincubated with 10^{-9} M 8-epi-PGF_{2 α} in the presence or absence of SQ29548 in a molar ratio ranging from 1:1 to 1:1000. After preincubating the cells for 18 h with these compounds, ^3H -thymidine (ICN; specific activity 6.7 Ci/mmol) was added at the concentration of 10 $\mu\text{Ci}/\text{ml}$, and incubation continued for 6 more hours. After this incubation period, cells were washed once with PBS, fixed with 5% trichloroacetic acid (TCA) for 20 min at 4°C and then solubilized in 0.1 N NaOH/Na₂CO₃ 2%. Radioactivity was counted in a Packard 2100 TriCarb liquid scintillation analyser.

Study of Collagen Synthesis

Collagen synthesis was measured using a ^3H -proline incorporation assay as described previously.¹⁹ Briefly, HSC were seeded in 12-well plates and grown to visual confluence. At 2 h before the treatment, HSC were made quiescent by incubation with serum-free DMEM. Cells were then treated with 8-epi-PGF_{2 α} (10^{-9} M) or 8-epi-PGF_{2 α} plus SQ29458 in a

molar ratio of 1:10 for 48 h. At 16 h before the end of the incubation period, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, 80 $\mu\text{g}/\text{ml}$ β -aminopropionitrile and 10 $\mu\text{Ci}/\text{ml}$ of ^3H -proline (Amersham International; specific activity 23 Ci/mmol) were added to each well to estimate the collagenous synthesis as described by Peterkovsky and Diegelmann.¹⁹ Media were harvested for the determination of ^3H -proline incorporation into collagen and noncollagen proteins, following the collagenase digestion method, using highly purified bacterial collagenase (Calbiochem Cod. 234134, 250 IU). Incorporation of radioactivity into collagen and noncollagen proteins was determined following precipitation with TCA. Collagen-incorporated radioactivity was recovered in the TCA acid-soluble fraction, whereas noncollagen radioactivity was recovered from TCA precipitate. Data were expressed as tritiated proline incorporation (dpm) per microgram of DNA.

Collagen Content Assay

The collagen content was evaluated in the media of HSC cultures treated with 8-epi-PGF_{2 α} (10^{-9} M) or 8-epi-PGF_{2 α} plus SQ29458 in a molar ratio of 1:10 for 48 h, using the Kit Sircol Collagen Assay (Biocolor, Tebu-Bio, Milan, Italy).

Binding Studies

Saturation curves

Cells were seeded on 12-well cell plates as 6×10^4 cells/ml and used when they reached confluence. After washing twice with PBS, cells were incubated with ^3H -SQ29548 (Perkin Elmer, Boston, MA, USA; specific activity 48.2 Ci/mmol) in the concentration range of 1.25–20 nM for 2.5 h at 4°C.

The reaction was terminated by four washes with ice-cold PBS to remove unbound ligand. Cells were dissolved in 1 ml 1 N NaOH followed by neutralization with 1 ml 1 N HCl. Radioactivity was counted in a Packard 2100 TriCarb liquid scintillation analyser.

Specific binding was calculated as the difference between binding measured in the absence and presence of unlabeled SQ29548 (10 μM).

The maximum number of binding sites (B_{max}) and the dissociation constant (K_d) were calculated by nonlinear regression analysis of the saturation curve performed by one-site curve fit.

Competitive displacement

For displacement studies, the binding of 15 nM ^3H -SQ29548 was investigated in the absence (total binding) or presence of different concentrations of unlabeled ligands, 8-epi-PGF_{2 α} or I-BOP. Nonspecific binding was determined in the presence of excess unlabeled SQ29548 (10 μM) and was subtracted from the total binding (100%) and from the binding measured in the presence of the competitors. K_i values were obtained by nonlinear regression analysis of competition curves performed by one-site competition.

Statistical Analysis

Nonlinear regression analysis and statistical analysis were performed using commercially available software (Prism 4.0; GraphPad Software Inc., San Diego, CA, USA). All data are expressed as mean $\pm$ s.e.m. Statistical comparisons were made using one-way ANOVA followed by *post hoc* Bonferroni's test ($P < 0.05$).

RESULTS

Identification and Localization of TxA_2r in HSC

The expression of TxA_2r on HSC was demonstrated by immunoblot analysis of membrane proteins using a polyclonal antibody raised against C-terminal 323–343 amino acids of human TxA_2r .

As shown in Figure 1, lane B, a single immunoreactive band was present at 55 kDa. The immunoreactive band of 55 kDa present in human platelet membrane extracts is shown as the positive control (Figure 1, lane A). No band was

Figure 1 Identification of the TxA_2r protein in HSC membrane lysates. Thirty micrograms of membrane proteins were analyzed as described in Materials and Methods. Incubation with TxA_2r (TP) antibody (1:1000 dilution) revealed immunoreactivity for a 55 kDa protein in membranes from HSC (lane B) that comigrated with a 55 kDa protein found in lysates obtained from human platelet membranes (HPM, lane A). Blots were reprobed with β -actin to assess equal loading.

detected when immunoblotting was performed on quiescent HSC (data not shown). These results demonstrate that rat HSC do not constitutively express TP receptor protein, but this receptor appears only in activated HSC.

These data are confirmed by immunocytochemistry studies that demonstrate that TxA_2r colocalize with α -SMA in activated HSC (Figure 2), whereas is not detectable in quiescent cells (data not shown). Confocal analysis (Figure 3) and colocalization studies (Figure 2) indicate that TP receptor is localized on both cell surface and inside of the cell. In particular, TP receptor immunoreactivity was predominantly detected in the region of cytoplasm surrounding the nucleus (Figure 3).

Experiments of DNA Synthesis

To investigate whether TxA_2r are involved in the effects of F_2 -isoprostanes on HSC, experiments on DNA synthesis were carried out in the presence of 8-epi-PGF $_{2\alpha}$ or the TxA_2r -specific agonist, I-BOP. Both compounds were used at 10^{-9} M concentration, because as previously reported,¹⁵

Figure 3 Confocal image showing the subcellular localization of TP receptor. Activated HSC were permeabilized and incubated with anti-TP antibody. The cells were further incubated with FITC-conjugated anti-rabbit IgG. The figure shows that TP receptor is predominantly expressed in the area around the nucleus (original magnification $\times 900$).

Figure 2 Colocalization of TP receptor and α -SMA in HSC. Activated cells expressed both TP receptor (red labeled) and α -SMA (green labeled). Merged images (yellow) show the degree of colocalization. All the pictures are at the same enlargement (original magnification $\times 400$).

Figure 4 Effect of F_2 -isoprostanes and I-BOP on DNA synthesis of HSC. Cells were treated for 24 h with 10^{-9} M 8-epi-PGF_{2α} or I-BOP in the presence or absence of SQ29548 in a molar ratio of 1:10. Results are expressed as 3 H-thymidine incorporation (dpm) per well. Each sample was run in quadruplicate. Data are the mean $\pm$ s.e.m. of three experiments. Statistical analysis indicated a significant difference between the groups ($F_{5,68} = 5.90$; $P < 0.001$). *Post hoc* analysis: * $P < 0.05$ vs control; ** $P < 0.05$ vs 8-epi-PGF_{2α} or I-BOP; *** $P < 0.001$ vs control. C, control.

8-epi-PGF_{2α} induced its maximum effect at this concentration. As shown in Figure 4, both agonists significantly stimulated 3 H-thymidine incorporation, even if I-BOP showed a somewhat lower effect as compared to 8-epi-PGF_{2α}. Moreover, the stimulation of DNA synthesis by both 8-epi-PGF_{2α} or I-BOP was almost completely inhibited by the TxA₂r-specific antagonist SQ29548 in a molar ratio of 1:10 (Figure 4).

On the other hand, SQ29548 by itself did not modify DNA synthesis. These results seem to suggest that much of the effect of 8-epi-PGF_{2α} is mediated by the TxA₂r.

To characterize the kind of inhibition exerted by SQ29548 (competitive or not), we evaluated DNA synthesis in HSC after the addition of the most stimulating dose of 8-epi-PGF_{2α} (10^{-9} M), in the presence or absence of SQ29548 (from 10^{-9} to 10^{-6} M). Figure 5 shows that increasing concentrations of SQ29548 progressively inhibited DNA synthesis, thus suggesting a possible competitive antagonism between the two molecules.

Experiments on Collagen Synthesis

On the light of the above results on DNA synthesis, we also investigated whether the stimulatory effect of 8-epi-PGF_{2α} on collagen synthesis and total collagen content could be mediated by TxA₂r. Thus, experiments were carried out using 10^{-9} M 8-epi-PGF_{2α} in the absence and presence of the TxA₂r antagonist SQ29548 at the concentration of 10^{-8} M. The results clearly indicate that 8-epi-PGF_{2α} significantly stimulated collagen synthesis (Figure 6a) and total collagen content

Figure 5 Effect of SQ29548 on 8-epi-PGF_{2α}-stimulated DNA synthesis in HSC. Cells were treated with 10^{-9} M 8-epi-PGF_{2α} in the presence or absence of SQ29548 in a molar ratio ranging from 1:1 to 1:1000. Results are expressed as 3 H-thymidine incorporation (dpm) per well. Each sample was run in quadruplicate. Data are the mean $\pm$ s.e.m. of three experiments. Statistical analysis indicated a significant difference between the groups ($F_{5,65} = 7.02$; $P < 0.001$). *Post hoc* analysis: * $P < 0.001$ vs control; ** $P < 0.05$, *** $P < 0.01$ and **** $P < 0.001$ vs 8-epi-PGF_{2α}. C, control.

(Figure 6b), whereas SQ29548 completely abolished its stimulatory effect.

These results, as well as those obtained on DNA synthesis, suggest that the effect of 8-epi-PGF_{2α} is mediated by the activation of TxA₂r.

Binding Studies

Binding studies were carried out to characterize the population of TxA₂r on HSC and to study their interactions with 8-epi-PGF_{2α}. Analysis of the saturation curve by nonlinear regression (Figure 7) indicated that 3 H-SQ29548 apparently binds to a single class of receptors on HSC, with a dissociation constant (K_d) of 1.42 ± 0.33 nM and a maximal binding capacity (B_{max}) of 5.91 ± 0.36 fmol/well.

In further experiments, the competition by 8-epi-PGF_{2α} and by the selective TxA₂r agonist, I-BOP for the specific binding of 3 H-SQ29548 was assayed in HSC. Figure 8 shows that both ligands were able to displace 3 H-SQ29548 binding, although with different potency. I-BOP, as expected, exhibited a high affinity for TxA₂r as indicated by the value of its inhibition constant ($K_i = 1.15 \pm 0.3 \times 10^{-9}$ M), whereas 8-epi-PGF_{2α} exhibited a much lower affinity ($K_i = 4.14 \pm 1.9 \times 10^{-6}$ M), in agreement to what has been reported by Fukunaga *et al*⁸ in the rat vascular smooth muscle cells.

DISCUSSION

Besides being markers of oxidative stress, F_2 -isoprostanes exert a variety of actions on many different cell types.^{20–22} Although it has been suggested that isoprostanes exert their biological action through activation of receptors analogous to

Figure 6 Effect of F₂-isoprostanes and SQ29548 on collagen synthesis and content in HSC. Cells were treated for 48 h with 10⁻⁹ M 8-epi-PGF_{2α} in the absence and presence of SQ29548 in a molar ratio of 1:10. (a) Data are expressed as ³H-proline incorporation (dpm) per microgram of DNA. (b) Collagen content was evaluated in the media of HSC using the Kit Sircol Collagen Assay. Each sample was run in quadruplicate. Data are the mean ± s.e.m. of three experiments. Statistical analysis indicated a difference between the groups ($F_{2,10} = 37.38$; $P < 0.001$). *Post hoc* analysis: * $P < 0.05$, ** $P < 0.001$ vs control; *** $P < 0.001$ vs 8-epi-PGF_{2α}. C, control; CP, collagen protein; NCP, noncollagen protein.

those for TxA₂,⁷⁻¹⁵ up to now there has been no evidence for the presence of such receptors on HSC.

Kawada *et al*²³ provided direct evidence of the reversible contractility of HSC after exposure to various vasoactive eicosanoids or to U46619 (another TxA₂ agonist). The cancellation of the U46619 effects by the TxA₂ antagonist BM13177²³ argues in favor of the presence of a TxA₂ on HSC.

In our previous work,¹⁵ we showed that TxA₂ antagonist SQ29548 was able to antagonize the stimulatory effect of 8-epi-PGF_{2α} on DNA synthesis in HSC, thus suggesting a possible role of TxA₂ receptors in mediating 8-epi-PGF_{2α} effects. In the present study, we confirmed that the 8-epi-PGF_{2α} stimulatory effect on DNA synthesis is inhibited by

Figure 7 Saturation curve of ³H-SQ29548 binding on HSC. Cells were incubated with ³H-SQ29548 (1.25–20 nM). Specific binding was calculated as the difference between binding measured in the absence and presence of unlabeled 10 μM SQ29548. Each point was run in quadruplicate. Data are the mean ± s.e.m. of three experiments.

Figure 8 Competitive binding assay in HSC. Experiments were carried out with ³H-SQ29548 (15 nM) in the absence or presence of different concentrations of unlabeled ligands, 8-epi-PGF_{2α} or I-BOP. Each point was run in quadruplicate. Data are the mean ± s.e.m. of three experiments.

SQ29548 coinubation, and we showed that the inhibition augmented with increasing concentrations of SQ29548, suggesting a competition at the binding site. Moreover, DNA synthesis in HSC was stimulated by the specific agonist of TxA₂r, I-BOP, but its effect at equimolar concentration (10⁻⁹ M) was lower than that of 8-epi-PGF_{2α}; I-BOP effect was also antagonized by SQ29548. We also found that the stimulatory effect exerted by 8-epi-PGF_{2α} on collagen synthesis and on total collagen content was antagonized by the TxA₂ antagonist SQ29548. These results strengthened our hypothesis that 8-epi-PGF_{2α} effects on HSC are due to its interaction with TxA₂ receptors and prompted us to search for the expression of TxA₂ receptors in the HSC. Immunoblotting studies indicated the occurrence in activated HSC of a 55 kDa protein reacting with an antibody raised against human TP receptor. Immunocytochemistry showed that TP receptor colocalize with α-SMA and it is present both

on cell surface and in the cytoplasm. In particular, the immunoreactivity was localized in the region of cytoplasm around the nucleus. This is in agreement with the observations of Sasaki *et al*²⁴ that demonstrated in a different type of cells (HEK293) that the TP receptor is detectable at both the cell surface and inside of the cell.

Moreover, binding studies demonstrated that HSC expresses a population of receptors that specifically bind to the TxA₂r antagonist ³H-SQ29548 showing an affinity in the nanomolar range. To our knowledge, these are the first studies demonstrating the presence of TxA₂ receptors on HSC. Competitive binding studies, using SQ29548 as the radioligand and I-BOP and 8-epi-PGF_{2α} as the displacing agents, indicated that both agents were able to displace ³H-SQ29548 binding, even though with a very different affinity. I-BOP was capable of displacing ³H-SQ29548 binding with a high affinity in the nanomolar range, whereas 8-epi-PGF_{2α} displaced ³H-SQ29548 binding with a much weaker affinity. These data indicated the presence, in HSC, of TxA₂ receptors to which SQ29548 and I-BOP are able to bind with high affinity. The much weaker affinity shown by 8-epi-PGF_{2α} for this binding site suggests that the stimulation of DNA and collagen synthesis induced by this agent was probably achieved through a separate receptor, which has close homology to that of TxA₂, as SQ29548 was able to antagonize such effects. Similar results have been reported by Fukunaga *et al*⁸ in rat vascular smooth muscle cells, where it was shown that, in contrast with the order of potency shown in competition for binding to the TxA₂r, 8-epi-PGF_{2α} stimulates inositol 1,4,5-P₃ production and DNA synthesis with greater potency than the specific agonists I-BOP and U46619.

Recently published papers^{24,25} report that TxA₂ receptors can be expressed as monomers or heterodimers of the α and β isoforms and that the heterodimerization produces alterations in receptor regulation and signaling in response to a TxA₂ agonist.²⁴ Moreover, it has been demonstrated that the expression of heterodimers (TPα/TPβ) significantly increases the biological response to isoprostanes without increasing the ability of isoprostanes to bind to the receptor.²⁴ These observations suggest that heterodimer formation may generate a modified TP receptor with different binding characteristics giving rise to inconsistency between their biological effect and the results of binding studies. To clarify this issue in our experimental conditions, binding studies were attempted using ³H-8-epi-PGF_{2α}. However, as the commercially available radioligand has a very low specific activity, no useful data were obtained.

In the present study, we demonstrate that in HSC some fibrogenic events (such as cell proliferation and collagen synthesis) are induced by isoprostanes through TxA₂r (TP receptor). How isoprostanes act to initiate these events is still unclear and signal transduction mechanisms should be investigated.

As mentioned above, in aortic smooth muscle cells, 8-epi-PGF_{2α} exerts its biological actions through TxA₂r by

stimulating Ins^{1,4,5} P₃ production⁸ with subsequent cell proliferation. In these cells, 8-epi-PGF_{2α} also dose-dependently activates MAP kinase,¹² which have been shown to influence collagen gene expression.²⁶ Furthermore, it has been demonstrated that certain proliferation-associated signaling pathways, including those leading to the activation of ERK1/2 and phosphatidyl inositol 3-kinase, are activated by oxidative stress in an epidermal growth factor receptor-dependent manner and may promote cell survival.²⁷ It has been shown that the stimulation of TP receptor causes a transactivation of epidermal growth factor receptor,²⁸ and results in the activation and phosphorylation of ERK 1/2.²⁹

As 8-epi-PGF_{2α} stimulates the production of TGF-β by U937 cells, as previously observed,¹⁵ a possible pathway linking isoprostane production to collagen synthesis may involve TGF-β, which is known as the fibrogenic master cytokine.³⁰ It must be considered that, in the kidney, 8-epi-PGF_{2α} increases TGF-β responses through the activation of TP receptor even if less potently than the TP agonist U-46 619.³¹ Thus, isoprostanes generated in hepatocytes may stimulate HSC through TP receptors to proliferate and produce excess collagen. Alternatively, they can indirectly stimulate HSC by increasing TGF-β release by Kupffer cells or macrophages.

In conclusion, the results reported in the present study demonstrate that (a) HSC on their surface and in their cytoplasm expresses high-affinity receptors for TxA₂ ligands, (b) the fibrogenic effects elicited in HSC by 8-epi-PGF_{2α} can also be induced, although with a lesser efficacy, by the specific TxA₂r agonist I-BOP. Moreover, our data suggest that the biological effects of 8-epi-PGF_{2α} could be mediated by the activation of a modified form of isoprostane receptor homologous to the classic TP-binding site, showing enhanced isoprostane sensitivity.

Further experiments will be carried out in HSC to investigate the signal transduction pathways set into motion by 8-epi-PGF_{2α}.

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DUALITY OF INTEREST

None declared.

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