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Review

Oxidative stress, isoprostanes and hepatic fibrosis

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Summary. An introduction to oxidative stress enlightening the spreading of interest in lipid peroxidation in the 60's and in the identification of cytotoxic aldehydes originating from it is given. The discovery of F_2-isoprostanes as specific markers of oxidative stress is described. Isoprostanes are also agonists of important biological effects. Since a relationship between oxidative stress and collagen hyperproduction has been previously suggested, and since lipid peroxidation products (aldehydes) have been proposed as possible mediators of liver fibrosis, we investigated whether collagen synthesis is induced by F_2-isoprostanes, which can posses receptors for signal transduction pathways. In a rat model of carbon tetrachloride-induced hepatic fibrosis, plasma isoprostanes were markedly elevated for the entire experimental period and hepatic collagen content was also increased. Moreover, when hepatic stellate cells (HSC) isolated from normal livers were cultured up to activation and then treated with F_2-isoprostanes (8-epi-$PGF_{2\alpha}$) in the concentration range found in the *in vivo* studies (10^{-9} to 10^{-8} M), a striking increase in DNA synthesis, in cell proliferation and in collagen synthesis was observed. F_2-isoprostanes also increased the production of transforming growth factor-ß1 by U937 cells, assumed as a model of Kupffer cells or liver macrophages. The hypothesis that F_2-isoprostanes generated by lipid peroxidation in hepatocytes mediate HSC proliferation and collagen hyperproduction, seen in this experimental hepatic fibrosis, was reinforced by the demonstration, by using immunoblot analysis, that isoprostane receptors identical or analogous to those for thromboxane A_2 (TxA_2r) are present in HSC. Immunocytochemical studies showed the major localization of TxA_2r in the perinuclear site and its colocalization with α-smooth muscle actin.

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Introduction

The topic of oxidative stress is deeply-rooted in some biological paradoxes: oxygen is necessary for the life of aerobic organisms, but the univalent reduction of oxygen leads to the formation of one of the reactive oxygen species, the superoxide anion (O_2^-). In the mitochondrial electron transport chain, about 95% of the consumed oxygen undergoes the cytochrome oxidase-catalysed tetravalent reduction to H_2O, but more than 5% of the consumed oxygen is released in the form of reactive species, such as O_2^- and hydrogen peroxide (Boveris and Chance, 1973; Chance et al., 1989). Thus, reactive oxygen species are produced even in the normal respiratory chain.

Iron is necessary for life too, being an essential component of vital enzyme and complexes. However, iron, when released from these complexes in a free form, can react with active oxygen species to yield oxy-radicals (Fenton Reaction). Iron is therefore potentially toxic to biological structures.

Similar considerations are true for cytochrome *P450*, a key component of the mixed function oxidase system. This system, which is basically involved in the detoxification of xenobiotics, can produce harmful radicals from xenobiotics themselves.

The mechanisms of free-radical induced cell injury include, in summary: (i) reactions with nucleic acids, nucleotides, polysaccarides, protein and non protein thiols; (ii) covalent binding to membrane components (proteins, lipids, enzymes, receptors, etc.); and (iii) still at the level of the membranes, initiation of lipid peroxidation. The latter has been suggested as a common mechanism in a large number of biopathological conditions. Yet, up to some decades ago lipid peroxidation was only known in the chemistry of oil and

fat rancidity. The spreading of interest in lipid peroxidation in the field of biopathology in the 60's was mainly due to (i) the knowledge that lipid peroxidation can be linked to the electron transport chain of drug metabolism (Hochstein and Ernster, 1963); (ii) the recognition that the metabolism of the prototype model toxic molecule, carbon tetrachloride (CCl_4), yields aloalkane free radicals (Recknagel, 1967; Slater, 1972); and (iii) our observation that CCl_4 in fact greatly stimulates the peroxidation of liver microsomal lipids (Comporti et al., 1965; Recknagel and Ghoshal, 1966).

Today it is well established that lipid peroxidation is only one of the reactions set in motion as a consequence of the formation of free radicals in cells and tissues. It was one of the first aspects of abnormal oxidative reactions to be recognized, probably because it represents the most prominent phenomenon of uncontrolled oxidative stress. With the discovery of superoxide dismutase (McCord and Fridovich, 1969) and the consequent acquirement that oxyradicals can be easily produced in living tissues, a much more complex spectrum of pathological oxidations has been progressively recognized, so that the term "oxidative stress" has been introduced (Sies, 1985), to signify any condition in which the prooxidant/antioxidant balance is shifted in favour of oxidations.

Our studies on lipid peroxidation of cellular membranes have been carried out through the use of various models of experimental pathology; I will only mention here that of CCl_4 hepatotoxicity (Comporti, 1985, 1989), that of GSH depletion and cell damage (Comporti, 1987) and that of iron release from iron complexes (Comporti et al., 2002).

The study of the mechanisms of the adverse effects of lipid peroxidation in biological systems led to the hypothesis that toxic products originate from lipid peroxidation of cellular membranes, diffuse into the cell and act at distant loci (Comporti, 1985). These products were identified as aldehydes of the class of 4-hydroxyalkenals, mainly 4-hydroxy-2,3-trans-nonenal (4-HNE) (Benedetti et al., 1980; Benedetti and Comporti, 1987; Comporti, 1993), which are provided with a very high reactivity with -SH groups or amino groups of cellular constituents. 4-HNE has been regarded as the model molecule of oxidative stress and has represented for several years the only specific marker of lipid peroxidation. However, 4-HNE is detectable mainly in tissues bound to cellular macromolecules, because its high reactivity reasonably preclude to a great extent the possibility for its release into the blood compartment.

Isoprostanes as markers of oxidative stress

In human pathology the biological materials available are blood and urine only, and in some cases small bioptic materials. Therefore, none of these studies on oxidative stress, although bringing forth very interesting results, had allowed the evaluation of oxidative stress in humans, at least on a large scale. This was due to the fact that a reliable and non invasive method to monitor lipid peroxidation in vivo with the use of only blood and urine was lacking. In fact, all the methods used (detection of conjugated dienes, lipoperoxides and aldehydes) are poorly reproducible and not reliable when carried out in plasma due, as mentioned above, to the extreme reactivity (Esterbauer et al., 1991) and instability of the species that are going to be dosed; or they imply the use of tissues and are therefore hardly feasible in humans. Some years ago, however, the group of Morrow and Roberts (Morrow et al., 1990) 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, a pathway which is independent of the cyclooxygenase pathway. Because isoprostanes, initially formed in situ on phospholipids (Morrow et al., 1992a), are released into the circulation and because these prostanoids are less reactive than other lipid peroxidation products, such as lipoperoxides and aldehydes, they can be found more easily in plasma and urine. Therefore, plasma F_2-isoprostanes can nowadays be considered as the most reliable markers of oxidative stress (lipid peroxidation) and can be used to evaluate the oxidative status in a number of human pathologies. Elevated levels of plasma or urinary isoprostanes have been reported in several diseases, such as alcoholic liver disease, hepato-renal syndrome, ischemia/reperfusion injury, diabetes, chronic obstructive lung disease, allergic asthma, adult respiratory distress syndrome, Alzheimer diseases, Huntington disease, retinopathy of prematurity and smoking (see Roberts and Morrow, 2000 for a review).

Some methodological statements and validations of the method used

A problem which is encountered when measuring F_2-isoprostanes is that concerned with the methodological procedure to be used. Generally the gas-chromatography mass spectrometry procedure is used even if some ELISA immunoassay methods have also been used. The results of the latter however have been repeatedly questioned (Proudfoot et al., 1999; Bessard et al., 2001). We followed the procedure of Nourooz-Zadeh (Nourooz-Zadeh et al., 1995) for the preparation of F_2-isoprostanes prior to gas-mass analysis. This procedure is similar to that classically described by the group of Morrow (Morrow et al., 1990). The latter involves solid phase extraction on an octadecylsilane (C_{18}) and silica cartridge, followed by thin layer chromatography (TLC). In the procedure of Nourooz-Zadeh, on the other hand, the silica cartridge and the TLC steps are replaced with an aminopropyl cartridge solid phase extraction. The recovery obtained with this method was higher than that obtained with the Morrow's method. The determinations were carried out by gas chromatography combined with

negative ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) with an ion trap. 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, and its tetradeuterated derivative, respectively. The detection limit was about 5 pg/ml (=0.014 nM) (Signorini et al., 2003).

With this methodological approach, the results of the acute CCl_4 intoxication of rats (0.2 ml/100 g body weight, intragastrically) showed extremely elevated levels (4520±477 vs 130±26 pg/ml in controls) of plasma F_2-isoprostanes in the intoxicated animals (Comporti et al., 2004), thus confirming both our early studies and the more recent data by Morrow's group (Morrow et al., 1992b).

Isoprostanes as agonists of biological effects

Besides being markers of oxidative stress, F_2-isoprostanes appeared to be mediators of important biological effects. The first one to be revealed (Takahashi et al., 1992) was the vasoconstriction of renal glomerular arterioles, as demonstrated by the direct infusion of 8-epi-$PGF_{2\alpha}$ (the most represented isomer of the series) into renal artery. It appears to act through the activation of receptors analogous or identical to those for thromboxane A_2 (TxA_2r) (Fukunaga et al., 1993). This effect is believed to be very important in the explanation of the hepato-renal syndrome, in which the initial production of F_2-isoprostanes would occur in the liver; they would then induce vasoconstrictory effects in the kidney, resulting in the full feature of renal failure.

Other biological effects of 8-epi-$PGF_{2\alpha}$ are those on muscle vascular cells (Fukunaga et al., 1993) and on endothelial cells (Yura et al., 1999) in which DNA synthesis and cell proliferation are stimulated. These effects too are probably due to activation of receptors related to TxA_2r. Eight-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 (Lahaie et al., 1998). 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 (Reynaud and Dorey, 1994), and the isoprostane-induced generation of thromboxane (Lahaie et al., 1998) may produce vasoconstriction with ischemia of the retina. Because ischemia and tissue hypoxia precede angiogenesis (Reynaud and Dorey, 1994), the

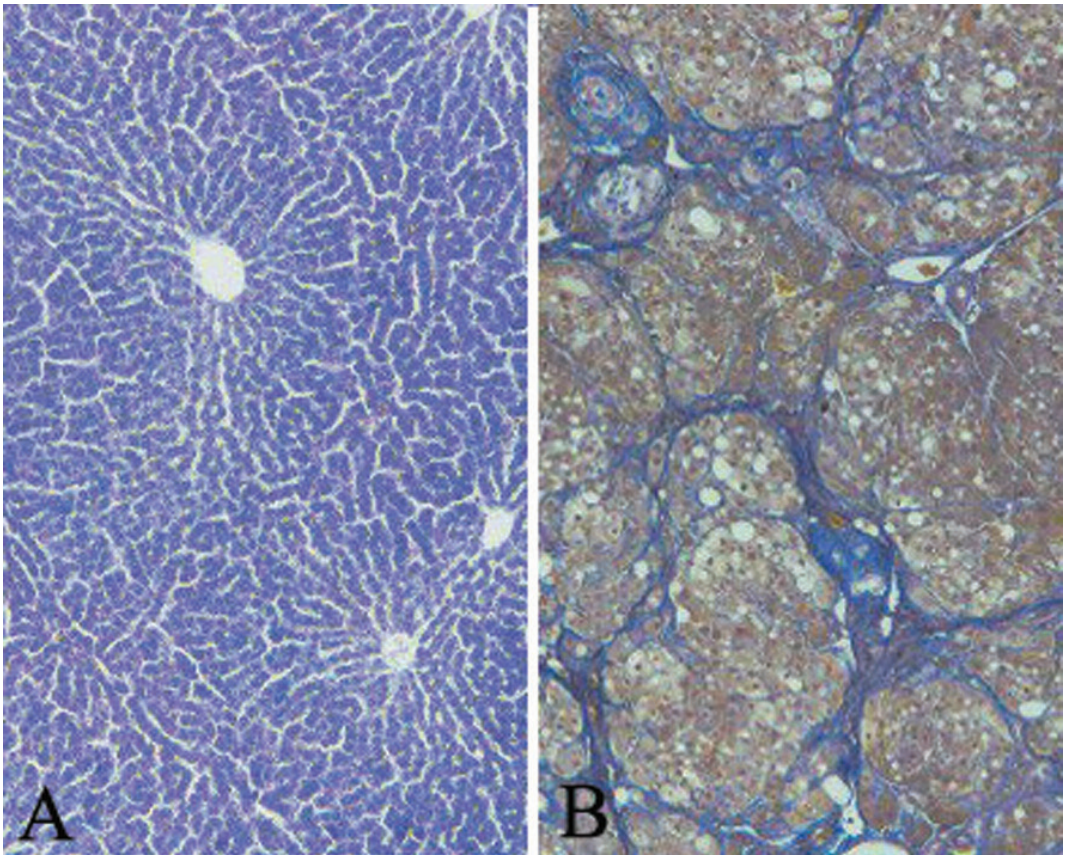

Fig. 1. Histological examination of rat liver after chronic CCl_4 treatment. **A.** Control rat livers show normal architecture. **B.** After 7 weeks of treatment, a marked collagen deposition and a clear cirrhosis was evident. Liver sections were stained with Mallory trichrome for collagen staining. Original magnification, x 100

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 (Lahaie et al., 1998; Davì et al., 1999). Finally, 8-epi-$PGF_{2\alpha}$ seems to mediate the increased production of transforming growth factor-ß1 (TGF-ß1) in kidney mesangial and glomerular cells exposed to high ambient glucose, such as that produced by streptozotocin-induced diabetes (Montero et al., 2000).

Isoprostanes as mediators of hepatic fibrosis

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 CCl_4 experimentally-induced hepatotoxicity, besides the classical steatonecrosis, fibrosis also develops and evolves into cirrhosis in chronic intoxication. CCl_4 hepatotoxicity is now considered a model of oxidative stress *in vivo* (Kadiiska et al. 2005).

The relationship between oxidative stress and collagen hyperproduction was first proposed by Chojkier and coworkers (Chojkier et al., 1989) 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.

One of the most effective fibrogenic mediators is TGF-ß1, which strongly stimulates the production of matrix proteins (particularly collagen) in various cellular types (Ignotz and Massagué, 1986). In chronic CCl_4 intoxication (Armendariz-Borunda et al., 1990) an increase in TGF-ß1 mRNA occurs in nonparenchymal cells. Among liver nonparenchymal cells, hepatic stellate cells (HSC) (lipocytes or Ito cells) 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, with expression of alpha-smooth muscle actin (α-SMA) (Friedman, 2000).

It has been reported (Parola et al., 1993) that lipid peroxidation induced *in vitro* in human HSC, or the treatment of the latter with 4-HNE, stimulates the expression of procollagen α1 (I) gene. Also, the treatment of various lineages of macrophages (Leonarduzzi et al., 1997) with 4-HNE has been reported to induce mRNA production and synthesis of TGF-ß1. Finally, 4-HNE added to cultured HSC up regulates the synthesis of procollagen α1 (I) (Zamara et al., 2004).

Since aldehydic lipid peroxidation products have been reported (as mentioned above) 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

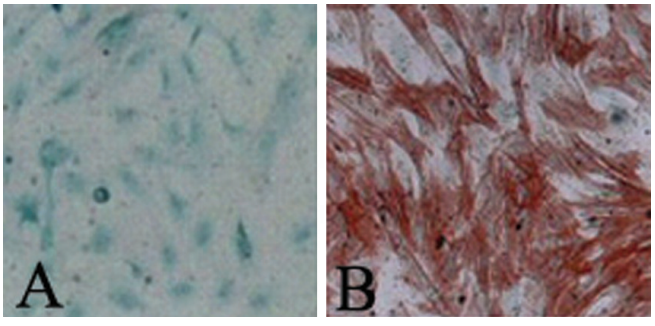

Fig. 2. Expression of α-SMA in activated HSC. **A.** After 1 day, control cells appear quiescent and negative for the marker. **B.** By 7 days in culture, all the cells are activated and show the marker α-SMA. Original magnification, x 120

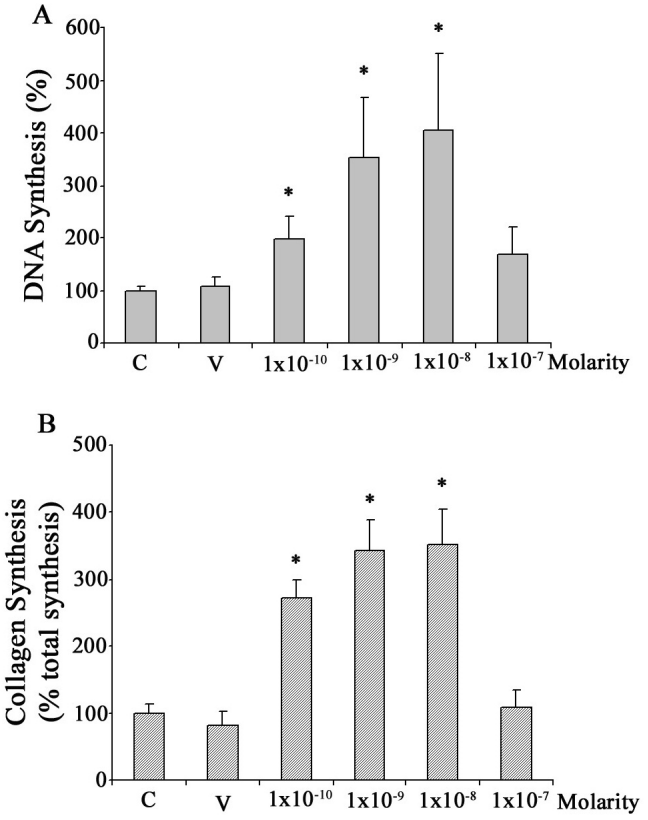

Fig. 3. Fibrogenic effects of 8-epi-$PGF_{2\alpha}$ on HSC. **A.** DNA synthesis was evaluated as ^{3}H-thymidine incorporation. Results are expressed as percentage assuming control as 100%. **B.** Collagen synthesis was estimated as ^{3}H-proline incorporation and data are expressed as percentage of total protein production (collagen plus noncollagen proteins) *p<0.05 vs control. C, control; V, vehicle. (Modified from Comporti et al., 2005).

isoprostanes over aldehydes is that, while aldehydes can interact with cellular macromolecules by addition processes only, isoprostanes could interact with receptors able to induce specific signal transduction pathways.

As previously mentioned, elevated levels of plasma F_2-isoprostanes have been found in acute CCl_4 intoxication. We therefore followed a model of chronic intoxication with CCl_4 leading to hepatic fibrosis and investigated whether the allegedly elevated levels of F_2-Isoprostanes could be related to the increased hepatic collagen production.

Rats were injected i.p. three times weekly with relatively small doses (62 μl/100g body wt) of CCl_4 for a period of 7 weeks. The histological aspects of the livers of the intoxicated animals showed a progressive fibrosis and finally a clear cirrhosis (Fig. 1) after 7 weeks of treatment (Comporti et al., 2005).

The levels of plasma F_2-isoprostanes, even if lower than in the acute intoxication, are maintained much elevated for the entire experimental period with a particular rise at 7 weeks, which seems to be paralleled by the amount of the hepatic collagen (measured as hydroxyproline) (Comporti et al., 2005).

Effect of F_2-isoprostanes on HSC

In parallel studies, we examined the effects of isoprostanes on cultured HSC isolated from normal rat liver. At the seventh day of culture, all the cells were activated to the myofibroblast-like phenotype (expression of α-smooth muscle actin, α-SMA) (Fig. 2). The cells were deprived of serum and then treated for 48 hr with isoprostanes (8-epi-$PGF_{2\alpha}$) in the range of concentrations seen in the *in vivo* experiment (10^{-8} to 10^{-10} M).

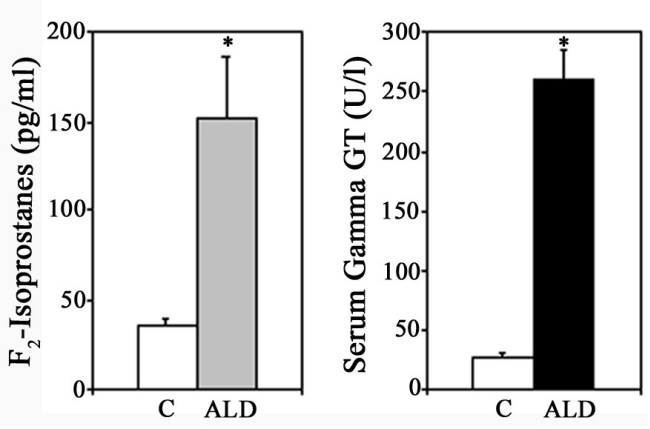

Fig. 4. Plasma F_2-isoprostanes and gamma GT in patients with alcoholic liver disease (ALD). Results are the means ± s.e.m. of 10 patients with alcoholic liver disease and 17 healthy adult controls. All the patients showed liver fibrosis or cirrhosis and were heavy drinkers. *p<0.001.

As can be seen in Figure 3A, the isoprostane addition induced a marked increase in DNA synthesis, as measured by tritiated thymidine incorporation and of cell proliferation (as measured by the cell counts, not shown in the Figure), as well as a striking increase in collagen synthesis, as measured by tritiated proline incorporation. The relative collagen production, i.e. the percentage of collagen production over total protein production (collagenic plus non collagenic proteins), was increased by 3.0-3.5 fold (Fig. 3B). Total collagen content of the culture (measured with Sirius Red) was similarly increased (Comporti et al., 2005). The most active concentrations were between 10^{-8} and 10^{-9} M (= 10 nM and 1 nM), exactly like those found in the *in vivo*

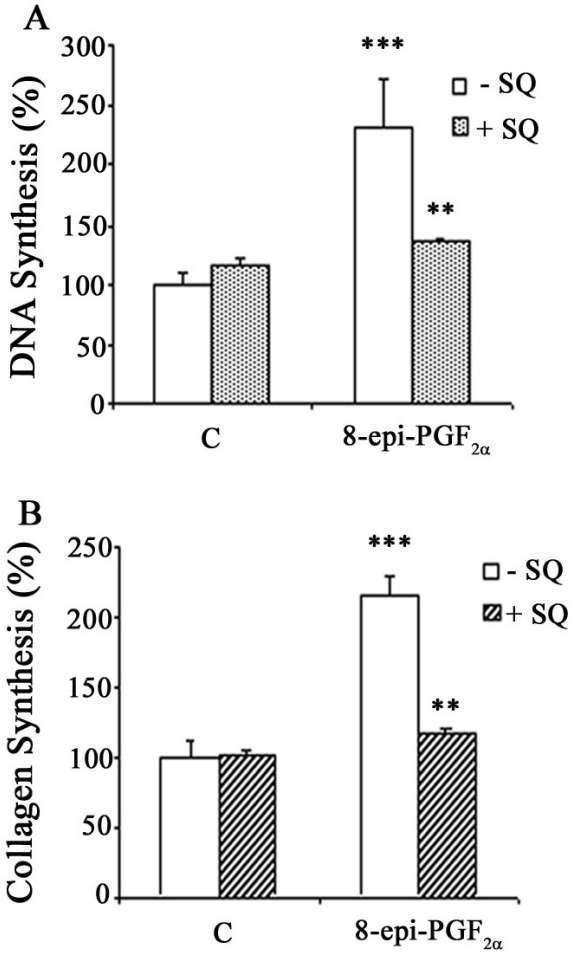

Fig. 5. Inhibition of F_2-isoprostane-evoked fibrogenic effects by SQ29548. Cells were treated with 8-epi-$PGF_{2\alpha}$ in the presence or absence of SQ29548, in a molar ratio of 1:10. **A.** DNA synthesis was evaluated as 3H-thymidine incorporation. Results are expressed as percentage assuming control as 100%. **B.** Collagen synthesis was estimated as 3H-proline incorporation and data were expressed as percentage of total protein production (collagen plus noncollagen proteins) ***p<0.001 vs control; **p<0.05 vs 8-epi-$PGF_{2\alpha}$. C: control; SQ: SQ29548. (Modified from Gardi et al., 2008).

intoxication (3000-500 pg/ml of plasma =9.0-1.5 pmol/ml → 9.0 -1.5 nM).

Since it is generally believed (De Bleser et al., 1997) that activation of HSC follows the release of soluble factors (cytokines, mainly TGF-ß1) by cells of macrophage lineages, the effects of F_2-isoprostanes on TGF-ß1 release by the human promonocyte cell line U937, assumed as a model for Kupffer cells or liver macrophages, was also studied. F_2-isoprostanes increased the production of TGF-ß1 by U937 cells (Comporti at al., 2005). This could suggest an alternative pathway of stimulation of HSC through TGF-ß1, with consequent collagen synthesis.

Finally, Figure 4 shows the results for plasma F_2-isoprostanes in a small group of patients (all heavy drinkers) with alcoholic liver disease (with initial or prominent fibrosis), showing a marked increase of isoprostanes in the alcoholic patients and of plasma GGT as marker of liver damage.

In summary, we propose that F_2-isoprostanes generated by lipid peroxidation in hepatocytes act on HSC and mediate collagen hyperproduction in this model of hepatic fibrosis.

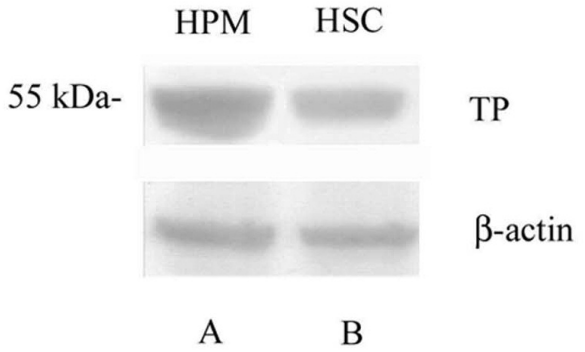

Fig. 6. Immunoblotting identification of the TxA_2r in HSC membrane lysates. Incubation with an antibody raised against the terminal aminoacids of human TxA_2r (TPr) (1:1000 dilution) revealed immunoreactivity for a 55 kDa protein in HSC membrane lysates (lane B). Lysates from human platelet membranes (HPM, lane A) were used as positive control. Blots were reprobed with ß-actin to assess equal loading (from Gardi et al., 2008).

Search for 8-epi-$PGF_{2\alpha}$ receptors on HSC

In view of the results obtained with the addition of isoprostanes to cultured HSC, in further studies receptors for 8-epi-$PGF_{2\alpha}$ on HSC were thoroughly searched for. Since, as previously stated, most of the known effects of isoprostanes appear to be mediated through receptors identical or analogous to those for thromboxane A_2 (TxA_2r), we investigated whether the specific antagonist of TxA_2r, SQ29548, is able to inhibit the effects of isoprostanes in HSC. As be can seen (Fig. 5A), the isoprostane-induced stimulation of DNA synthesis is almost completely abolished in the presence of SQ29548. Such inhibition seems to be of competitive type since it increases progressively with the increase of concentrations of SQ29548 (Comporti et al., 2005).

Moreover, we have seen that an effect similar to that of 8-epi-$PGF_{2\alpha}$ (even if to a lower level, but anyhow statistically significant) is produced by I-BOP, the specific agonist of TxA_2r, and that even this effect, still on DNA synthesis, is abrogated by SQ29548 (Gardi et al., 2008).

In addition, we have seen (Fig. 5B) that even the other effect of 8-epi-$PGF_{2\alpha}$ on HSC, i.e. the stimulation of collagen synthesis, is completely antagonized by SQ29548 (Gardi et al., 2008). Therefore, the effects of isoprostanes on HSC seem to be mediated by TxA_2r.

Finally, the expression of TxA_2r on HSC was demonstrated by immunoblot analysis of membrane proteins using a polyclonal antibody raised against C-terminal aminoacids of human TxA_2r (TPr). A single immunoreactive band was present at 55 kDa (Fig. 6) (Gardi et al., 2008).

These data were confirmed by immunocyto-

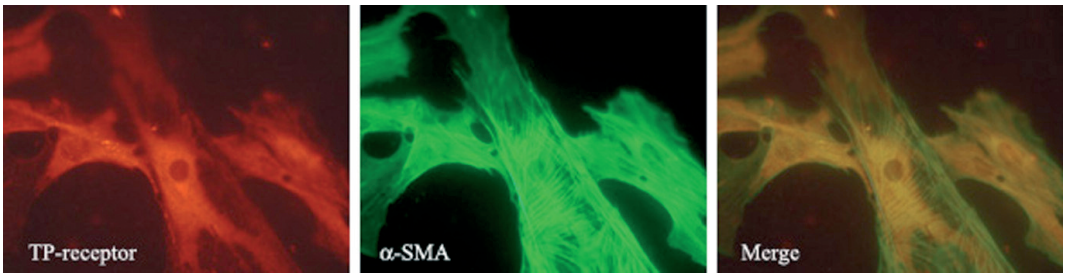

Fig. 7. Co-localization of TxA_2r (TPr) and α-SMA in HSC. Activated HSC cells expressed both TxA_2r (TPr) revealed by TRITC (crystalline tetramethylrhodamine isothiocyanate) (red labeled) and α-SMA revealed by FITC (fluorescein isothiocyanate) (green labeled). Merged images (yellow) show the degree of co-localization. Original magnification, x 400

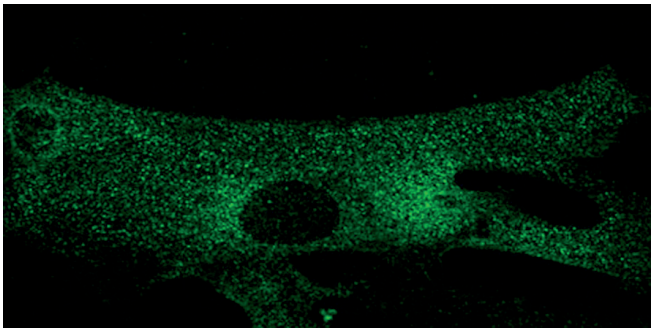

Fig. 8. Subcellular localization of TxA_2r (TPr) in HSC. Cells were permeabilized, incubated with anti-TxA_2r antibody and then with FITC (fluorescein isothiocyanate) conjugated anti-rabbit IgG. Confocal image shows that TxA_2r (TPr) is predominantly expressed in the area around the nucleus. Original magnification, x 900

chemistry studies that demonstrated that TxA_2r colocalize with α-SMA (Fig. 7). Confocal analysis (Fig. 8) and colocalization studies (Fig. 7) indicated that TxA_2r is localized on both cell surface and inside of the cell. In particular TPr immunoreactivity was predominantly detected in the region of cytoplasm surrounding the nucleus (Fig. 8). All these immunocytochemical studies gave negative results on quiescent (non activated) HSC, indicating that these cells do not constitutively express TPr but that this receptor appears only in activated cells.

Binding studies have been carried out in our laboratory to characterize the population of TxA_2 receptors in HSC and the results have been reported elsewhere (Gardi et al., 2008).

Studies on the signal transduction pathways involved in the effects of 8-epi-$PGF_{2\alpha}$ on HSC are ongoing. Since in vascular smooth muscle cells 8-epi-$PGF_{2\alpha}$ exerts its biological effects through TxA_2r by stimulating the phosphoinositide pathway, we studied this pathway in activated HSC stimulated by 8-epi-$PGF_{2\alpha}$ (10^{-9} M). Preliminary results indicated a striking increase (about 6 times) in Inositol triphosphate (IP_3) production 5 min after isoprostane addition (unpublished data). Therefore, it is likely that this is one of the main signal transduction pathways involved in the effects of isoprostanes on HSC.

In conclusion, our studies show that: i) 8-epi-$PGF_{2\alpha}$ stimulates DNA and collagen synthesis in activated HSC; ii) these cells express receptors for TxA_2r ligands; iii) these receptors mediate the 8-epi-$PGF_{2\alpha}$ effects; iv) one of the signal transduction pathways involved seems to be that of phosphoinositide.

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