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F_2-isoprostanes stimulate collagen synthesis in activated hepatic stellate cells: a link with liver fibrosis?

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Carbon tetrachloride (CCl_4)-induced hepatic fibrosis has been considered to be linked to oxidative stress and mediated by aldehydic lipid peroxidation products. In the present study, we investigated whether collagen synthesis is induced by F_2-isoprostanes, the most proximal products of lipid peroxidation and known mediators of important biological effects. By contrast with aldehydes, F_2-isoprostanes act through receptors able to elicit definite signal transduction pathways. In a rat model of CCl_4-induced hepatic fibrosis, plasma F_2-isoprostanes were markedly elevated for the entire experimental period; hepatic collagen content also increased. When hepatic stellate cells (HSCs) from normal liver were cultured with F_2-isoprostanes in the concentration range found in the *in vivo* studies (10^{-9}–10^{-8} M), a striking increase in DNA synthesis (reversed by the thromboxane A_2 antagonist SQ 29 548), in cell proliferation and in collagen synthesis was observed. Total collagen content was similarly increased. Moreover, F_2-isoprostanes markedly increased the production of transforming growth factor-β1 by U937 cells, considered a model of liver macrophages. The data provide evidence for the possibility that F_2-isoprostanes generated by lipid peroxidation in hepatocytes mediate HSC proliferation and collagen production seen in hepatic fibrosis.
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Increased deposition of collagen and other extracellular matrix-proteins is a feature of many chronic diseases affecting the liver, lung, arteries and nervous systems. In carbon tetrachloride (CCl_4) experimentally-induced hepatotoxicity, besides the classical steatonecrosis, fibrosis also develops and evolves into cirrhosis in the chronic intoxication. CCl_4 hepatotoxicity is now considered 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 alpha 1 (I) mRNA; the effects are reproduced by the addition to the same fibroblasts of malonaldehyde, one of the end products of lipid peroxidation.

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One of the most effective fibrogenic mediators is transforming growth factor (TGF)-β, which strongly stimulates the production of matrix proteins (particularly collagen) in various cellular types.[2–6] In chronic CCl_4 intoxication,[7] an increase in TGF-β mRNA occurs in nonparenchymal cells. Among liver nonparenchymal cells, hepatic stellate cells (HSC) represent a very important source of production of matrix proteins. The activation of HSC, which occurs quickly even in culture, is accompanied by an increased production of matrix proteins, by cellular proliferation and by the typical change from the resting to the myofibroblast-like phenotype (expression of smooth muscle-alpha actin; α-SMA).

It has been reported[8] that lipid peroxidation induced *in vitro* in human HSC or the treatment of the latter with 4-hydroxynonenal (the most cytotoxic aldehyde originating from lipid peroxidation[9]) stimulates the expression of procollagen α1 (I) gene. Also, the treatment of various lineages of macrophages[10] with 4-hydroxynonenal induces mRNA production and synthesis of TGF-β1. Finally, 4-hydroxynonenal added to cultured HSC upregulates the synthesis of procollagen α1 (I).[11]

Despite the many studies carried out on oxidative stress, there has not been a reliable and noninvasive

method to monitor lipid peroxidation *in vivo*, as with the use of blood and urine. Some years ago, however, the group of Morrow *et al*[12] demonstrated the production of a series of prostaglandin F_2-like compounds, named F_2-isoprostanes, which are formed *in vivo* and *in vitro* by free radical-catalyzed peroxidation of phospholipid-bound arachidonic acid,[13] a pathway that is independent of the cyclooxygenase pathway. F_2-isoprostanes might therefore be considered a reliable marker of oxidative stress (lipid peroxidation)[14] and thus be used to evaluate the oxidative status in a number of human pathologies, such as alcoholic liver disease and diabetes.[15]

Since aldehydic lipid peroxidation products, like 4-hydroxynonenal, have been reported[8–11] to induce collagen expression and synthesis, we have investigated whether analogous effects can be obtained with F_2-isoprostanes, the most proximal derivatives of peroxidizing arachidonic acid. One potential advantage of isoprostanes over aldehydes is that while aldehydes can interact with cellular macromolecules by addition processes only, isoprostanes could possess receptors able to induce specific signal transduction pathways. Indeed, F_2-isoprostanes, besides being markers of oxidative stress, are also known to mediate important biological effects[16] such as vasoconstriction of renal glomerular arterioles,[17] an effect that appears to be mainly mediated through thromboxane A_2 receptor (TxA_2r) activation.

An elevated level of plasma F_2-isoprostanes has been reported[18,19] to be associated with acute CCl_4 intoxication. We therefore investigated whether elevated isoprostane levels are maintained throughout the experimental period. In parallel studies, isolated HSC were cultured and treated with F_2-isoprostanes in the range of concentrations found in the *in vivo* studies in order to evaluate the effects of these prostanoids on cell proliferation and collagen synthesis. Since it is generally believed[5,6,20,21] that activation of HSC follows the release of soluble factors (cytokines, mainly TGF-β) by cells of macrophage lineages such as Kupffer cells or liver macrophages, the effects of F_2-isoprostanes on TGF-β release by the human promonocyte cell line U937 was also studied. The results support the idea that F_2-isoprostanes are among the active agonists inducing increase in collagen deposition, at least in CCl_4-induced liver fibrosis.

Materials and methods

In Vivo Studies

The experimentation was carried out by following guidelines prescribed by Italian D.L. 27 January 1992, no. 116, with the approval of Siena University Ethic-Deontological Committee.

Male Sprague–Dawley rats (Harlan-Nossan, Correzzana, Italy), initial weight 80 g, were maintained in a temperature- and light-controlled environment, and fed Harlan Global diet and water *ad libitum*.

In all, 20 rats were injected intraperitoneally two times weekly with a CCl_4-mineral oil solution (1:1, v/v) at the dose of 125 μl/100 g body weight. Eight saline-treated animals, age and weight matched, served as controls. The increase in body weight of the treated animals was slower as compared to the respective controls (−13, −24, −20, −26 and −31% at the 2nd, 3rd, 5th, 6th and 7th week, respectively), while the liver weight to body weight ratio significantly increased. No mortality occurred during the experimental period of time. Groups of three animals were killed after CCl_4 treatment for 1, 2, 3, 5, 6 and 7 weeks, respectively. Killing was performed 24 h after the last injection with the exception of a group killed 4 h after the first injection and a group killed 48 h after the third injection. Blood and livers were processed for biochemical and morphological analysis.

Preparation procedure and gas-mass analysis of plasma F_2-isoprostanes

For F_2-isoprostane determination, platelet poor plasma was obtained from heparinized blood by centrifugation at 2400 *g* and butylated hydroxytoluene (90 μM) was added to plasma as an antioxidant. Aliquots of plasma were stored under nitrogen at −70°C until analysis (within 2 months). Immediately after thawing, plasma (1 ml) was spiked with tetradeuterated $PGF_{2\alpha}$ (500 pg in 50 μl of ethanol) as an internal standard.

The procedure of Nourooz-Zadeh *et al*[22] was used for the preparation of plasma F_2-isoprostanes (free isoprostanes) prior to gas-mass analysis. This procedure is similar to that classically described by the group of Morrow and Roberts,[23] which involves solid-phase extraction on an octadecylsilane (C_{18}) and silica cartridge, followed by thin-layer chromatography. In the procedure of Nourooz-Zadeh, on the other hand, the silica cartridge and the thin-layer chromatography steps were replaced with an aminopropyl (NH_2) cartridge solid-phase extraction. Such aminopropyl solid-phase extraction was shown[22,24] to be highly efficient in retaining tritium-labelled 9α, 11α-$PGF_{2\alpha}$, with which recovery studies were performed and allowed a higher (nearly 70%) recovery.

The determinations were carried out by gas chromatography/negative ion chemical ionization tandem mass spectrometry analysis. An ion trap was used as mass analyser (ThermoFinnigan Instrument) as reported previously.[25] Samples (1 μl) were injected into the gas chromatograph in undecane containing 10% *N,O*-bis (trimethylsilyl) trifluoroacetamide. The carrier gas was ultrapure helium and methane was used as reagent gas (flow 1.2 ml/min). The collision energy used was 1.3 eV. The measured ions were *m*/*z* 299 and *m*/*z* 303 derived from the typical ions (*m*/*z* 569 and *m*/*z* 573) produced from 8-epi-$PGF_{2\alpha}$ also referred to as 15-F_{2t}-IsoP (the most

represented and the generally evaluated isomer for F_2-isoprostane measurement) and the tetradeuterated derivative of $PGF_{2\alpha}$, respectively.

Hydroxyproline content
Total collagen was determined by hydrolyzing liver samples in 6 N HCl and by measuring hydroxyproline according to the method of Kivirikko *et al*.[26] Collagen content was estimated by multiplying the amount of hydroxyproline by a factor of 7.69. Results are expressed as milligram of collagen per gram of liver (wet weight).

In Vitro Studies

HSC isolation and culture
HSC were prepared by means of sequential pronase/collagenase digestion method as reported[27] 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 cm^2 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 autofluorescence of vitamin A (Figure 1a), respectively. Cells were incubated in 95% air–5% CO_2 at 37°C and allowed to adhere. After 1 day, the cells appeared quiescent and negative for the marker α-SMA (Figure 1b), detected by means of Smooth muscle-alpha actin Immunoistology Kit (IMMH-2, Sigma, St Louis, USA). Experiments were performed at the second serial passage when the cells exhibited the α-SMA marker.

Culture treatment with F_2-isoprostanes
HSC were seeded at density of 6×10^4 cells/ml in DMEM supplemented with 10% fetal bovine serum and allowed to grow to confluence. At 2 h before treatment, the medium was changed with serum-free DMEM. HSC were treated for 48 h with 8-epi-$PGF_{2\alpha}$ (BIOMOL Research Laboratories Inc., USA) in the range of concentrations seen in the *in vivo* experiments (10^{-7}–10^{-10} M). Stock solution of 8-epi-$PGF_{2\alpha}$ (1 mg/ml in ethanol) was diluted to a 10^{-5} M concentration and then further diluted with DMEM to obtain final concentrations ranging from 10^{-7} to 10^{-10} M. Ethanol diluted with serum-free DMEM (1%) was used as vehicle.

DNA synthesis and cell proliferation
DNA synthesis was evaluated by measuring ^{3}H-thymidine incorporation in HSC, according to Boscoboinik *et al*.[28] Subconfluent cells were incubated with 8-epi-$PGF_{2\alpha}$ as mentioned above. At 6 h before assay, 10 μCi/ml of ^{3}H-thymidine (ICN; specific activity 6.7 Ci/mmol) was added. Radioactivity was counted in a Packard 2100 TriCarb liquid scintillation analyser. DNA content was measured in HSC according to Taylor *et al*.[29] Cell proliferation was evaluated by cell counting and trypan blue exclusion, after 24 h of treatment with 8-epi-$PGF_{2\alpha}$. Data are expressed as the number of cells per well.

Collagen synthesis assay
HSC were seeded onto 12-well plates and grown to visual confluence. At 2 h before treatment, the medium was changed to serum-free DMEM to allow the cells to become relatively quiescent. Collagen

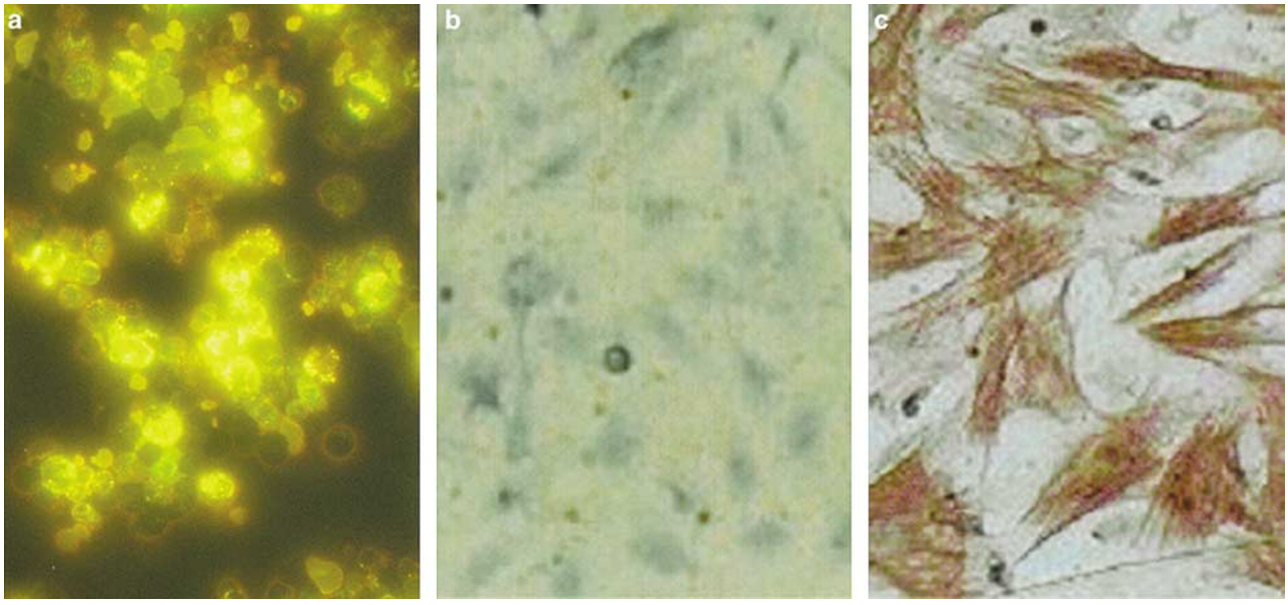


Figure 1 (**a**) Autofluorescence of freshly isolated HSC from rat liver. Cells examined under ultraviolet illumination at 328 nm revealed yellow fluorescence indicating the presence of vitamin A; expression of α-SMA. (**b**) Control: after 1 day, cells appear quiescent and negative for the marker; (**c**) by 7 days in culture, all the cells are activated. (original magnification ×120).

synthesis was assessed as reported.[30] Briefly, 16 h before the end of the 48 h treatment with 8-epi-$PGF_{2\alpha}$, 10 μCi/ml of ^{3}H-proline (Amersham International; specific activity 23 Ci/mmol) were added to each well. Media were harvested for determination of ^{3}H-proline incorporation into collagen and non-collagen proteins following the collagenase digestion method, by using highly purified bacterial collagenase (Calbiochem Cod. 234134, 250 IU). Incorporation of radioactivity into collagen and noncollagen proteins was determined following precipitation with trichloroacetic acid. Collagen-incorporated radioactivity was recovered in the trichloroacetic acid-soluble fraction, while noncollagen radioactivity was recovered from trichloroacetic acid precipitate. Data were expressed as tritiated proline incorporation (dpm) per microgram of DNA. Percentage collagen synthesis was estimated using the formula of Diegelman and Peterkofsky.[31]

Collagen content assay
The collagen content was evaluated in the media of HSC cultures treated with 8-epi-$PGF_{2\alpha}$ for 48 h, using the Kit Sircol Collagen Assay (Biocolor, Tebu-Bio, Milan, Italy).

Inhibition experiments
DNA synthesis was estimated as mentioned above under 'DNA synthesis and cell proliferation' paragraph. Subconfluent cells were made quiescent by incubation in serum-free DMEM medium for 2 h and then treated for 24 h with the following agents: 10^{-10}–10^{-8} M 8-epi-$PGF_{2\alpha}$; 10^{-9}–10^{-7} M SQ 29 548; and 8-epi-$PGF_{2\alpha}$ plus SQ 29 548 in a molar ratio 1:10. The ^{3}H-thymidine incorporation was determined as above.

Evaluation of TGF-β1 in macrophage cell line
The human promonocyte cell line U937 (ATCC, Rockville, MD, USA) was used. The cells were seeded at 10^6/ml and cultured in RPMI-1640 plus 10% fetal bovine serum in the presence of 150 nM phorbol 12-myristate 13-acetate for 24 h to induce the macrophage-like phenotype of adherence. At 2 h before treatment, the medium was removed and replaced with serum-free medium. Cells were treated for 24 h with 8-epi-$PGF_{2\alpha}$ in the range of concentrations of 10^{-6}–10^{-10} M. The concentration of TGF-β1 in the media was measured with an ELISA method using a Quantikine Kit from R&D System.

Statistical analysis
Results were reported as means $\pm$ s.e.m. Comparisons between groups were carried out by Student's *t*-tests. Correlation coefficients were determined by Pearson's test. All tests were two-tailed. The value of $P<0.05$ was considered statistically significant.

Results

Plasma Isoprostanes in the CCl_4 Treatment

In the model of chronic CCl_4 intoxication, the levels of plasma F_2-isoprostanes, even if lower than in the

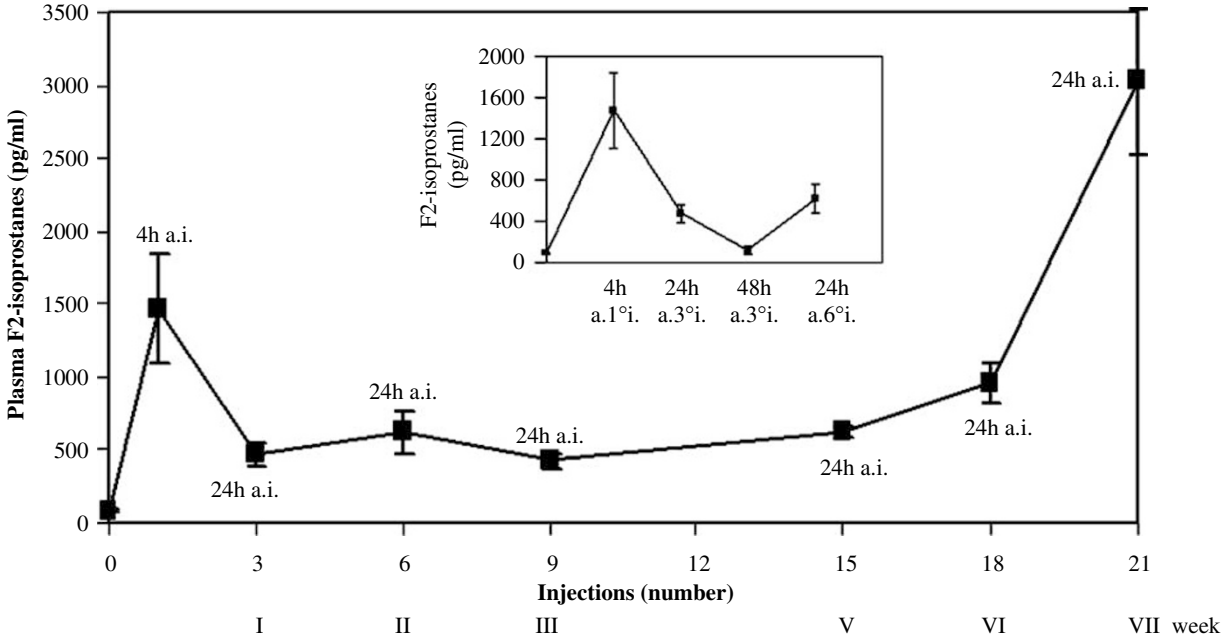


Figure 2 Plasma F_2-isoprostanes in rats chronically intoxicated with CCl_4. Measurement were carried out 4 h after the first injection and 24 h after the third, sixth, ninth, 15th, 18th and 21st injection (I, II, III, V, VI and VII week of treatment, respectively). In the inset, the values for 4 h after the first injection, 24 and 48 h after the third injection and 24 h after the sixth injection are reported. h, hours; a.i., after injection; a.1°i., after the first injection; a.3°i., after the third injection; and a.6°i., after the sixth injection.

acute intoxication (in which concentrations of over 4000 pg/ml have been found[18,19]), were maintained elevated (Figure 2) for the entire experimental period (with a sharp peak at 4 h and decrease at 48 h, Figure 2 inset, with respect to the usual time of measurement, that is, 24 h after the last injection). In particular, very high levels of F_2-isoprostanes were found at 7 weeks (the end of the experiment, Figure 3a) and this was accompanied by a marked increase of hepatic collagen content (measured as hydroxyproline, Figure 3b). Plasma alanine aminotransferase (ALT) activity was also increased (Figure 3c), although with a pattern different from that of plasma isoprostanes.

The histological aspects of the livers at 1, 2, 3 and 7 weeks of treatment showed a progressive fibrosis and finally a clear cirrhosis (Figure 4).

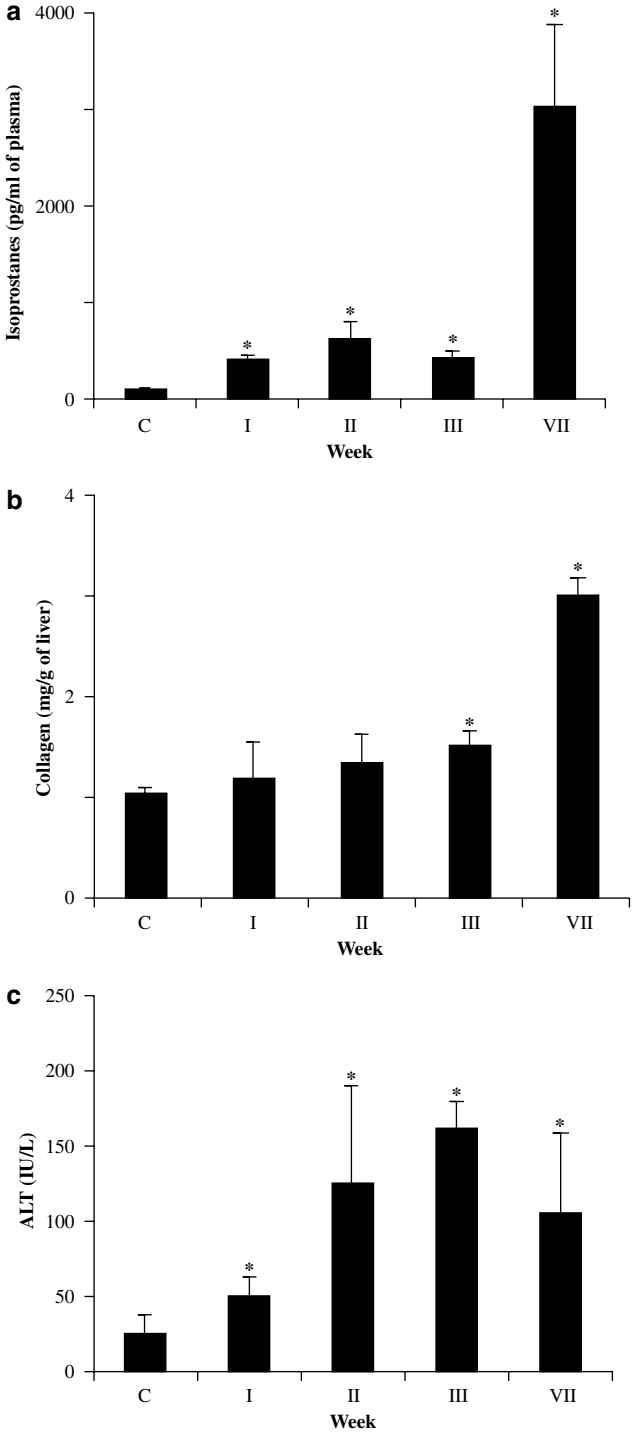


Figure 3 Plasma F_2-isoprostanes, hepatic collagen content and plasma ALT in rats chronically treated with CCl_4. (**a**) Plasma F_2-isoprostanes (pg/ml) (see Figure 2). (**b**) Hepatic collagen content (evaluated as hydroxyproline) after I, II, III and VII weeks of CCl_4 treatment. (**c**) Serum ALT activity (Sigma Alanine aminotransferase Test). Means $\pm$ s.e.m. $^*P<0.05$ *vs* control.

Effect of F_2-Isoprostanes on HSC

The addition of F_2-isoprostanes (10^{-10}–10^{-8} M) to cultured activated HSC induced a two- to four-fold increase in DNA synthesis (Figure 5), as measured by tritiated thymidine incorporation. Also, the cell number was increased (231 ± 26 and 197 ± 42% with 10^{-9} and 10^{-8} M isoprostanes, respectively, as compared to vehicle control).

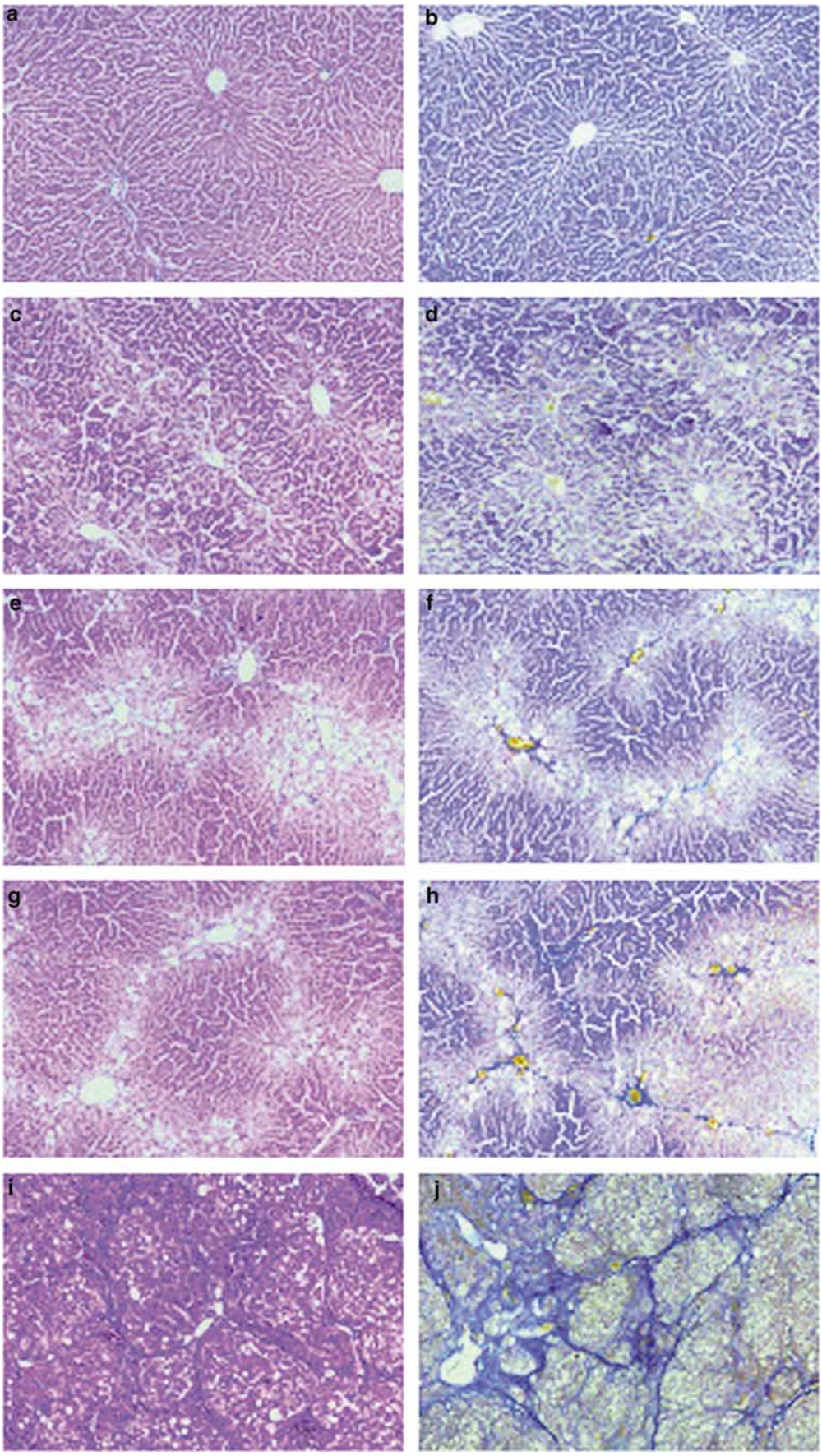
A similar 2.5-fold increase was also seen in collagen synthesis, as measured by tritiated proline incorporation (Figure 6a). The relative collagen production, that is, the percentage of collagen production over the total protein production (collagenic plus noncollagenic proteins), was increased by 3.0- to 3.5-fold (Figure 6b). Total collagen content (Figure 6c) was similarly increased. The lack of effect at 10^{-7} M is unclear, but most likely associated to toxic effects.

The most active concentrations were between 10^{-8} and 10^{-9} M ($=10$ and 1 nM), exactly as those found in the *in vivo* intoxication (3000–500 pg/ml = 9.0–1.5 pmol/ml = 9.0–1.5 nM). No changes at all in HSC viability were seen (not shown).

On the other hand, 4-hydroxynonenal similarly added to cultured HSC at much higher (0.1–5 μM) concentrations stimulated DNA synthesis (Figure 7) to a much lower extent (nearly 1.6-fold at 1 μM concentration) as compared to isoprostanes. No effect was seen on collagen synthesis (not shown).

Inhibition Experiments

To investigate whether TxA_2r are involved in the effects of F_2-isoprostanes on HSC, activated HSC were incubated in the presence of both 8-epi-$PGF_{2\alpha}$ and the specific TxA_2r antagonist SQ 29 548, in a molar ratio of 1:10. After the addition of tritiated thymidine, the incorporation of the latter in HSC was measured. As shown in Figure 8, the stimulatory effect of 8-epi-$PGF_{2\alpha}$ was almost completely abolished, with only a small increase when 10^{-9} M concentration of the isoprostane was used. This could suggest that much of the effect of 8-epi-$PGF_{2\alpha}$ is mediated by TxA_2r.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Effect of F_2-Isoprostanes on TGF-β1 Production by U937 Cells

F_2-isoprostanes also increased the production of TGF-β1 by U937 cells (Figure 9). The increase, although statistically significant (10^{-9}–10^{-7}), was however lower (~2-fold) than that seen for other parameters in HSC and was maximal at higher concentrations (10^{-7} M). No effect was seen on TGF-β1 production by HSC (not shown).

Discussion

Besides being markers of oxidative stress, F_2-isoprostanes appear to be mediators of important biological effects. The first one of these to be revealed[17,32,33] was the vasoconstriction of renal glomerular arterioles, as demonstrated by the direct infusion of 8-epi-$PGF_{2\alpha}$ into the renal artery. It

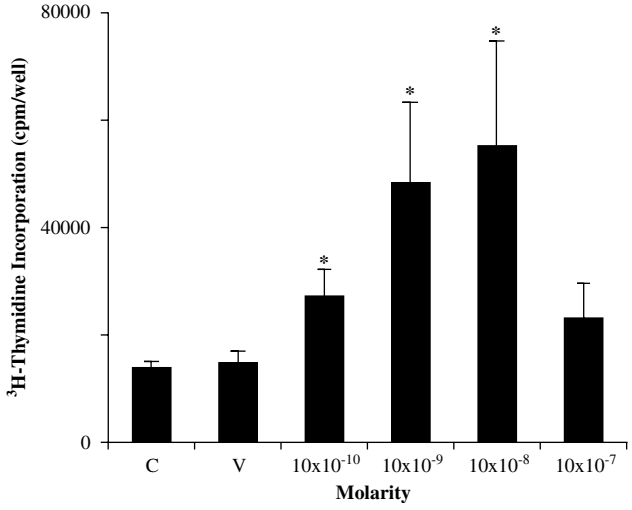

Figure 5 Effect of F_2-isoprostanes on DNA synthesis of HSC. Cells were treated with 8-epi-$PGF_{2\alpha}$ for 48 h. (**a**) Tritiated thymidine was added to the incubation medium 6 h before the end of the treatment. Results are expressed as ^{3}H-thymidine incorporation (cpm) per well. Each sample was run in quadruplicate. Data are the means ± s.e.m. of three experiments. *$P<0.05$ *vs* control. C, control; V, vehicle.

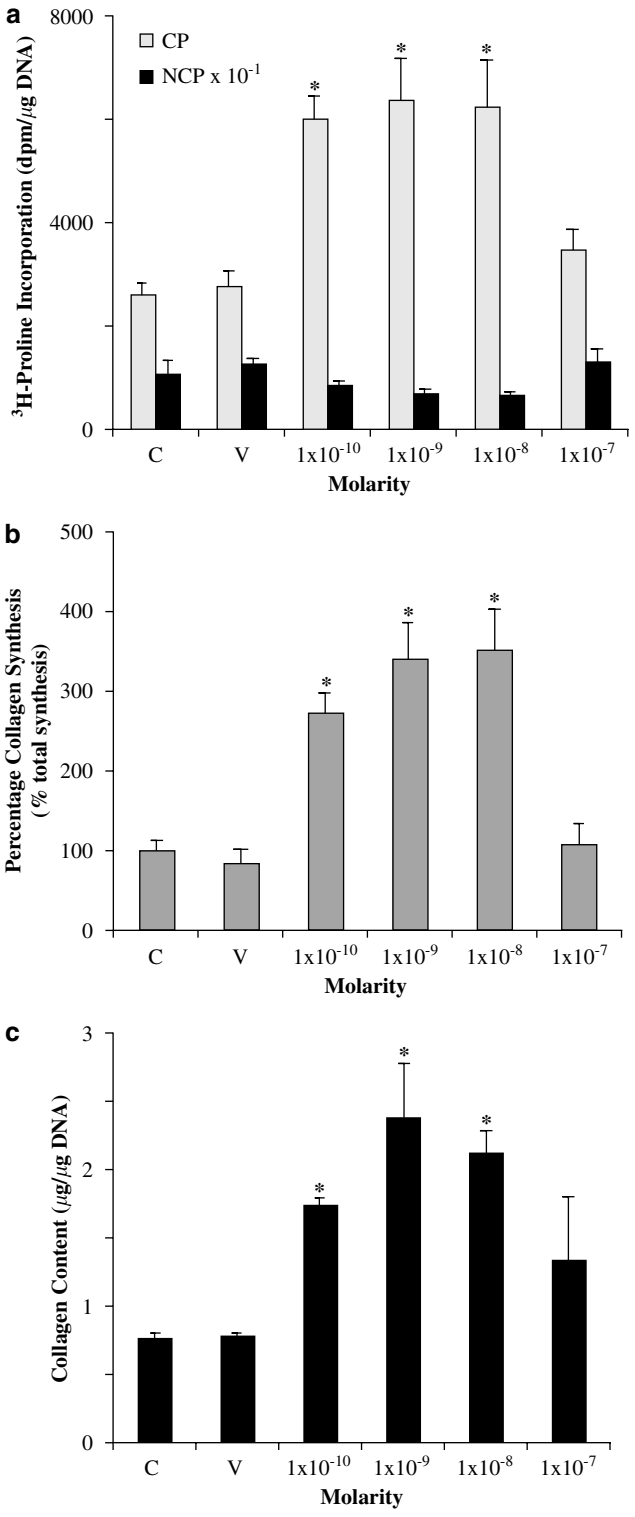

Figure 6 Effect of F_2-isoprostanes on synthesis and content of collagen in HSC treated with 8-epi-$PGF_{2\alpha}$ for 48 h. (**a**) Data are expressed as ^{3}H-proline incorporation (dpm) per microgram of DNA. (**b**) Percentage collagen synthesis determined by calculating collagen production as percentage of total protein production (means ± s.e.m.), using the formula of Diegelmann and Peterkofsky.[31] (**c**) Collagen content as evaluated in the media of HSC using the Kit Sircol Collagen Assay. Each sample was run in quadruplicate. Data are the means ± s.e.m. of three experiments. *$P<0.05$ *vs* control. C, control; V, vehicle; CP, collagen protein; NCP, noncollagen protein.

Figure 4 Hepatic histology in rats after chronic CCl_4 treatment. Control rat livers show a normal architecture. In the treated animals, a progressive injury with hepatocellular necrosis and marked collagen deposition was seen. In particular, at the VII week, a frank cirrhosis was evident. Liver sections were stained with haematoxylin–eosin (**a**) and Mallory trichrome for collagen staining (**b**). (Original magnification ×100).

appears to act through the activation of receptors analogous to those for TXA_2r,[17] an effect believed to be important in explaining the Hepato-Renal Syndrome.[34]

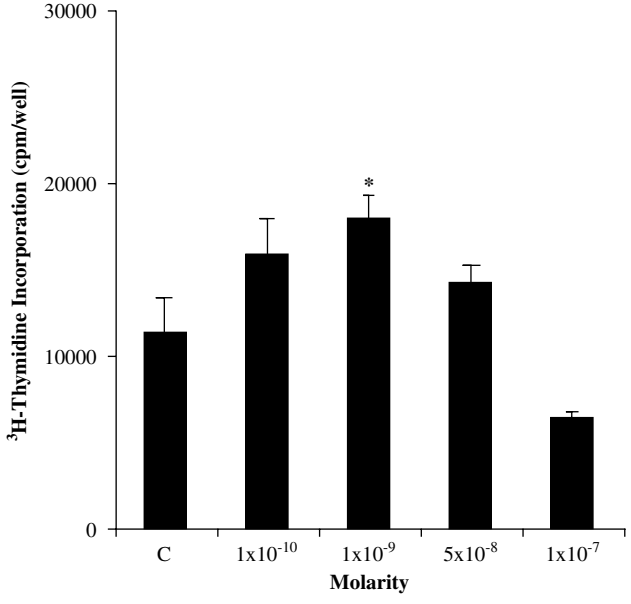

Figure 7 Effect of 4-hydroxynonenal on HSC proliferation. Cells were incubated for 48 h with 4-hydroxynonenal. Tritiated thymidine was added to the incubation medium 6 h before the end of the treatment. Results are expressed as ^{3}H-thymidine incorporation (cpm) per well. Each sample was run in quadruplicate. Data are the means ± s.e.m. of three experiments. $*P<0.05$ *vs* control. C, control.

Other biological effects of 8-epi-$PGF_{2\alpha}$ are those on muscle vascular cells and on endothelial cells in which DNA synthesis and cell proliferation is stimulated.[33,35] These effects also are probably due to activation of receptors related to TXA_2r. Finally, 8-epi-$PGF_{2\alpha}$ seems to mediate the increased production of TGF-β1 in kidney mesangial and glomerular cells exposed to high ambient glucose such as that produced by streptozotocin-induced diabetes.[36]

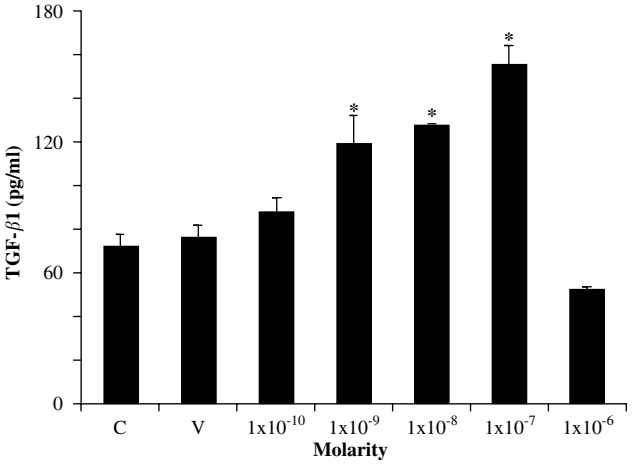

Figure 9 Effect of F_2-isoprostanes (8-epi-$PGF_{2\alpha}$) on TGF-β1 production by U937 human promonocytic cells. The cells were incubated for 24 h with 8-epi-$PGF_{2\alpha}$. TGF-β1 was assayed in the media with an ELISA method. Each sample was run in quadruplicate. Data are the means ± s.e.m. of three experiments. $*P<0.05$ *vs* control. C, control; V, vehicle.

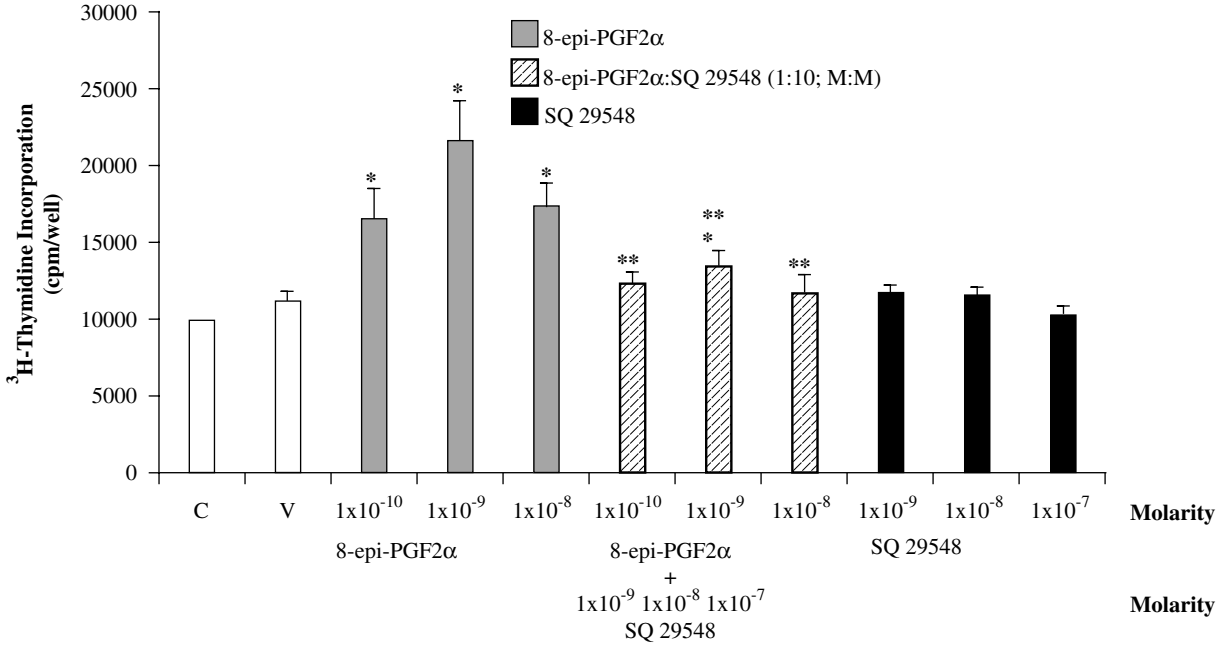

Figure 8 Effect of SQ 29 548 on 8-epi-$PGF_{2\alpha}$-stimulated DNA synthesis by HSC. Tritiated thymidine was added to the incubation medium 6 h before the end of the treatment. Cells were treated with 1×10^{-10} to 1×10^{-8} M 8-epi-$PGF_{2\alpha}$; 1×10^{-9} to 1×10^{-7} M SQ 29 548; and 8-epi-$PGF_{2\alpha}$ plus SQ 29 548 in a molar ratio 1:10. Results are expressed as ^{3}H-thymidine incorporation (cpm) per well. Data are the means ± s.e.m. of three experiments. $*P<0.05$ *vs* vehicle, $**P<0.05$ *vs* respective 8-epi concentration. C, control; V, vehicle.

Therefore, it seemed likely that HSC, which have been reported to respond to lipid peroxidation products, could be activated by F_2-isoprostanes, the direct derivatives of arachidonic acid. The present study demonstrates that 8-epi-$PGF_{2\alpha}$, added to cultured activated HSC in the range of concentrations found in chronic CCl_4 intoxication, stimulates cell proliferation and DNA and collagen synthesis (as measured by ^{3}H-thymidine and ^{3}H-proline incorporation, respectively) and markedly increases the percentage of collagen production over total protein production (collagenic plus noncollagenic proteins). Total collagen content is also increased. The most effective concentrations were between 10^{-8} and 10^{-9} M, exactly as those found in plasma in the *in vivo* intoxication (9.0–1.5 nM). Therefore, we suggest that F_2-isoprostanes generated by lipid peroxidation in hepatocytes stimulate one of the main functions of HSC that is collagen production.

The assumption that F_2-isoprostanes are generated in hepatocytes is supported by a 40-year long-lasting literature,[37–41] demonstrating (*in vivo* and *in vitro* studies) that CCl_4 induces lipid peroxidation in the liver and in particular in the endoplasmic reticulum of hepatocytes. Lipid peroxidation is induced in both acute and chronic[42] CCl_4 intoxication and there is no indication that the process occurs in mesenchymal (nonparenchymal) cells. Moreover, negligible amounts of F_2-isoprostanes are released by cultured Kupffer cells when challenged with CCl_4.[43] Also, it has been shown[18,19] that both free and total (sum of free plus esterified) isoprostanes are dramatically increased in the liver after CCl_4 intoxication.

The results of inhibition studies would suggest that F_2-isoprostanes stimulate collagen production through activation of HSC receptors related to those for TxA_2. In addition, F_2-isoprostanes stimulate the production of TGF-β1 by U937 cells, assumed as a model for Kupffer cells or liver macrophages. This would suggest an additional paracrine pathway for stimulation of HSC and consequent synthesis of collagen. The receptor for 8-epi-$PGF_{2\alpha}$ has been extensively investigated and whether such a receptor is identical or analogous to[17] or distinct from[33,44–46] that for TxA_2 on HSC is not definitively clarified.

The 8-epi-$PGF_{2\alpha}$ potently contracts retinal vessels, elicits endothelin 1 release from retinal preparation, increases thromboxane production in the retina and cultured endothelial cells and also increases Ca^{2+} transients in retinal endothelial cells.[47] All these effects may play a role in the retinopathy of prematurity, since it has been suggested that oxidative stress such as reoxygenation after an asphyxic episode is frequently encountered in premature newborns[48,49] and the isoprostane-induced generation of thromboxane[47] may produce vasoconstriction with ischemia of the retina. As ischemia and tissue hypoxia precede angiogenesis,[48,49] the overall pathway may be relevant in the revascularization of the retinopathy of prematurity and, with the obvious changes, in the revascularization of the retinopathy of diabetes; in both cases, increased levels of plasma isoprostanes have been reported.[50–52]

In the liver, a sequence of similar events can be even more easily guessed: the liver is endowed with the appropriate cells (HSC) to produce extracellular matrix proteins, particularly collagen and it is endowed with Kupffer cells and macrophages able to synthesize cytokines (TGF-β, mainly), which in turn are able to stimulate HSC. Other cytokines (FGF) may contribute to the proliferation of fibroblasts, thus perpetuating collagen production.[53] The activation of HSC and the consequent collagen hyperproduction appears to be an important step in liver fibrosis. The production of isoprostanes may be the initial step of the puzzle, at least when an oxidative stress can be recognized (CCl_4 is able to generate isoprostanes even in minimal doses (1–10 μl/rat)). The isoprostanes so generated will stimulate HSC through receptors related to TXA_2r. HSC will thus produce excess of collagen; alternatively or in addition, HSC can be stimulated by the increased production of TGF-β by Kupffer cells or macrophages. In any case, the cytokine-mediated recruitment of inflammatory cells is needed for the full feature of fibrosis.

Although other liver cell types are also able to produce collagen,[54–59] the difficulty and the complexity in isolating them precludes a definitive allocation to HSC alone of the data seen in the *in vivo* experiments. However, the *in vitro* stimulation of collagen synthesis by HSC under the same conditions (isoprostane concentrations) found in the *in vivo* experiments seems to warrant that at least a consistent part of collagen production is due to the HSC.

Whether or not such a cascade of events might also induce human fibrosis is unclear. However, the fact that plasma isoprostanes are elevated not only in experimental ethanol-induced liver damage (not shown) generally recognized as an oxidative stress injury[60] but also in alcoholic patients[61–64] lends some support to such a possibility. Finally, thiacetamide, which also induces an increased level of plasma isoprostanes,[18] has long been known as a cirrhotic drug.

Future studies are aimed at clarifying the signalling pathways in HSC in this experimentally induced liver fibrosis.

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Duality of interest

None declared.

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