FREE RADICALS, OXIDATIVE STRESS AND ANTIOXIDANTS

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The topic of oxidative stress is deep-routed in some biological paradoxes.
Oxygen is necessary for the life of aerobic organism and acts as terminal oxidant in the mitochondrial respiratory chain, that is the main source of energy. However the univalent reduction of oxygen leads to the formation of one of the reactive oxygen species, the superoxide anion ($O_2^{-\bullet}$). In the mitochondrial electron transport chain about 95% of the consumed oxygen undergoes the cytochrome oxidase catalysed tetravalent reduction to H_2O, but over than 3-5% of the consumed oxygen is released in the form of reactive species, such as $O_2^{-\bullet}$ and hydrogen peroxide (1). Thus reactive oxygen species are produced even in the normal respiratory chain.
Iron is necessary for life too, being an essential component of vital enzymes, such as cytochromes, cytochrome oxidase, catalase and of equally vital complexes such as hemoglobin, myoglobin, ferritin, etc. However iron, when released from these complexes in a free form, as it will be seen below, can react with active oxygen species to yield additional oxy-radicals (through the Fenton reaction). Iron is therefore potentially toxic to biological structures.
Similar considerations are true for cytochrome P_{450} (cyt P_{450}) a key component of the mixed function oxidase system, which is utilized, in its microsomal electron transport chain, by the drug metabolising system. This system, that is basically involved in the detoxification of xenobiotics, can produce harmful radicals or electrophilic intermediates from xenobiotics themselves.
The mechanisms of free-radical induced cell injury include, in summary, (i) reactions with nucleic acids, nucletides, polysaccharides, protein and non protein thiols (thiol oxidation); (ii) covalent binding to membrane components (proteins, lipids, enzymes, receptors, transport systems, etc.); and (iii) initiation of lipid peroxidation, schematically illustrated in Fig.1 (see the legend for summary explanations).
Lipid peroxidation of cellular membranes has been suggested as a common mechanism in a large number

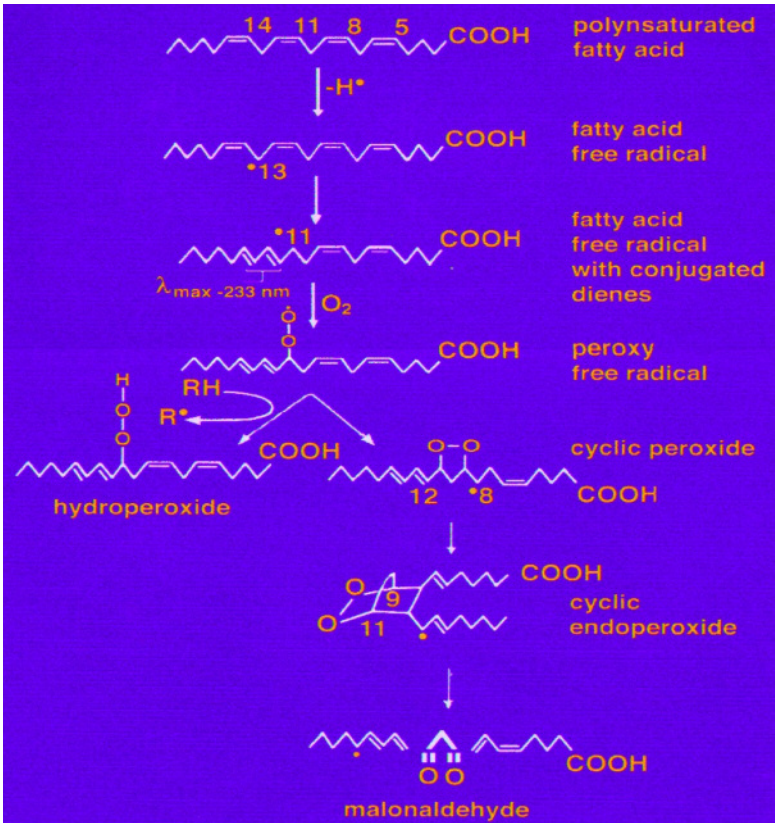

Fig.1 - Scheme of lipid peroxidtion
The free radical attack at a carbon of the methylenic group adiacent to a double bond of the polyunsaturated fatty acid is shown with the consequent formation of a fatty acid free radical. After shift of the double bond because of resonance, conjugated dienes are formed which show both characteristic U.V. absorption at 233 nm and change in the steric conformation of the olefinic chain from the cis to the trans form. The fatty acids free radical then interacts with molecular oxygen and the peroxy free radical is formed which undergoes various pathways shown in the scheme. Finally the fatty acid molecule is broken into various fragments one of which is malonaldehyde as shown in the scheme.

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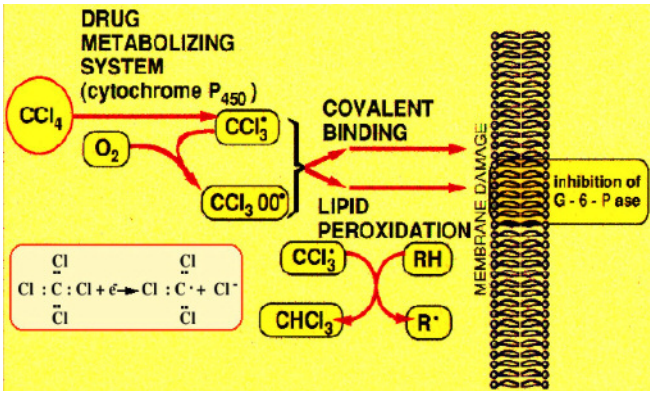
of biopathological conditions. Yet, up to some decades ago lipid peroxidation was only known in the chemistry of oil and fat rancidity, and its interest was confined mainly to the field of food technology. The spreading out of interest in lipid peroxidation in the field of biopathology in the half of the 60's was mainly due to (i) the knowledge that lipid peroxidation can be linked to the microsomal electron transport chain of drug metabolism (2); (ii) the recognition that the metabolism of the model molecule, prototype of experimental pathology of that time, carbon tetrachloride, yields aloalkane free radicals (3); and (iii) our observation that CCl_4 in fact greatly stimulates the peroxidation of liver microsomal lipids (4).

Today it is well established that lipid peroxidation is only one of the reactions set into 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 SOD (5) and with the consequent acquisition that oxy radicals can be rather easily produced in the living tissues, a much more complex spectrum of pathophysiological and pathological oxidations has been progressively recognized, so that the term of "oxidative stress" has been introduced (6), to signify any condition in which the prooxidant/antioxidant balance is shifted in favour of oxidations.

When over fifty years ago, I started to work in oxidative stress (but at that time only the term lipid peroxidation or the "thiobarbituric acid reaction" was mentioned) it was only known (7) that liver homogenates from vit. E deficient animals produced, after aerobic incubation, a higher amount of malonic dialdehyde (MDA) with respect to controls. Today formation of free radicals, particularly from oxygen, and initiation of oxidation and peroxidation processes are implicated in a very large number of conditions of cellular damage belonging not only to experimental pathology but even to human spontaneous pathology (8).

Our studies of oxidative stress have been carried out through the use of various models of experimental pathology; for the sake of brevity, I will only give some flashes on that of carbon tetrachloride hepatotoxicity, on that of GSH depleting agents' toxicity and on that of the release of iron in a free form and its relationships with erythrocyte ageing. With regard to the CCl_4-model, Fig.2 shows the homolytic cleavage of the carbon-halogen bond occurring during the metabolism of the molecule in the drug metabolism of the hepatocyte, with formation of CCl_4 radicals (3) (you can see the one electron reduction of the molecule catalysed by cytochrome P_{450}, the latter being maintained in the reduced form by NADPH, the general cofactor of drug metabolism).

***Fig.2** - Metabolism of CCl_4 showing the homolytic cleavage of the carbon-halogen bond and the consequent formation of the trichloromethyl radical ($CCl3^{\bullet}$).*



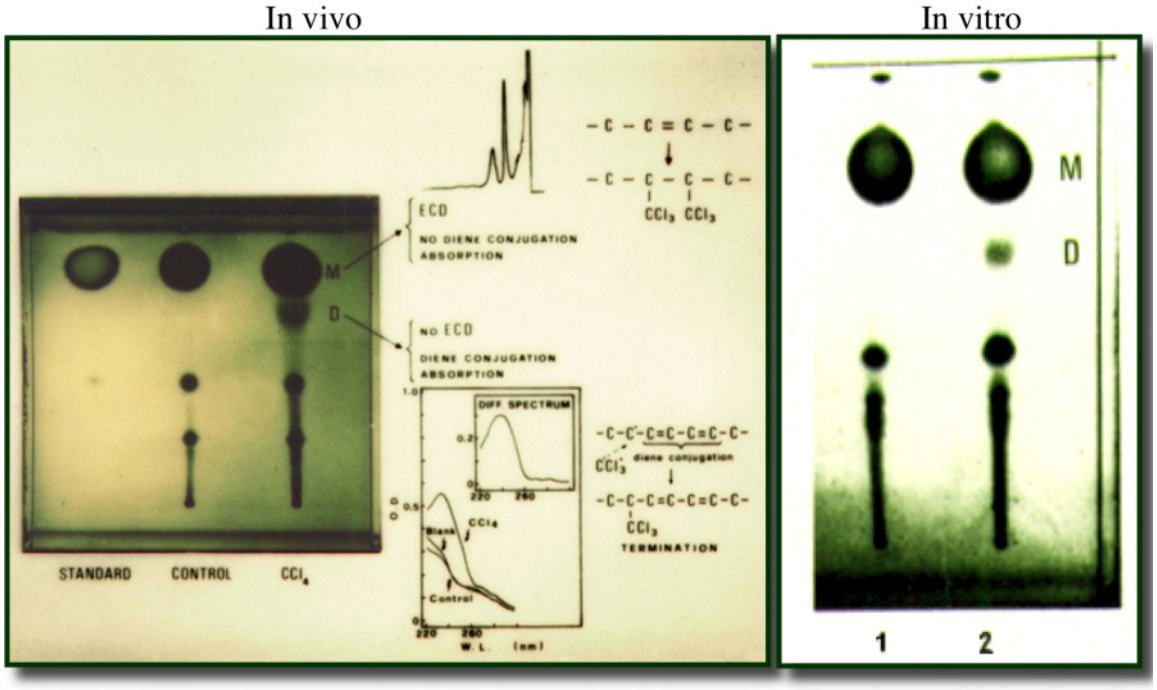


***Fig.3** - **In vivo:** thin layer chromathography (TLC) of fatty acids methyl esters (M) of liver microsomal lipids of control and CCl4-intoxicated (250 µl/100 g body wt) rats (sacrifice at 1 hr). The GLC analysis with electron capture detection (ECD) is reported as well as the absolute absence of U.V. absorption of conjugated dienes. (D) The spot containing the entire U.V. absorption of conjugated dienes and the absolute absence of the response of GLC with ECD is shown.*
***In vitro:** TLC of fatty acid methyl esters (M) of lipids of liver microsomes incubated under anaerobic conditions in the presence of CCl_4 (~150 µg/ml of incubation mixture). Both band M and band D are clearly visible.It must be pointed out that in this anaerobic in vitro system no decrease in microsomal G-6-Pase was observed. (See refs. 12, 13, 14)*

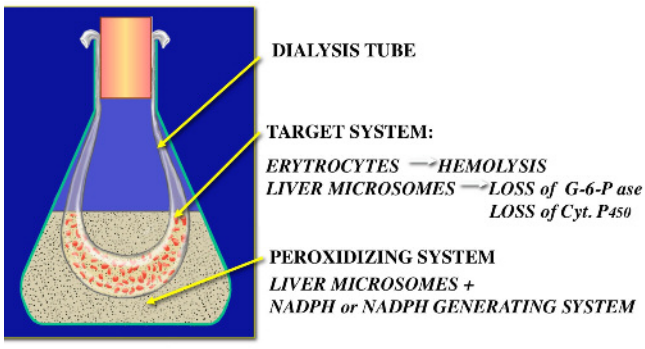
The trichloromethyl radical (CCl3·) is thus generated, which in the presence of oxygen can form the trichloromethyl peroxy radical (Fig. 2). These radicals, whose formation has been then demonstrated in vitro and in vivo (9-11), can act both by covalently binding to lipids and proteins of endoplasmatic reticulum and by initiating lipid peroxidation, which was in fact our pioneristic demonstration (4,12) with liver preparations containing the microsomal fraction.

By dissociating the two pathways in vitro, we showed that at least some early effects of CCl_4, such as the inactivation of microsomal glucose-6-phosphatase (G-6-Pase) (assumed as a test membrane enzyme) are mediated by lipid peroxidation rather than by covalent binding. This because in anaerobic systems it was possible to reproduce (Fig. 3) the molecular alterations induced by CCl_4 in the fatty acids of microsomal lipids, such as conjugated dienes and chloromethyldienes (13,14), without any enzyme inactivation (15).

Therefore some product downstream conjugated dienes in the peroxidative cascade after the intervention of oxygen, seemed to be responsible for the enzyme inactivation (16). These studies, therefore, led to the hypothesis that in addition to the local membrane damage, toxic products originate from lipid peroxidation of cellular membranes, diffuse and act at distant loci, as second toxicological messengers of cell injury (16).

In this line of studies, we showed (17) that products originating from the peroxidation of liver microsomal lipids are capable of inducing cytopathological effects (hemolysis, inhibition of microsomal enzymes, such as G-6-Pase and cyt. P_{450}) in revealing or target systems (red blood cells, or liver microsomes, respectively), which were separated from the peroxidizing system by a dialysis membrane (Fig. 4).

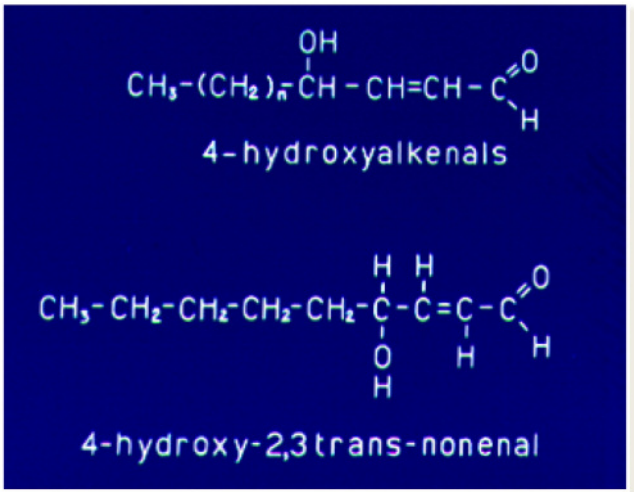
Fig.4 *- Scheme of the cytopathological effects brought about by dialysable products originayting from the peroxidation of liver microsomal lipids.*



Thus we had to do with metastable products (much more stable than the CCl_4 radicals), capable of crossing a dialysis membrane and of inducing pathological effects at a distance.

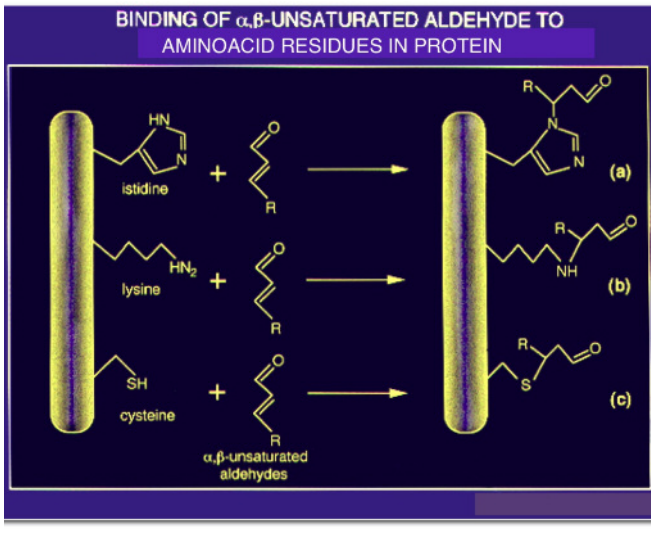
In further studies (18) some of these toxic products were separated from the dialysate and characterized. Of particular importance was the identification (19) of an aldehyde of the class of 4-hydroxyalkenals, namely 4-hydroxy-2,3-trans-nonenal (4-HNE) (Fig. 5).

Fig.5 *- Four-hydroxyalkenals and 4-hydroxynonenal (4-HNE)*



These aldehydes are provided with an extremely high biological reactivity, mainly due to their capacity to bind to nucleophilic groups by their double bonds in α-β position; and the reaction, that is a Micheal addition, occurs whit –SH groups (thioether linkage with C_3), with -NH_2 groups and with - NH groups (himidazolic ring of histidine) (Fig. 6).

Fig.6 *- Reactivity of 4-hydroxyalkenals with -SH and amino groups of mcromolecules*

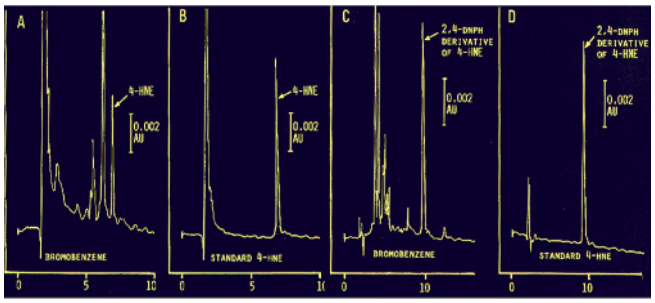


4-HNE and related aldehydes show inhibitory effects on an extremely large number of biological functions (20) and can be considered mediators of the cellular damage produced by agents which, like CCl_4, promote lipid peroxidation. In further studies (21,22) we demonstrated (Fig. 7) that 4-HNE and related aldehydes are in fact formed in vivo in the liver of animals intoxicated with various prooxidants. Also, this aldehyde

(4-HNE) has been extensively studied (20) and regarded as the model molecule of oxidative stress.

Fig.7 *- Detection by high pressure liquid chromatography (HPLC) of 4-hydroxynonenal (4-HNE), as free aldehyde (A,B) or 2,4-dinitrophenylhydrazone (2,4-DNP) derivative (C,D), in the liver (≈5mg of protein) of bromobenzene-poisoned mice. See ref.21,22 for operating conditions.*

(A) 4-HNE in the liver of bromobenzene-treated mice.
(B) Standard 4-NHE (5pmol).
(C) 2,4-DNP derivative of 4-HNE in the liver of bromobenzene-treated mice.
(D) 2,4-DNPH derivative of standard 4-HNE.
Control mice showed no 4-HNE



Coming to the second model of oxidative stress mentioned above, that concerned with GSH depleting agents (Fig. 8), we have studied (23,24) the intoxications with bromobenzene and analogues, allyl alcohol and diethylmaleate, which (as shown in Fig. 8), follow different metabolic pathways in the hepatocyte, all producing GSH depletion.

Fig.8 *- Glutathione (GSH) depleting agents used as experimental models of oxydative stress and the relative pathways of liver metabolism.*

In a typical experiment of bromobenzene intoxication of the mouse (Fig. 9) a very marked hepatic GSH depletion occurs within the first hours (or minutes in the case of allyl alcohol) (23,24). Subsequently (15-18 hr with bromobenzene, 2-4 hr in the case of allyl alcohol) severe lipid peroxidation develops and is accompanied by a likewise severe liver necrosis.

Fig.9 *- Typical experiments of bromobenzene or allyl alcohol intoxications in the mouse with the time-course of hepatic GSH depletion, hepatic lipid peroxidation (MDA) and liver necrosis (serum SGPT).*

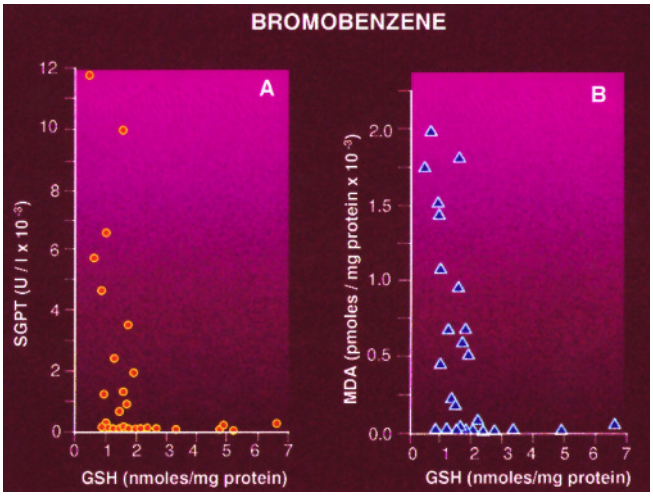
BROMOBENZENE
(13 mmol/Kg b.w., per os)

Time after intoxication	0 time	3 hr	12 hr	15 hr	18 hr
GSH (nmol/mg protein)	24.3 ± 1.7	3.7 ± 0.2	2.2 ± 0.2	2.4 ± 0.3	1.9 ± 0.3
Hepatic MDA content (pmol/mg protein)	---	0	3 ± 2	189 ± 85	1097 ± 406
SGPT (units/L)	46 ± 5	48 ± 30	63 ± 15	2578 ± 1389	4669 ± 1545

ALLYL ALCOHOL
(1.5 mmol/Kg b.w., per i.p.)

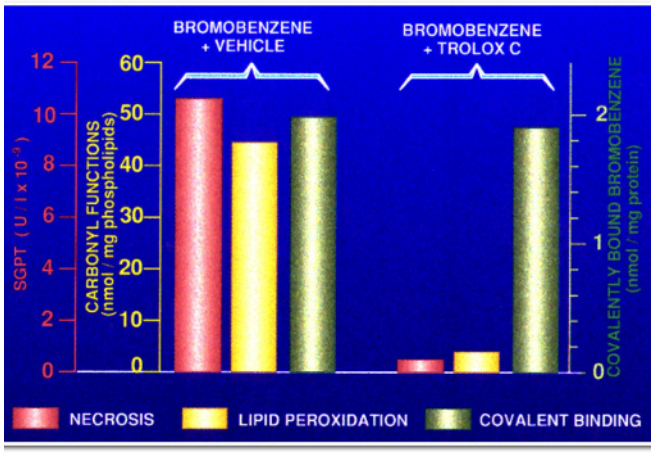
Time after intoxication	0 time	30 min	2 hr	4 hr
GSH (nmol/mg protein)	22.3 ± 0.8	2.4 ± 0.2	3.3 ± 0.4	6.3 ± 1.0
Hepatic MDA content (pmol/mg protein)	---	12 ± 2	55 ± 16	225 ± 63
SGPT (units/L)	36 ± 7	116 ± 39	591 ± 206	1639 ± 310

Fig.10 *- Plot of the individual values for lipid peroxidation (MDA) or liver necrosis (serum GPT) against the corresponding hepatic GSH levels in bromobenzene-intoxicated mice.*



The plot (Fig. 10) of the individual values for lipid peroxidation and for serum transaminases (liver necrosis) against the corresponding hepatic GSH levels shows that the two phenomena develop only when the hepatic GSH depletion has reached critical or threshold values (23). The treatment of the animals (Fig. 11), even after the intoxication, with antioxidants (Trolox C in this case, but even with other antioxidants) completely prevents both lipid peroxidation and liver necrosis, while not changing at all the extent of the covalent binding of bromobenzene metabolites to liver protein (23).

Fig.11 - Effects of the treatment with the antioxidant Trolox C on liver necrosis (serum GPT), lipid peroxidation (carbonyl functions in liver phospholipids) and covalently bound bromobenzene to liver protein in bromobenzene-intoxicated mice.



These studies therefore indicated that, contrary to what assumed for a long time, covalent binding is not a factor in this type of hepatotoxicity, rather that lipid peroxidation seems to be implicated.

The third model of oxidative stress mentioned above is that related to iron release from its macromolecular complexes. Iron redox cycling, in fact, is at the base of the Fenton reaction which produces the potent oxidant, hydroxyl radical.

Normally iron is transported and stored in specific proteins (transferrin, ferritin, haem proteins) that prevent its reaction with reduced oxygen species. Thus, to be redox cycling active, iron has to be released from these complexes. Studies from our laboratory have shown (25) that iron is released in a free (desferrioxamine (DFO) chelatable) form when mouse erythrocytes are incubated with a number of oxidizing agents, such as phenylhydrazine, divicine, isouramil, acrolein, phenylhydroxylamine and dapsone hydroxylamine.

The results (25) obtained with phenylhydrazine are reported in Fig.12: as can be seen, a consistent release of iron occurs; iron is released from hemoglobin or heme (26) and the release is accompanied by methemoglobin formation. If the erythrocytes are depleted of GSH (which can be easily obtained by a short preincubation with diethylmaleate, DEM), the release of iron is also accompanied by lipid peroxidation and hemolysis (25).

A similar release of iron also occurs (Fig.13) during the erythrocyte ageing, here experimentally reproduced by a prolonged (24-48-60hr) aerobic incubation in buffer (a model of rapid in vitro ageing of erythrocytes), and it is accompanied by methemoglobin formation (27); whereas no substantial iron release nor so massive methemoglobin formation occurs when the incubation is carried out under anaerobic conditions (Fig.13), which again suggests the involvement of oxidative stress in iron release.

Fig.13 - Iron release and methemoglobin (Met-Hb) formatioin during erythrocyte ageing, experimentally reproduced by a prolonged aerobic incubation in buffer (model of rapid in vitro ageing of erythrocytes). The results of the anaerobic incubation are reported for comparison.

Release of iron (DFO-chelatable iron) and methemoglobin (Met-Hb) formation in calf erytrocytes incubated in phosphate buffer under aerobic or anaerobic condition

	Incubation time (h)	"free iron" (nmol/ml)	Met-Hb (nmol/ml)
Aerobic Incubation	0	1.7 ± 0.3	169 ±6
	24	5.2 ± 0.9	460 ±30
	48	11.6 ±1.7	1690 ±60
	60	36.0 ± 2.0	3866 ±121
Anaerobic incubation	24	2.2 ± 0.3	130 ±30
	48	3.1 ± 0.9	247± 37
	60	5.0 ± 0.9	324± 45

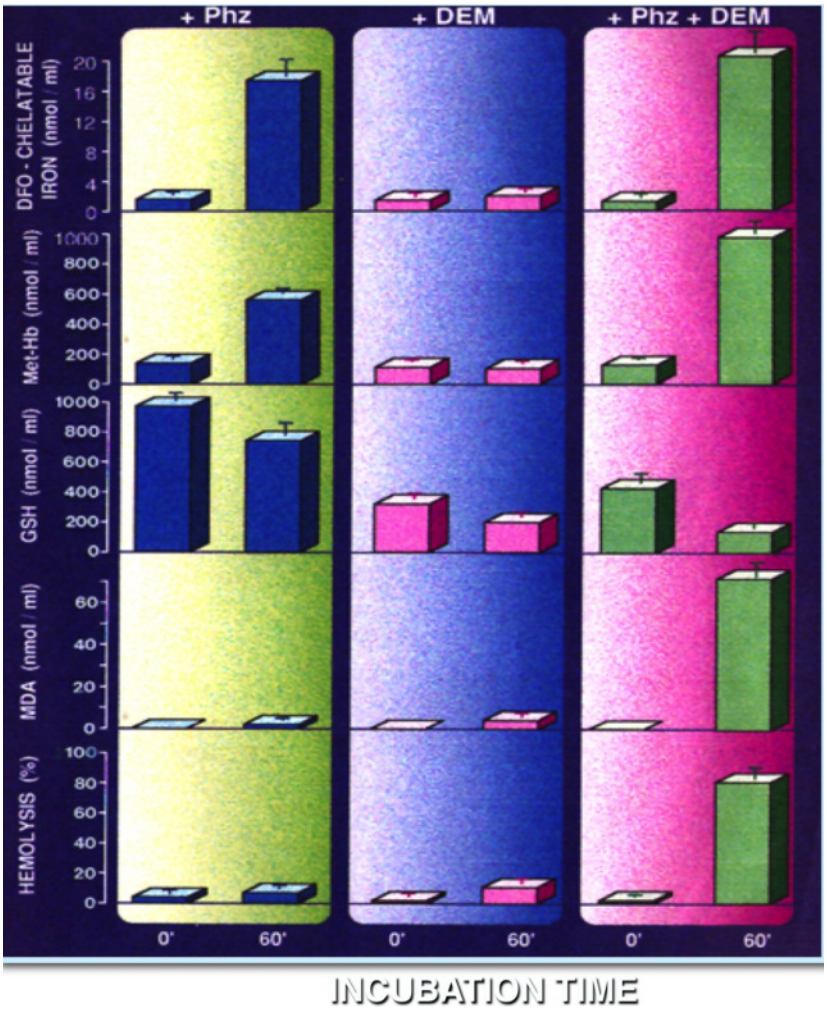
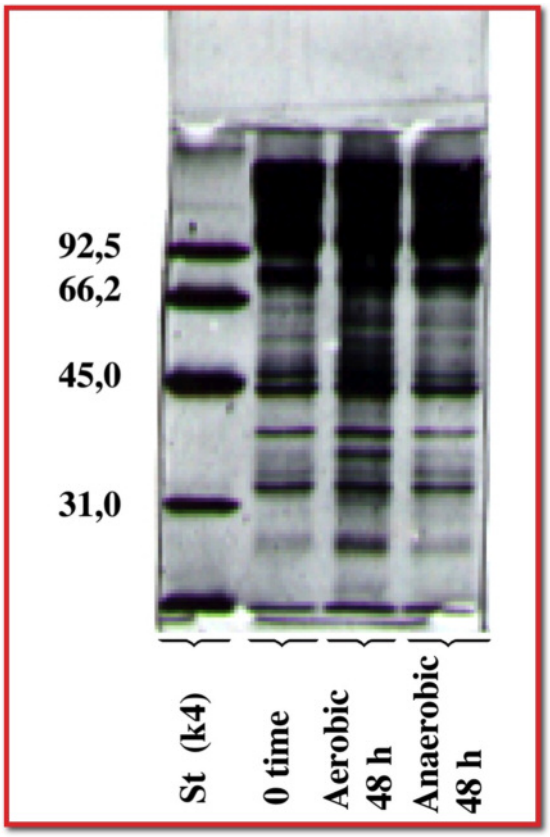


Fig.12 (right) - Iron release (DFO-chelatable iron), methemoglobin (Met-HB), gluthatione (GSH) decrease, lipid peroxidation (MDA) and hemolysis in erithrocytes incubated with phenylhydrazine (Phz) or preincubated with diethylmaleate (DEM) and then incubated with phenylhydrazine.

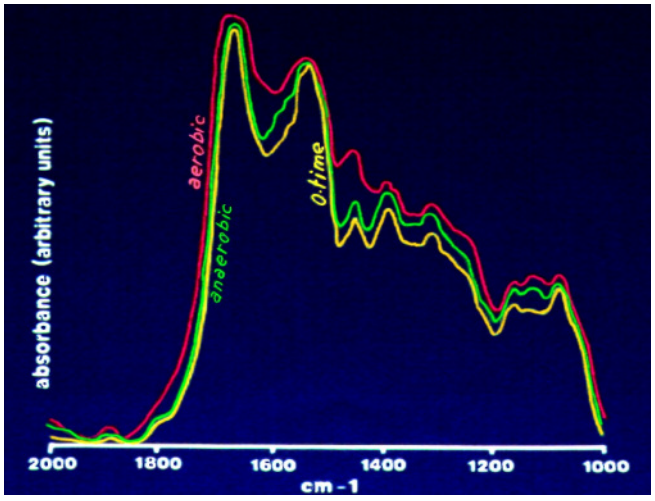
The release of iron is also accompanied by oxidative alterations of membrane proteins (27) and these alterations have been detected by both PAGE electrophoresis (Fig.14) and infrared spectroscopy (IR).

Fig.14 - PAGE electrophoresis of membrane proteins of erythocytes incubated aerobically or anaerobically as in Fig.13



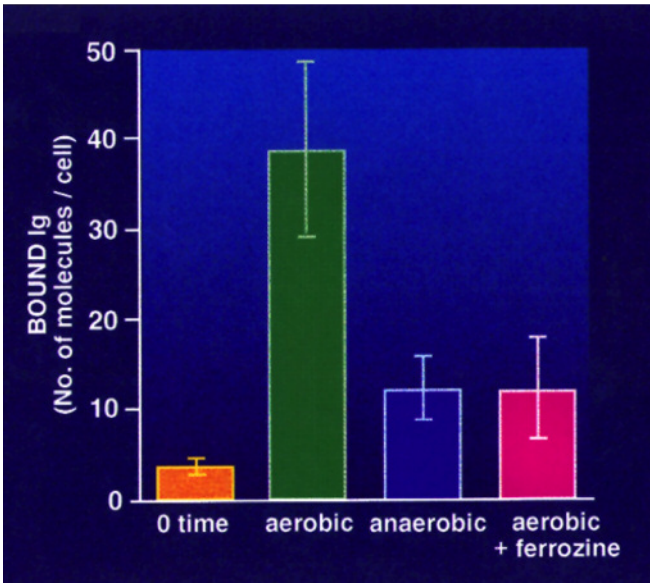
The latter (Fig.15) shows (27) a consistent increase in carbonyl groups (absorption increase in the 1685-1650 cm^{-1} range) and other alterations of the spectrum all related to protein oxidation.

Fig.15 - Infrared (IR) spectroscopy of membrane proteins of erythrocytes incubated under aerobic or anaerobic conditions, as in Fig.13



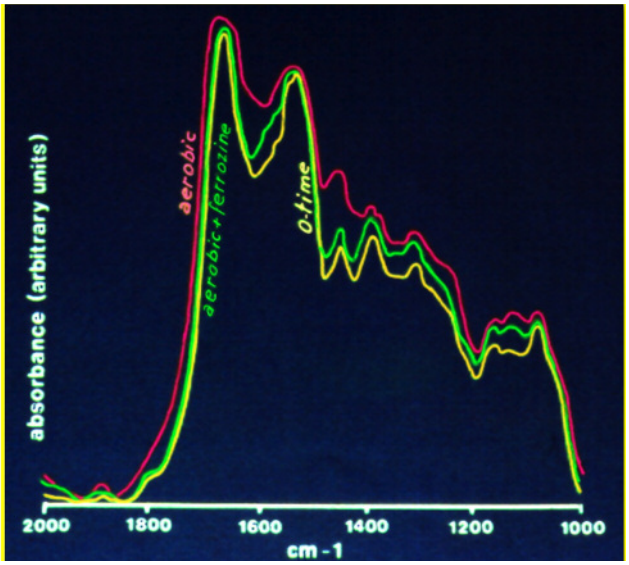
It is generally accepted that senescent cell antigen is generated as a result of oxidative modification of some membrane proteins, particularly band 3, and acts as a specific signal for termination of old cells, by initiating the binding of autologous IgG and subsequent removal by phagocytes.

Fig.16 - Binding of autologous IgG to membrane proteins of erythrocytes incubated aerobically or anaerobically, as in Fig. 13, or incubated aerobically in the presence of the iron chelator ferrozine



As can be seen in Fig.16, in the membranes of aerobically incubated cell (as reported in Fig. 13), the senescent antigen is formed as shown by the binding of autologous IgG, which substantially does not occur after the anaerobic incubation (27). The addition of the iron chelator ferrozine (which freely enters the cells) during the aerobic incubation prevents both the formation of senescent antigen (27), that is the binding of autologous IgG (Fig.16), and the membrane protein oxidative alterations previously (Fig.15) seen in the infrared spectroscopy (the green spectrum of the aerobic plus ferrozine sample is very close to the yellow one of the control, 0 time sample (Fig.17)) (27).

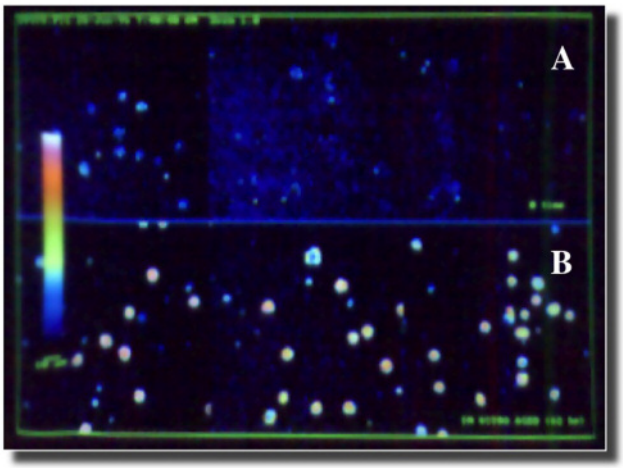
Fig.17 - Infrared (IR) spectroscopy of membrane proteins of erythrocytes incubated aerobically in the presence of the iron chelator ferrozine



These results strongly suggest that ferrozine is capable of chelating, at intracellular level, the iron released during the aerobic incubation and to prevent in such way the alterations of membrane proteins, probably produced by the redox cycling of the released iron and related to the formation of senescent antigen.
Moreover, even more suggestive results have been obtained (28) by using, as protective agent, a synthetic acyl hydrazone, pyridoxal fluor benzoyl hydrazone, which is an iron chelator (as shown in Fig. 18) and enters the cells.

***Fig.18** - Pyridoxal fluor benzoyl hydrazone (PFBH) and its iron chelation.*

This chemical prevented the formation of senescent cell antigen in human erythrocytes incubated aerobically for 48-60 hr. The autologous IgG binding was detected, in these experiments (28), by using an anti-IgG antibody labelled with fluorescein and by examing the cells for fluorescence with confocal microscopy. Fig. 19 shows the non-incubated (control, 0 time) cells (panel A) (only minor fluorescence can be seen); the aerobically incubated cells (panel B) showing extensive fluorescence that is extensive binding; and the cells incubated aerobically in the presence of the acyl hydrazone (panel C and D), which show much less fluorescence (much less binding).
Finally, a progressive iron release (Fig. 20) occurs in human erythrocytes stored in their own plasma at 4°C for 15 and 35 days (29). Therefore an iron release seems to be really related to the ageing of red blood cells and it is likely to occur even under physiological conditions.
In summary the overall scheme would be the following: an oxidative stress in the erythrocytes will promote iron release, which in turn will promote oxidation of membrane proteins and the consequent formation of senescent cell antigen, that is the ageing of erythrocytes.



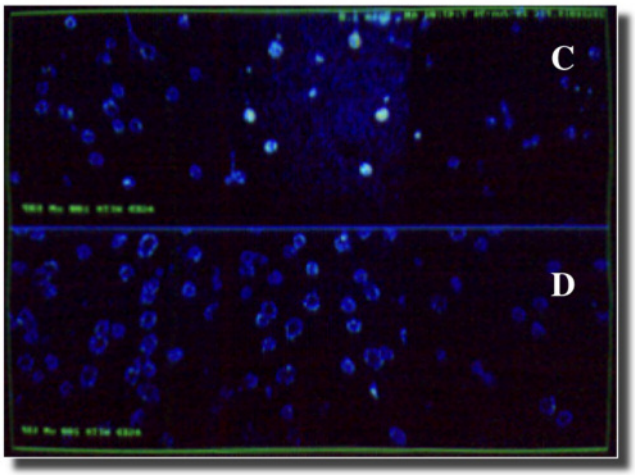


***Fig.19** - Protection by PFBH of erythrocyte ageing. The erythocytes were incubated aerobically for 48-60 h. The autologous IgG binding was detected, in these experiments, by using an anti-IgG antibody labelled with fluorescein and by examining the cells for fluorescence at confocal microscopy.*

A) non incubated (0 time, control) cells;
B) aerobically incubated cells;
C) aerobically incubated cells in the presence of PFBH (100 µM);
D) aerobically incubated cells in the presence of PFBH (200 µM).
A', B', C'and D', same experiment at higher magnification.

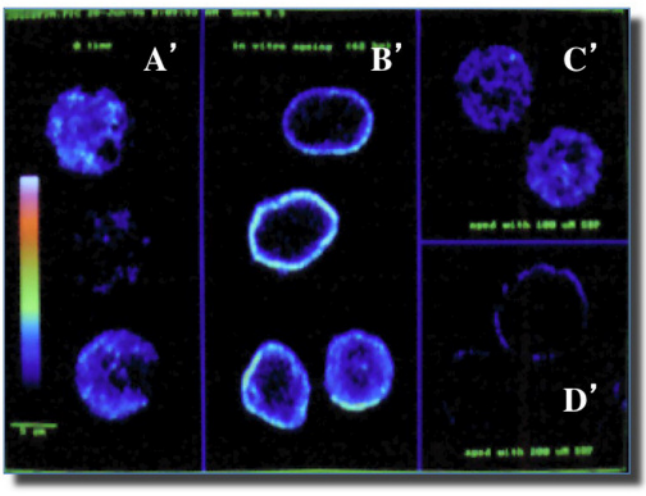


Fig.20 - Release of DFO-chelatable iron and methemoglobin (Met-HB) formation in human erythrocytes stored in their own plasma at 4°C

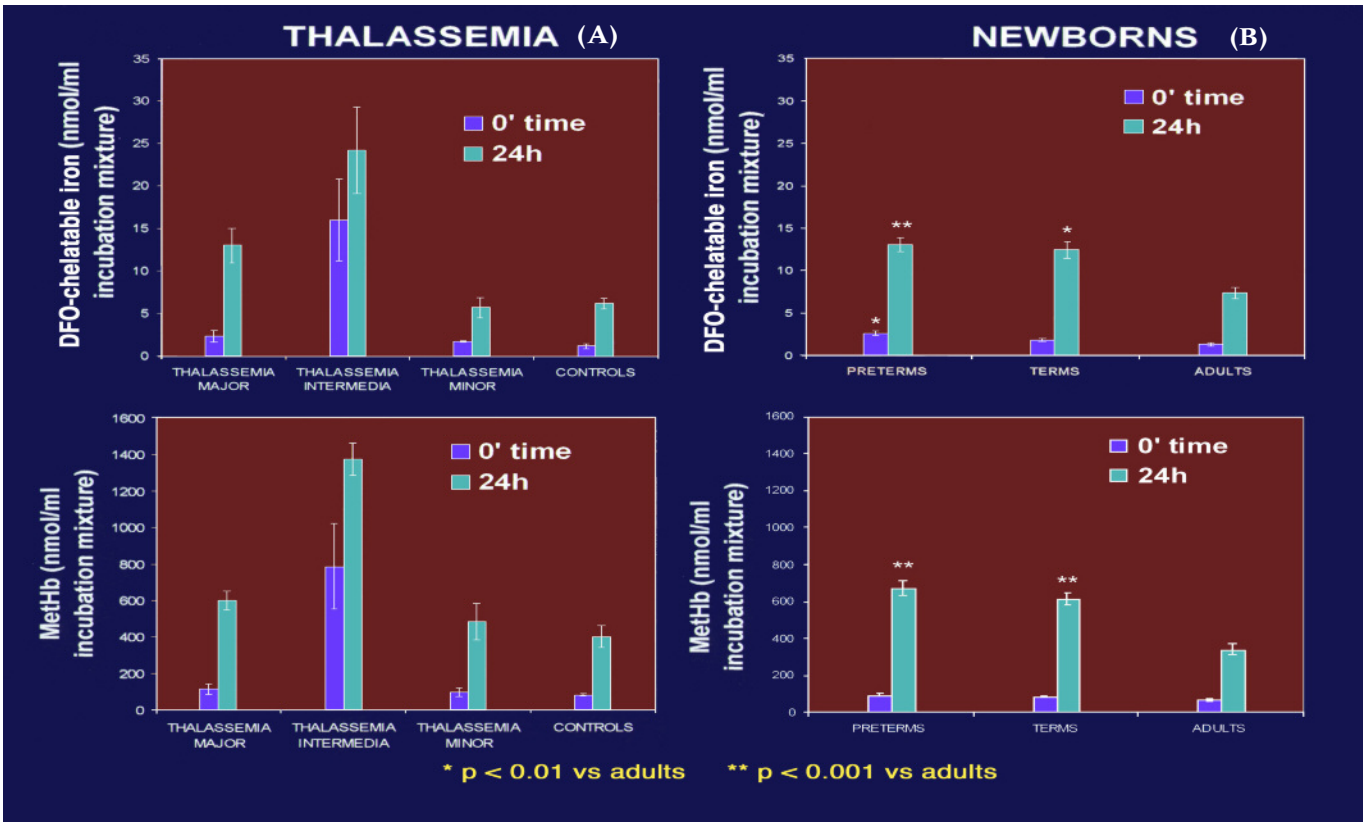
	Ageing Time (days)	Free Iron (nmol/ml)	MetHb (nmol/ml)
Storing in plasma at 4°C	0	1,7±0,03	115±7
	15	6,6±1,6	141±24
	35	15,4±3,2	179±40

An increased release of iron after aerobic incubation is also observed (30) in erythrocytes from subjects with β-thalassemia (major and intermedia) (Fig.21) and in erythrocytes from neonates (31). In both cases an increased susceptibility to oxidative stress, an accelerated removal from the blood stream and a marked increase of hemoglobin F (HbF) occurs. In thalassemic erythrocytes, besides the release (24 h incubation), the content of free iron (at 0 time) is also increased (Fig.21A). Both values are correlated with HbF content (30). In the erythrocytes from newborns (31) the release is increased in both term and preterm newborns (Fig.21B) and it is correlated (31) with the levels of plasma non protein-bound iron (NPBI), a form of non bound iron of uncertain origin, which appears in plasma of newborns, thalassemic and hemochromatotic patients. Both iron release and non protein bound iron are inversely correlated to the pH values of cord blood (31), which suggests that both values are more elevated when hypoxic conditions occur.

Recently the so called "senescent cell antigen" binding autologous IgG has been shown (32) to consist of dimers or larger aggregates of band 3. We have shown (33) that such an oxidatively modified band 3 is present in as much as 74% of preterm, 21% of term newborns and 10% of adults, which explains the accelerated removal of erythrocytes in the perinatal period. After in vitro ageing of erythrocytes (that is aerobic incubation), the band increases in frequency and intensity in all the erythrocytes (33) and the increase is almost completely paralleled by iron release.

The great deal of studies on oxidative stress, although bringing forth very interesting results, had not however allowed the evaluation of oxidative stress in human pathology, 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 only use of blood and urine was lacking. In fact all the methods used (detection of conjugated dienes, lipoperoxides and aldehydes) are poorly reproducible and reliable when carried out in plasma due to the extreme reactivity and instability of the species which are going to be detected; or they imply the use of tissues and therefore hardly feasible in men. Some years ago, however, the group of Morrow and Roberts in U.S.A. demonstrated (34,35) the production of a series of prostaglandin F_2-like compounds, named F2-isoprostanes (Fig. 22), that

Fig.21 - . Iron release and Meth-Hb formation in aerobically incubated erythrocytes from subjects with β-thalassemia (major, intermedia and minor) (A) and from newborns (preterm and term) (B). In thalassemic erythrocytes, besides the release, the content of free iron (at 0 time) in also increased. Both values are correlated with the HbF levels.



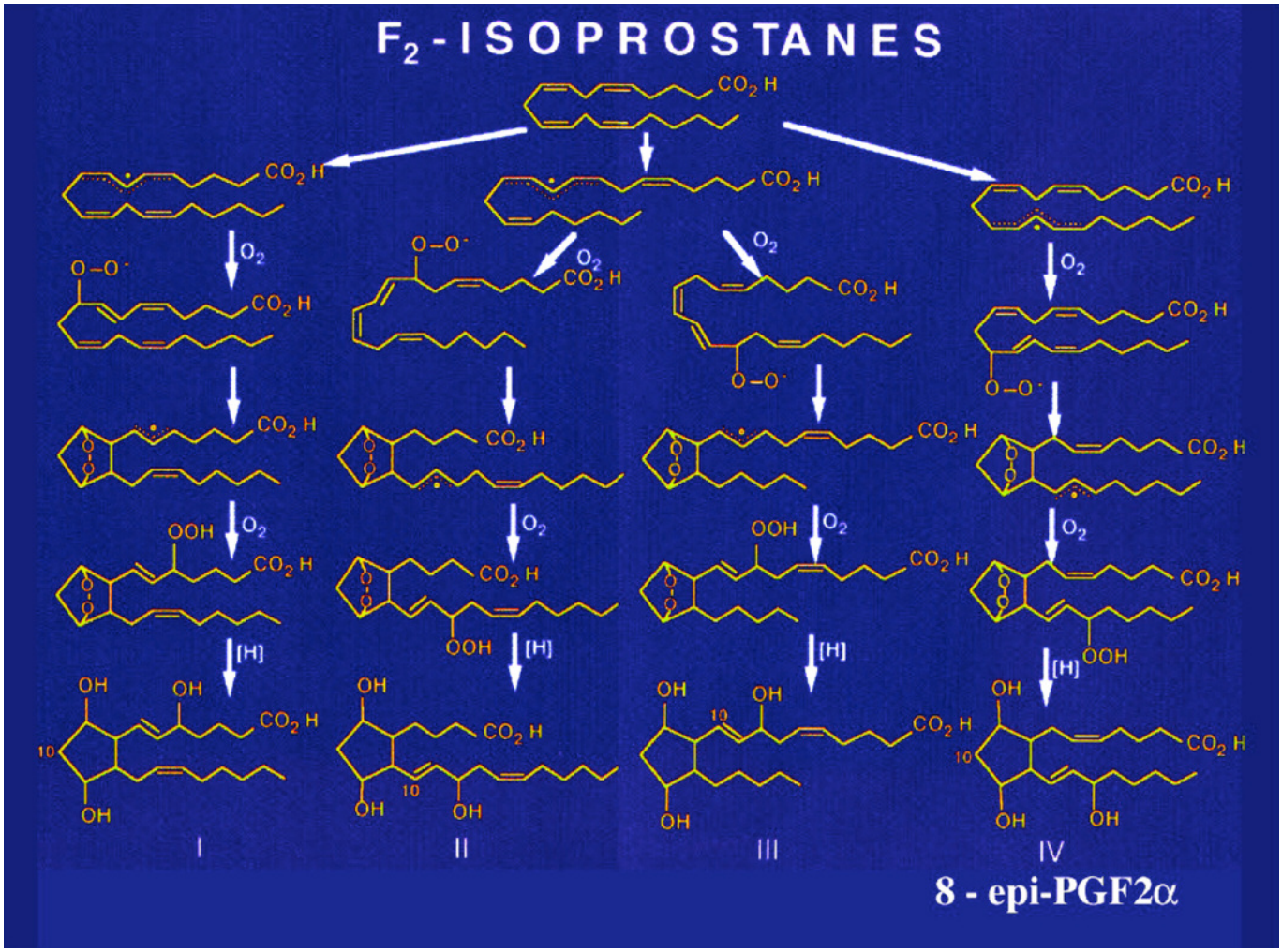


Fig.22 *- F_2-isoprostanes (F_2-iso) derived from the peroxidation of arachidonic acid (from Morrow et al. Analyt. Biochem., 184, 1-10, 1990).*

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. Since isoprostanes, initially formed in situ on phospholipids (36), are released into the circulation and since these prostanoids are less reactive than other lipid peroxidation products such as lipid hydroperoxides and aldehydes, they can be found more easily in plasma and urine. Therefore, 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 and/or urinary isoprostanes have been reported (37) in several diseases such as diabetes, alcoholic liver disease, ARDS, Alzheimer disease, rethinopathy of prematurity and many others.

We currently use, for the determination of F_2-isoprostanes, the gass-mass technique which, as also recommended by Roberts and Morrow (37), is the most reliable method to detect these products. The procedure of Noouroz-Zadeh (38) for the preparative technique before gass-mass is also used. Yet, before any approach to human pathology, we investigated whether elevated levels of plasma F_2-isoprostanes could be measured in our experimental models of oxidative stress, mentioned above. The results have shown (Fig. 23) dramatically elevated levels of plasma isoprostanes in the acute CCl4 intoxication (39) and even (although to a lesser extent) in the acute ethanol intoxication, thus confirming both our previous studies and the more recent results of Morrow et al. (40) (and also our early studies on oxidative stress in ethanol toxicity (41)).

Fig.23

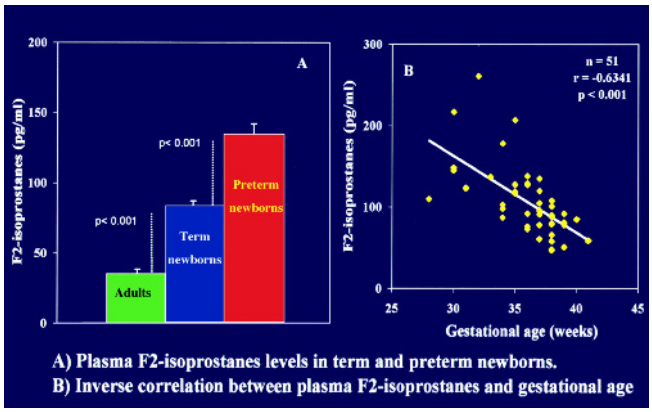
PLASMA F_2-ISOPROSTANE LEVELS IN CONTROL AND CCl_4 OR ETHANOL INTOXICATED RATS

	F_2-ISOPROSTANES (pg/ml)
controls	126 ± 24 (11)
CCl_4	4506 ± 451 (11)*
Ethanol	882 ± 185 (6)*

Starved rats were intoxicated intragastrically with CCl_4 (0.2 ml/100 g body weight) or with ethanol (7 g/kg body weight) and killed at 4 or 6 hr after the intoxication, respectively. Control rats received the same volume of saline. *$p < 0.001$ versus controls values

Since it has been repeatedly suggested that newborns are exposed to conditions of oxidative stress resulting from the change from a low oxygen pressure in utero to a relatively high oxygen pressure at birth, we have evaluated the oxidative status in human newborns (42). Plasma isoprostanes were significantly higher in newborns as compared to healthy adults, and the highest values were found in preterm newborns in whom F_2-isoprostanes were higher as compared to term babies (Fig.24A). Moreover, a highly significant inverse correlation was found (42) between the plasma levels of isoprostanes and the gestational age (Fig.24B).

Fig.24 (A) - (B)



A) Plasma F2-isoprostanes levels in term and preterm newborns.
B) Inverse correlation between plasma F2-isoprostanes and gestational age

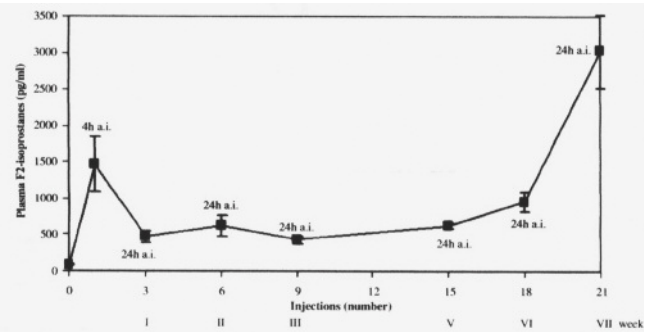
This suggests that some form of oxidative stress is active during the prenatal life and that it is going to attenuate during the last periods of gestation. This also confirms the occurrence of the so called "free radical disease of the neonate".

Finally we have recently suggested (43) that F_2-isoprostanes are potent agonists in experimentally induced liver fibrosis. The connection between oxidative stress and collagen hyperproduction was firstly proposed by Chojker et al. (44) who observed that the addition of ascorbic acid and iron to human cultured fibroblasts strongly stimulates lipid peroxidation and, at the same time, the production of collagen and procollagen α-1 (I) mRNA; the effects are reproduced by the addition to the same fibroblasts of malonaldeyde (MDA), one of the end products of lipid peroxidation. Also, 4-HNE has been reported (45) to stimulate collagen synthesis in hepatic stellate cells (HSC) (previously known as Ito cells or lipocytes, the most important source of collagen and other matrix proteins in the liver) and TGF β synthesis in cultured lineages of macrophages (46). Since F_2-isoprostanes proved to be mediators of important biological effects, we investigated whether collagen synthesis was stimulated in HSC by F_2-isoprostanes, which possess receptors able to induce specific signal transduction pathways, while aldehydes (such as 4-HNE) can interact with cellular macromolecules by addition or alkylation processes only.

Since, as above mentioned, we have shown that plasma F_2-isoprostanes are extremely elevated in the acute CCl_4 intoxication, we examined the levels of plasma F_2-isoprostanes in a model of chronic CCl_4 intoxication (47) leading to liver cirrhosis, we observed (43) that such levels are maintained elevated during the whole period of experimental treatment (Fig. 25) and correlated to the hepatic content of collagen and in parallel studies we investigated (43) the effects of isoprostanes on cultured HSC.

Fig.25 - Model of chronic intoxication with CCL_4 leading to fibrosis

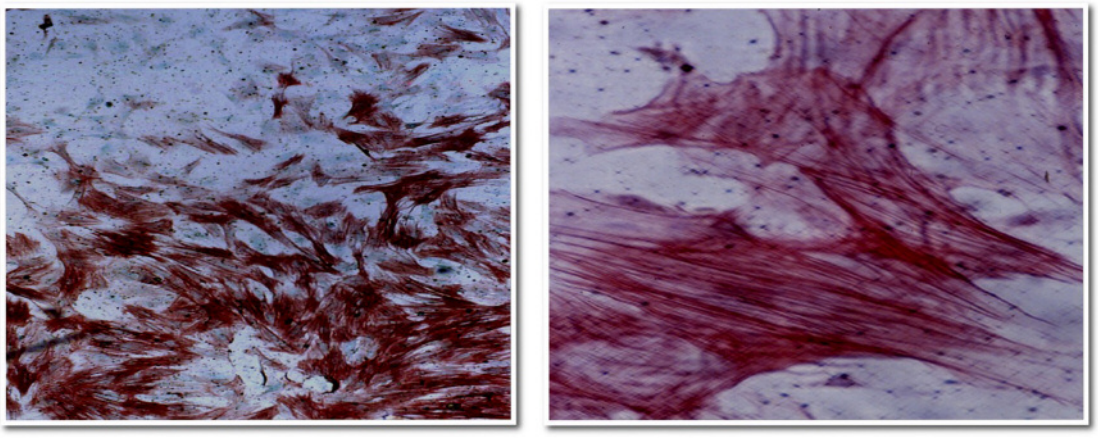
Rats were injected intraperitoneally three times weekly with 125µl/100g body wt of a CCl_4/mineral oil solution (1:1, v/v) (= 62.5µl CCl_4/100g body wt). Groups of 4 animals were sacrificed 24hr after the last injection at 1, 2, 3, 5, 6 and 7 weeks, respectively. Blood was drawn from the abdominal aorta and analysed for plasma F_2-iso.



Plasma F2-isoprostanes in rats chronically intoxicated CCl4

The latter were isolated (48) from normal livers and cultured in suitable media. At the seventh day of culture all the cells showed (Fig. 26) the typical transformation to the myofibroblast-like phenotype

***Fig.26** - Hepatic stellate cells (HSC) isolated from normal rat livers and their activation (expression of α-smooth muscle actin (α-SMA)) at 7 days of culture.*



(expression of α-smooth muscle actin (α-SMA)). The cells were deprived of serum and then treated for 48 hr with F_2-isoprostanes in the range of concentrations seen in the in vivo experiments (10^{-8} to 10^{-10} M).

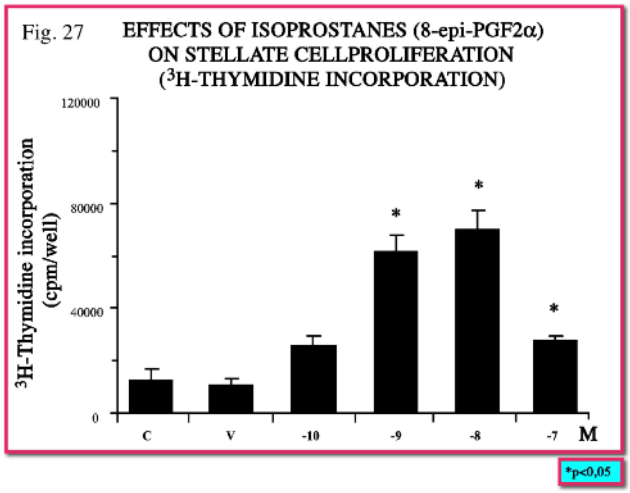
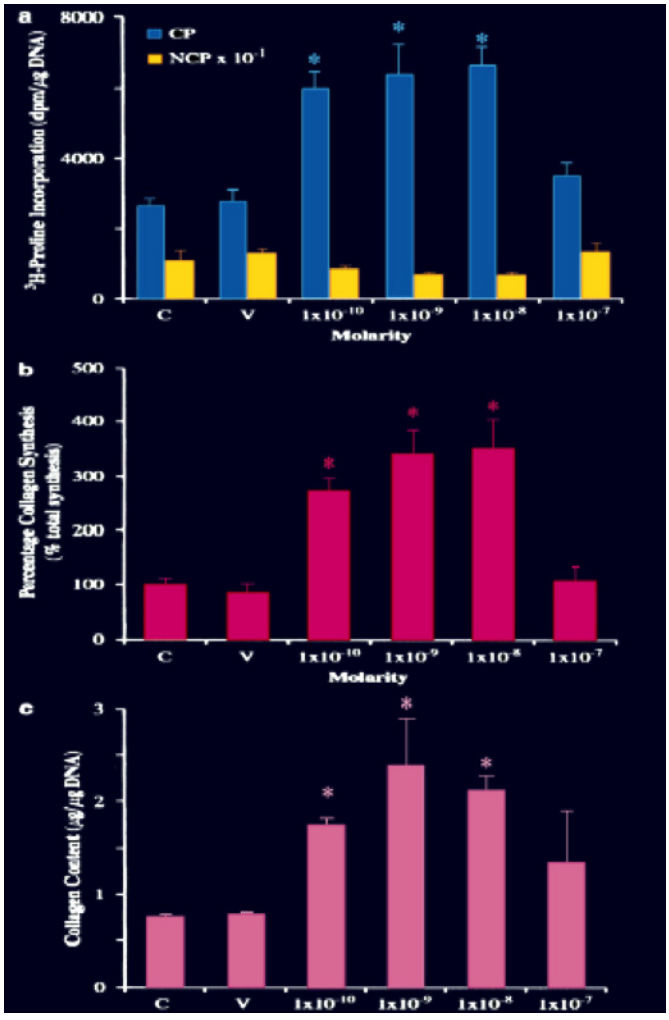
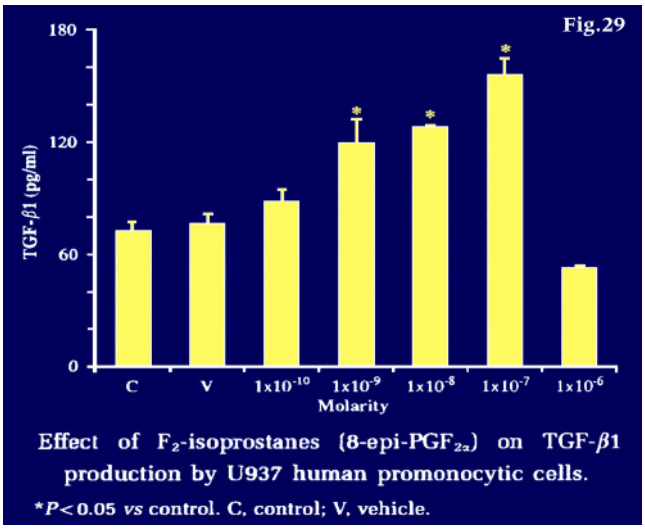


Fig.28 (a),(b),(c) - Effects of F_2-iso (8-epi-$PGF_{2\alpha}$) addition to HSC on collagen synthesis as measured by 3H-proline incorporation.
a) proline incorporation
b) percentage of collagen production overtotal protein production (collagenic plus non collagenic protein)
c) total collagen contents of the cultures.



The isoprostane (actually 8-epi-PGF2α the most represented isomer of the series) addition to HSC induced (43) a marked increase in DNA synthesis (Fig.27), as measured by tritiated thymidine incorporation, and of cell proliferation (as measured by the cell count, not shown in the Figure), as well as a striking increase (Fig.28A,B,C) in collagen synthesis, as measured by tritiated proline incorporation (43). The relative collagen production, that is the percentage of collagen production over the total protein production (collagenic plus non collagenic proteins) was increased by 3.0-3.5 fold (Fig. 28B). Total collagen content of the culture was similarly increased (Fig. 28C). The most active concentrations were between 10^{-8} and 10^{-9} M (10 nM 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).

Since it is generally believed (49-51) that activation of HSC follows the release of soluble factors (cytokines, mainly TGF-β1) by cells of macrophage linkages, the effects of F_2-isoprostanes on TGF-β1 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 (43) the production of TGF-β1 by U937 cells (Fig.29) and this would suggest an alternative pathway of stimulation of HSC, through TGF-β1, with consequent increase in collagen synthesis.

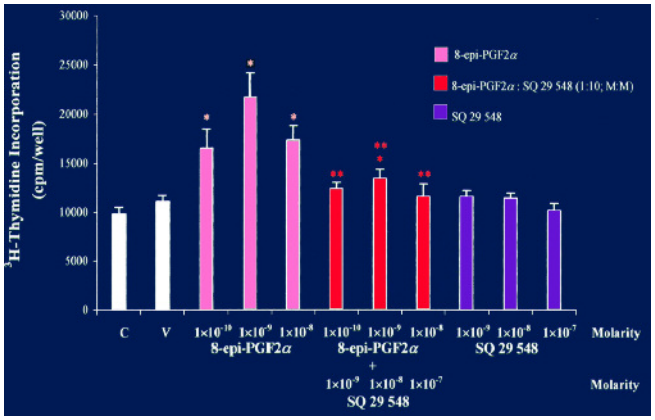


In summary we propose here that the plasma F_2-isoprostanes generated by lipid peroxidation in hepatocytes mediate the collagen hyperproduction in this model of hepatic fibrosis.

For such an hypothesis to stand up it had to be demonstrated that receptors for F_2-isoprostanes are present in HSC. Besides being markers of oxidative stress, F2-isoprostanes appeared to be mediators of important biological effects. The first one to be revealed (52) was the vasoconstriction of renal glomerular arterioles, an effect which is believed to be very important in the explanation of the hepato-renal syndrome. The effect is mediated through the activation of receptors analogous or identical to those for tromboxane A_2 (TxA_2r) (53).

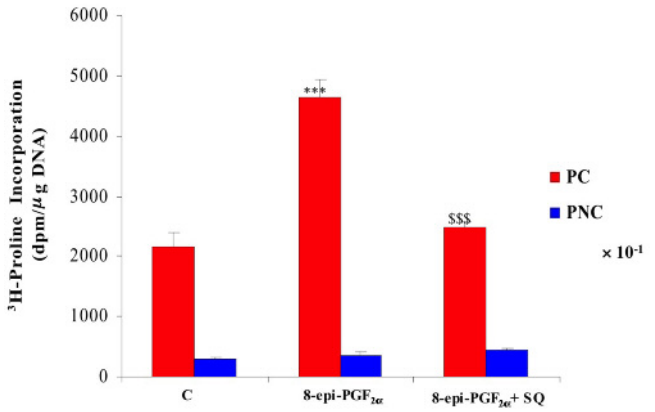
Many other biological effects of F_2-isoprostanes have been described and most of them are very likely due to activation of receptors related to TxA_2r (54-59). Therefore, we investigated whether the specific antagonist of TxA_2r, the molecule named SQ29548, was able to antagonize the effects of isoprostanes in HSC. As can be seen (Fig.30) the isoprostane-induced stimulation of DNA synthesis is almost completely abolished by SQ29548 (43,60).

Fig.30 - *Effect of SQ29 548 on the stimulation of DNA synthesis (^{3}H-thymidine incorporation) in HSC treated with F_2-isoprostane (8-epi-PGF2α).*



Also the other effect of isoprostanes (8-epi-PGF2α) on HSC, i.e. the stimulation of collagen synthesis is completely antagonized (Fig.31) by SQ29548 (60).

Fig.31 - *Effect of SQ 29 548 on the stimolation of collagen synthesis (^{3}H-thymidine incorporation) in HSC treated with F2-iso (8-epi-PGF2α).*



Moreover, an effect similar to that of 8-epi-PGF2α (although to a lower extent, but anyhow statistically significant) is brought about by the molecule named I-BOP (Fig.32), the specific agonist of TxA_2r, and even this effect, still on DNA synthesis, is abrogated by SQ29548 (60). The effect of isoprostanes on HSC seemed therefore to be mediated by TxA_2r and then this receptor should occur in HSC.

Finally by using an antibody raised against C-terminal aminoacids of human TxA_2r (TPr) we carried an immunoblot analysis of membrane proteins of HSC and we obtained (60) a single band of 55 kd (Fig.33), quite analogous to that obtained (still at 55 kd) with membrane proteins of human platelets used here as positive control (Fig.33) since it is known (61) that such membranes are rich of TxA_2r.

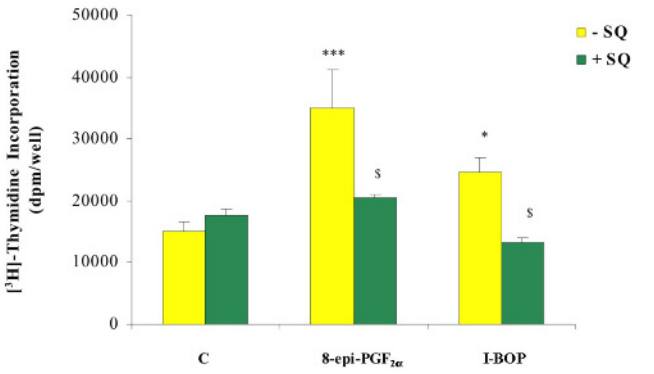
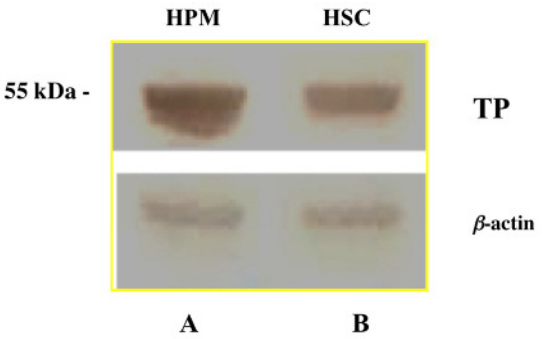


Fig.32 (above) - *Effect of F2-iso (8-epi-PGF2α) and of I-BOP on DNA synthesis (3H-tymidine incorporation) in HSC and the antagonizing activity of SQ 29 548.*

Fig.33 (below) - *Immunoblot identification of TxA_2r in HSC membrane protein lysates. Incubation with an antibody raised against the terminal aminoacids of human TxA_2r (TPr) (1:1000 dilution) revealed immunoreactivy for a 55 kDa protein in HSC membrane lysates (lane B). Lysates from human platelet membranes (HPM) (lane A) were used as positive control. Blot were reprobed with β-actin to assess equal loading.*



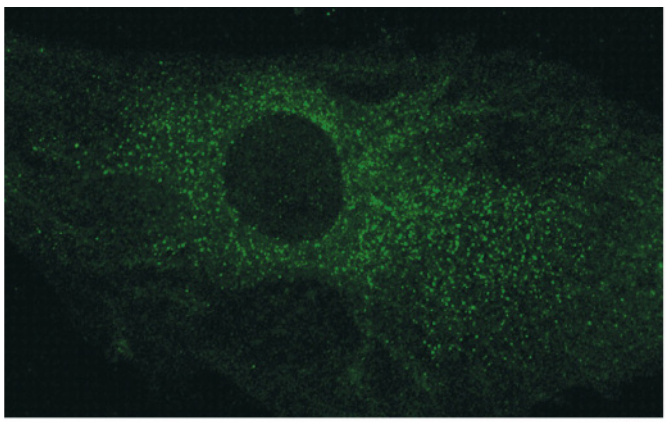
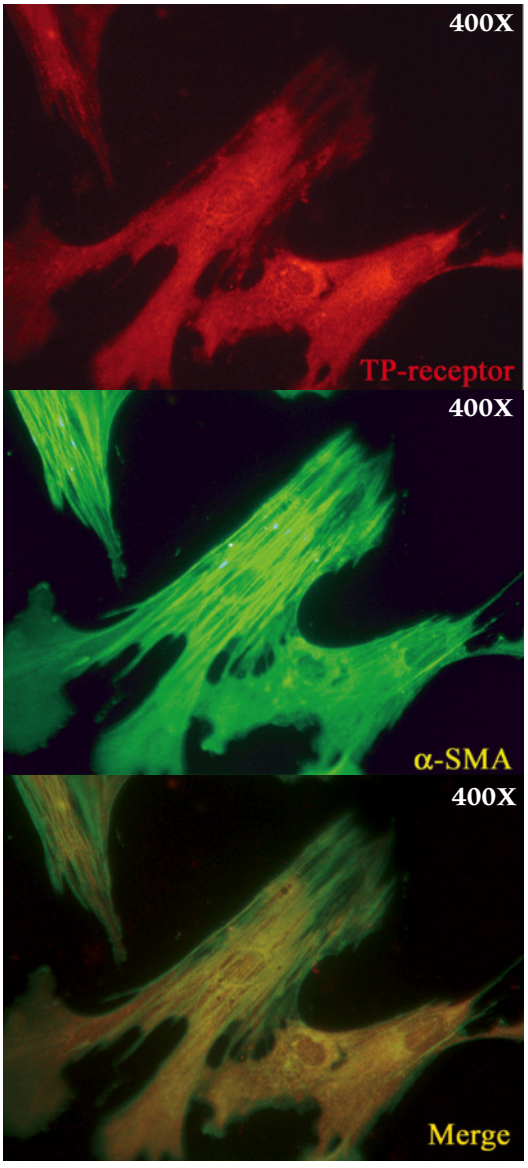


Fig.34 - *Subcellular localization of TxA_2r (TPr) in HSC. Cells were permeabilized, incubated with anti-TxA_2r antibody and then with FITC (fluorescein isothiocyanate) conjugated anti-rabbit IgG. Confocal image shows that TxA_2r (TPr) is predominantly expressed in the area around the nucleus.*

Immunocytochemical studies (60) carried out by using the same antibody (as primary antibody) together with a secundary anti-rabbit IgG antibody conjugated with the fluorescent dye FITC (fluoresceine isothyocanate) showed at confocal microscopy the presence of the same receptor (TxA_2r) in HSC (Fig.34) and its major expression in the perinuclear region. Lastly, in colocalization studies (Fig.35) carried out by using the same primary antibody together with a secundary anti-rabbit IgG antibody conjugated with the fluorescent dye TRITC (tetramethylrodamine esothyocianate) (red) we obtained (60) in red the TxA_2r (or TP receptor); and by using another primary antibody (anti α-SMA) together with a secundary anti-mouse IgG antibody conjugated with FITC we obtained (60) α-SMA in green. In merge images (Fig. 35) it was possible to appreciate the exact colocalization. It must therefore be concluded that not only HSC express the TxA_2r but also that such receptor is expressed at the same extent as α-SMA, which is the protein marking the activation of these cells.

Fig.35 *- Co-localization of TxA_2r (TPr) and α-SMA in HSC. Activated HSC expressed both TxA_2r (TPr),revealed by TRITC (tetrametylrhodamine isothiocyanate) (red labeled) and α-SMA, revealed by FITC (fluorescein isothiocyanate) (green labeled). Merged images (yellow) show the high degree of co-localization.*



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