

Université de Montréal

**Vue sur les mécanismes de contrôle du cholestérol hépatique suite  
au stress peroxydatif induit par le fer.**

par

Sylvain Brunet

Département de Nutrition

Faculté de Médecine

Thèse présentée à la Faculté des études supérieures

en vue de l'obtention du grade de

Philosophiæ Docteur (Ph.D.)

En Nutrition

Novembre 1999

© Sylvain Brunet, 1999



QU  
145  
U58  
2000  
V. 001

Université de Montréal

Les auteurs reconnaissent la contribution de certains de leurs collègues

au succès de ce projet de recherche.

par

Sylvain Bouchard

Département de Psychologie

Faculté de Médecine

Tiède présentée à la Faculté des études supérieures

en vue de l'obtention du grade de

Philosophie (Doctorat) (Ph.D.)

En Psychologie

Montréal, 1992

© 1992 Université de Montréal



Université de Montréal

Faculté des études supérieures

Cette thèse intitulée :

**Vue sur les mécanismes de contrôle du cholestérol hépatique suite  
au stress peroxydatif induit par le fer.**

Présenté par :

Sylvain Brunet

a été évaluée par un jury composé des personnes suivantes :

Dr Michelle Houde Nadeau	Présidente du jury
Dr Emile Levy	Directeur de recherche
Dr Marielle Ledoux	Membre du jury
Dr Angelo Tremblay	Examineur externe
Dr Pierre Haddad	Représentant du doyen

**Table des matières:**

SOMMAIRE .....	V
LISTE DES TABLEAUX.....	IX
LISTE DES FIGURES .....	XI
LISTE DES ABRÉVIATIONS .....	XIII
DÉDICACE .....	XIV
REMERCIEMENTS .....	XV
I. INTRODUCTION .....	1
1.1 CONTEXTE .....	2
1.2 ASPECTS BÉNÉFIQUES DE LA PEROXYDATION .....	4
1.3 ASPECTS NÉFASTES DE LA LIPOPEROXYDATION .....	13
1.4 LES RESPONSABLES DE LA PEROXYDATION .....	15
1.5 DÉFENSES ANTIOXYDANTES .....	20
1.5.1 Les défenses antioxydantes enzymatiques : .....	20
1.5.2 Les défenses antioxydantes non-enzymatiques .....	24
1.6 EXEMPLE DE L'ATHÉROSCÉROSE .....	29
1.7 SENSIBILITÉ DU FOIE AU STRESS OXYDATIF .....	36
1.8 CONTEXTE HÉPATIQUE DU FER .....	41
1.9 FONCTIONS HÉPATIQUES RELIÉES AU CHOLESTÉROL .....	43
II. HYPOTHÈSE.....	54

## III. ARTICLES ORIGINAUX ..... 56

- 1) Sylvain Brunet, France Guertin, Louise Thibault, Victor Gavino, Edgard Delvin and Emile Levy. Iron-salicylate complex induces peroxidation, alters hepatic lipid profile and affects plasma lipoprotein composition. *Atherosclerosis* : 129, 159-168, 1997
- 2) Brunet S, Thibault L, Lepage G, Seidman EG, Dubé N and Levy E. Modulation of endoplasmic reticulum-bound cholesterol regulatory enzymes by iron/ascorbate-mediated lipid peroxidation. (*Free radicals Biology and Medicine*, sous presse).
- 3) Sylvain Brunet, Louise Thibault, Edgar Delvin, Wagner Yotov, Moïse Bendayan and Emile Levy. Dietary iron overload and induced lipid peroxidation are associated with impaired plasma lipid transport and hepatic sterol metabolism in rats. *Hepatology*, 29 : 1809-1817, 1999
- 4) Sylvain Brunet, Isabelle Suc, Nadia Dubé, Louise Thibault and Emile Levy. Lipoprotein metabolism alterations in iron-loaded HepG2 cells. (*Biochemical & Biophysical Research Communications*., 4 novembre 1999).

## IV. DISCUSSION ..... 122

## V. CONCLUSIONS ET PERSPECTIVES ..... 134

## VI. BIBLIOGRAPHIE ..... 136

## VII. CURRICULUM VITAE ..... XIV

## SOMMAIRE

L'excès cellulaire ou tissulaire en cholestérol revêt un caractère délétère pour la physiologie de l'ensemble des mammifères. Pour éviter cette situation, l'organisme dispose de moyens biochimiques qui assurent un contrôle strict des niveaux de cholestérol. Le centre névralgique de cette régulation se situe au niveau du foie. En effet, ce dernier est le seul organe qui affiche la capacité de synthétiser le cholestérol grâce à l'HMG CoA réductase, de le mettre en réserve sous l'action de l'ACAT, de le transformer en acides biliaires à l'aide de la cholestérol 7  $\alpha$ -hydroxylase, et finalement de l'excréter hors de l'organisme par la voie biliaire.

D'un autre côté, il est maintenant bien établi que la peroxydation exerce une influence directe sur plusieurs systèmes biochimiques et physiologiques. Étonnamment, la régulation du cholestérol, face à un stress oxydatif, demeure obscure. C'est précisément l'objectif de nos études : comprendre l'impact de la peroxydation sur le système hépatobiliaire régissant le carrefour métabolique du cholestérol. Afin d'élucider cette question importante, nous avons opté pour le fer en tant qu'agent peroxydatif, puisqu'il fait partie intégrale de notre physiologie, est associé à plusieurs fonctions et possède un caractère pro-oxydant puissant dans un contexte de surcharge.

Nous avons développé quatre modèles de peroxydation pour évaluer les voies métaboliques régissant l'homéostasie du cholestérol au niveau du foie et de la périphérie. Chaque modèle fait appel à un niveau organisationnel et à un degré de peroxydation variable. Deux des modèles *in vivo* et *in vitro* sont associés à une induction peroxydative aiguë de grande intensité: le premier est représenté par le rat soumis à une peroxydation suite à un traitement combiné de fer et de salicylate, alors que le second illustre des microsomes de foie de rat soumis à l'influence du mélange fer / ascorbate. Les deux autres modèles chroniques consistent à explorer les mécanismes d'homéostasie altérés par la peroxydation chez le rat maintenu sur une diète enrichie en fer carbonyle ou dans des hépatocytes incubés avec le fer.

Avec le rat exposé au fer/salicylate, nous avons pu observer une élévation marquée ( $p < 0.001$ ) des TBARS et des dienes conjugués ( $p < 0.05$ ) au niveau du plasma et du foie, en parallèle avec la diminution du cholestérol total (25%,  $p < 0.0001$ ), cholestérol ester (34%,  $p < 0.001$ ) et HDL-Cholestérol (50%,  $p < 0.001$ ). Des altérations intenses sont apparues dans la composition des VLDL, IDL, LDL, HDL<sub>2</sub> et HDL<sub>3</sub>. De son côté, le foie dévoilait des modifications de lipides tels que le cholestérol total et le cholestérol ester.

Le deuxième modèle, à savoir des microsomes traités au fer, a permis de souligner les mécanismes responsables des changements lipidiques observés *in vivo*. Nos résultats ont pu mettre en évidence l'inactivation

de l'HMG CoA reductase (46%,  $p < 0.01$ ), l'ACAT (80%,  $p < 0.000$ ) et la cholestérol 7 $\alpha$ -hydroxylase (48%,  $p < 0.001$ ) par trois mécanismes distincts: une attaque radicalaire directe sur les protéines enzymatiques, la perturbation de la fluidité membranaire abritant ces enzymes et l'action propre au fer. Ainsi, sans protection antioxydante ou correction de la fluidité membranaire, la sensibilité suit le patron décroissant :  
ACAT > cholestérol 7  $\alpha$ -hydroxylase > HMG CoA réductase.

L'étude avec le rat maintenu sous un régime chronique de peroxydation, nous a permis de dégager les changements dans le profil lipidique, la composition des lipoprotéines et le métabolisme hépatique des stérols. En particulier, des différences ont été notées dans chacune des unités enzymatiques catalysant la transformation des stérols. Ainsi, l'HMG CoA réductase (66%,  $p < 0.005$ ) et la cholestérol 7  $\alpha$ -hydroxylase (58%,  $p < 0.0004$ ) voient toutes deux leur activité réduite significativement. Par contre, la voie d'estérification du cholestérol présente l'augmentation de ACAT (62%,  $p < 0.002$ ). Les résultats obtenus ont permis d'établir des associations entre la diminution de l'activité des enzymes HMG CoA réductase et cholestérol 7  $\alpha$ -hydroxylase d'une part et l'abaissement des stérols biliaires et les concentrations des lipoprotéines circulantes d'autre part.



Finalement, à l'aide d'un modèle de cellules hépatocytaires en culture (HepG2), nous avons reproduit les manifestations observées in vivo dans un contexte chronique de peroxydation (perturbation de la composition chimique des lipoprotéines et augmentation de la synthèse et de la sécrétion des lipoprotéines), tout en isolant la contribution potentielle du foie des interférences possibles de la périphérie. Les résultats démontrent un dérèglement de l'efficacité du récepteur LDL à soutenir l'augmentation du cholestérol extra hépatique.

L'ensemble des observations indique la sensibilité du système hépatobiliaire au stress oxydatif et l'incontestable efficacité du fer en tant qu'agent peroxydant. Toutes les réactions impliquées dans le contrôle métabolique du cholestérol au niveau du foie sont affectées par la peroxydation. Les mécanismes qui régissent ces réactions permettent d'entrevoir les stratégies variées élaborées par l'organisme pour assister la défense contre la pression peroxydante du fer au niveau du foie. De plus, nos résultats constituent une preuve supplémentaire de la nécessité de considérer le foie dans l'évaluation des désordres impliquant la peroxydation et le cholestérol (cholestases, athérosclérose, etc.).

## LISTE DES TABLEAUX

<b>Tableau 1</b> : Demi-vie de quelques radicaux libres .....	11
<b>Tableau 2</b> : Antioxydants chez l'être humain.....	23
<b>Tableau 3</b> : Pathologies humaines et lésions pour lesquelles une contribution des radicaux libres est suggérée.....	31
<b>Tableau 4</b> : Composés et événements susceptibles d'induire une peroxydation hépatique in vivo. ....	40

## ARTICLES ORIGINAUX :

### ARTICLE 1 :

Tableau 1	Assesment of lipid peroxidation and liver toxicity.....	59
Tableau 2	Chemical composition of lipoproteins.....	61
Tableau 3	Total plasma fatty acid composition of control and iron-treated rats.....	62
Tableau 4	Fatty acid composition of liver homogenate.....	63

### ARTICLE 2 :

Tableau 1	Microsomal fatty acid composition in normal conditions and following various treatments.....	90
Tableau 2	Microsomal fatty acid families and ratios.....	91

**ARTICLE 3 :**

Tableau 1	Charracteristics of iron overload and control rats.....	97
Tableau 2	Hepatobiliary glutathione levels.....	99
Tableau 3	Effect of iron treatment on plasma lipids and lipoproteins.....	99
Tableau 4	Chemical composition of lipoproteins.....	100
Tableau 5	Lipid content in liver and microsomes from iron overload rats and controls.....	100
Tableau 6	Membrane fluidity in microsomal membranes.....	101
Tableau 7	Correlation between hepatic MDA and various parameters.....	102

**ARTICLE 4 :**

Tableau 1	Distribution of lipid constituents in secreted lipoproteins particles from HepG2 cells exposed to iron-induced lipid peroxidation.....	117
Tableau 2	Membrane fluidity of HepG2 cells exposed to iron-induced lipid peroxidation.....	118

## LISTE DES FIGURES

<b>Figure 1</b> : Notion de stress oxydatif.....	3
<b>Figure 2</b> : Synthèse des écosanoïdes .....	5
<b>Figure 3</b> : Compétition pour la delta 6-désaturase .....	7
<b>Figure 4</b> : Flambée oxydative.....	8
<b>Figure 5</b> : Potentiel pro-oxydant du fer.....	12
<b>Figure 6</b> : Lipoperoxydation non enzymatique. ....	15
<b>Figure 7</b> : Sources intracellulaires pro-oxydantes .....	17
<b>Figure 8</b> : Régénération du tocophérol .....	26
<b>Figure 9</b> : Athérogenèse.....	34
<b>Figure 10</b> : Inter-relations enzymatiques au foie. ....	47
<b>Figure 11</b> : Voie de synthèse du cholestérol .....	48
<b>Figure 12</b> : Régulation post-transcriptionnelle de l'HMG CoA red.....	49
<b>Figure 13</b> : Voie de synthèse des acides biliaires. ....	53

### Articles Originaux :

#### Article 1 :

Figure 1	TBARS concentration in the plasma and liver of control, salicylate and Fe-salicylate treated rats.....	59
Figure 2	Plasma lipids and lipoproteins in control and Fe-salicylate treated-rats.....	60

Figure 3	Sodium dodecyl sulfate polyacrilamide gel electrophoresis showing the apolipoprotein composition of VLDL and LDL from Fe-salicylate treated and control rats.....	62
Figure 4	Apolipoprotein patterns of HDL2 and HDL3 fractions in Fe-salicylate and control rats.....	63
Figure 5	Lipid composition in the liver of control and Fe-salicylate treated rats.....	63
Figure 6	LCAT and ACAT activity in control and Fe-salicylate treated rats.....	64

## **Article 2 :**

Figure 1	Lipid peroxidation in microsomes challenged with Fe <sup>2+</sup> / ascorbate.....	92
Figure 2	Effect of Fe <sup>2+</sup> / ascorbate on key sterol regulatory enzymes.....	93
Figure 3	Influence of $\alpha$ -tocopherol on Fe <sup>2+</sup> / ascorbate mediated inactivation of HMG-CoA reductase, cholesterol 7 $\alpha$ -hydroxylase and ACAT.....	94
Figure 4	Microsomal enzymes involved in liver cholesterol homeostasis.....	95

**Article 3 :**

Figure 1	Light and electron micrographs of liver tissues from control and experimental animals.....	98
Figure 2	Malondialdehyde levels in plasma and hepatobiliary system.....	99
Figure 3	SDS-PAGE showing the apolipoprotein composition of lipoproteins .....	101
Figure 4	Effect of iron overload on biliary lipids and bile acids following bile duct cannulation.....	102
Figure 5	Activity of liver HMG-CoA reductase, cholesterol 7 $\alpha$ -hydroxylase and ACAT.....	102

**Article 4 :**

Figure 1	Cellular iron uptake and lipid peroxidation in HepG2 cells.....	119
Figure 2	Effect of iron on de novo lipid synthesis by hepG2 cells.....	120
Figure 3	LDL binding and internalization for determination of receptor activity and LDL receptor number.....	121

**LISTE DES ABREVIATIONS**

ACAT	Acyl-CoA :cholesterol acyltransferase
HDL	Lipoprotéine de haute densité (High density lipoprotein)
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
LDL	Lipoprotéine de basse densité (Low density lipoprotein)
MDA	Malondialdehyde
VLDL	Lipoprotéine de très basse densité (Very low density lipoprotein)

**DEDICACE**

*A Geneviève,*

*Mon rempart dans la tempête,*

*mon phare dans la nuit.....*



## REMERCIEMENTS

À mon directeur de thèse, Dr Emile Levy

qui m'a ouvert large l'avenue de la science et de la recherche  
et dont l'appui constant et l'enthousiasme renouvelé

ont jeté sur ma démarche scientifique

la lumière et la confiance

Mes plus sincères et plus chaleureux remerciements.

À mes autres précieux collaborateurs,

Ma reconnaissance et mes remerciements

À l'équipe du laboratoire du Dr. Emile Levy, en particulier  
Mesdames Louise A. Thibault, Carole Garofalo et Manon  
Chartré, pour leur aide et assistance technique et leur  
amitié.

À mes collègues, le Dr France Guertin, Monika Zoltowska,  
Simona Stan et le Dr Isabelle Suc pour les nombreux  
échanges scientifique et conseils toujours stimulants.

À mon épouse, Geneviève Oigny, dont l'appui et l'aide m'ont été  
indispensables dans la poursuite de mon travail.

## I. INTRODUCTION

Les recherches, portant sur les bienfaits et les répercussions des radicaux libres et de la peroxydation, représentent actuellement l'un des vastes domaines de recherche les plus étudiés. Historiquement, les études se sont attardées à déterminer les contributions réelles des phénomènes peroxydatifs dans l'étiologie de plusieurs maladies frappant les sociétés industrialisées (telles que le cancer, les maladies cardio-vasculaires et l'athérosclérose, le diabète, la maladie de Parkinson, etc.).

A mesure que notre compréhension évolue, grâce aux multiples efforts déployés, on se rend compte du rôle déterminant de la peroxydation dans la genèse de ces maladies dégénératives. En parallèle, de plus en plus de mécanismes, induits par le stress oxydatif, sont mis à jour. Il est à parier qu'une meilleure connaissance des effets au niveau cellulaire et moléculaire et des voies métaboliques empruntées, pourrait conduire à développer des moyens d'intervention plus efficaces face aux désordres mentionnés.

On peut supposer que particulièrement la pharmacologie et le domaine nutritionnel offriront les outils antioxydants efficaces pour prévenir et dans une certaine mesure, corriger les dommages peroxydatifs qui

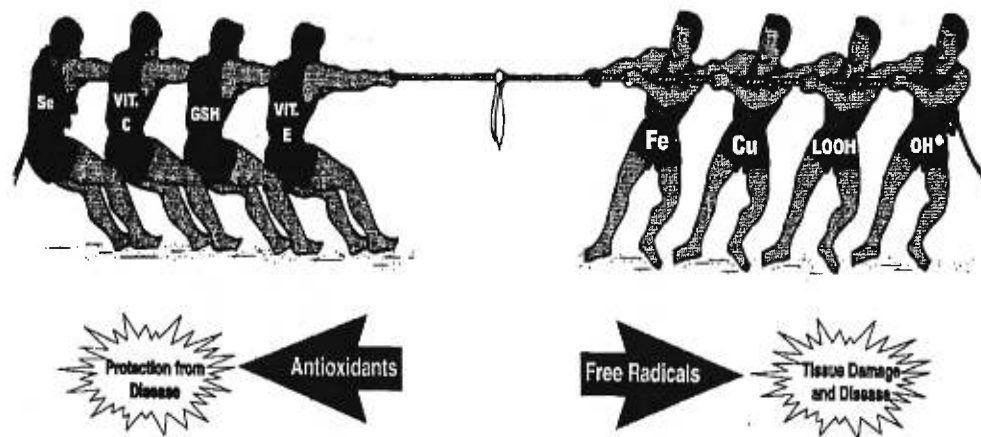
surviennent dans le développement des maladies en question. Dans ce contexte, il est de toute première importance que les recherches ne se limitent pas uniquement à l'étude d'un organe, mais plutôt à plusieurs systèmes physiologiques. Ce faisant, elles arriveraient à élucider les interrelations et les mécanismes impliqués.

La présente étude s'inscrit dans cette optique : dégager les effets d'un stress oxydatif sur les voies de régulation du cholestérol tant au niveau du foie et du système hépatobiliaire que dans tout le système circulatoire.

## **1.1 CONTEXTE**

Quotidiennement, notre organisme doit faire face à la pression oxydante des radicaux libres. Qu'elles soient d'origine alimentaire, environnementale ou endogène, ces molécules détiennent un potentiel oxydant plus ou moins puissant, qui les incitent à s'approprier des électrons pour stabiliser leur déficit électronique. Cette avidité des radicaux libres pour les électrons se manifeste au dépend d'autres molécules environnantes tels que les acides nucléiques, les protéines, les glucides et les lipides[1]. Dans ce dernier cas, on parle de lipoperoxydation. Les changements électroniques consécutifs à cette abstraction d'un électron pour ces molécules biologiques entraîne généralement leur dégradation ou la perte de fonctionnalité[2].

En conséquence, ces modifications devront être prises en charge par l'organisme et, selon la gravité de l'atteinte, les molécules devront être remplacées ou réparées lorsque se présentent de telles possibilités. Pour lutter contre les dommages oxydatifs, l'organisme dispose de moyens ou systèmes antioxydants. Le déséquilibre entre les deux forces opposées en faveur des oxydants réalise ce que l'on appelle le «stress oxydatif» (figure 1) et exige, conséquemment une vigilance face à ses effets néfastes.

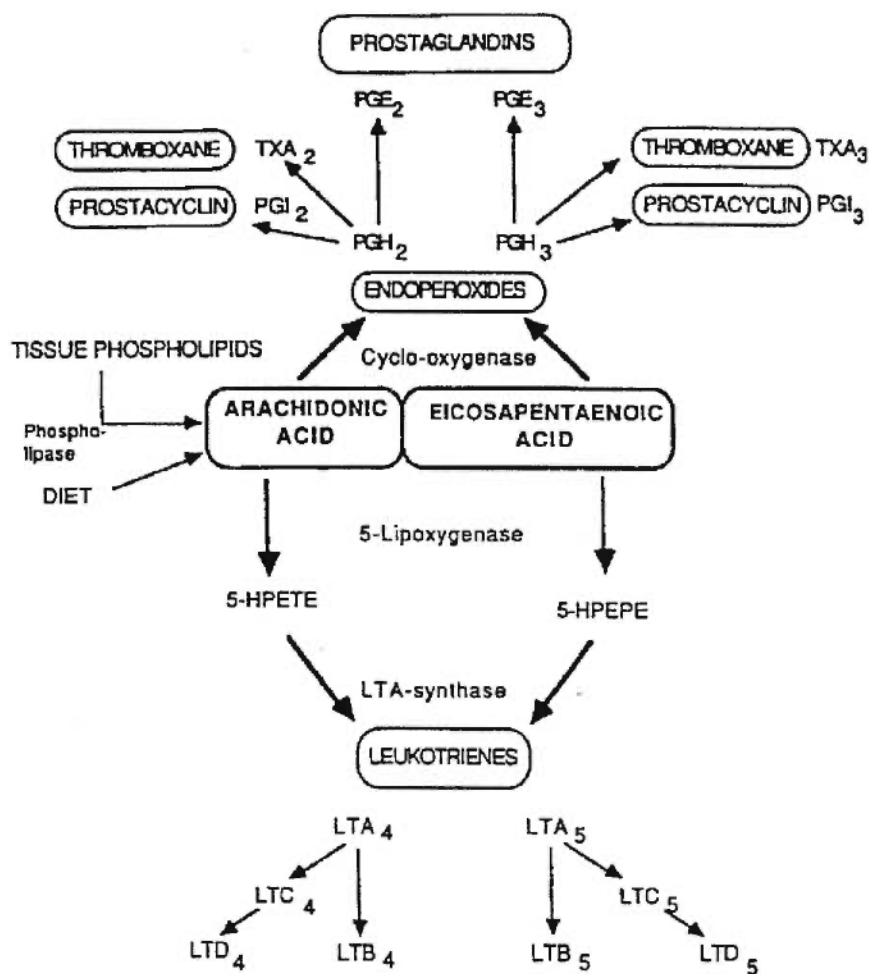


**Figure 1** :Schématisation de la notion de stress oxydatif, illustré par l'équilibre entre les forces pro-oxydantes et les forces antioxydantes.

## 1.2 ASPECTS BÉNÉFIQUES DE LA PEROXYDATION

Dans la situation physiologique normale, la lipoperoxydation, un phénomène «ubiquitaire» dans l'organisme humain, peut être bénéfique. Certaines fonctions reposent sur des réactions enzymatiques de lipoperoxydation. L'exemple, sans doute le plus cité, est la formation des écosanoïdes composés des leucotriènes, des prostaglandines et prostacyclines et des thromboxanes[3].

A partir des acides gras arachidonique et linoléinique, l'organisme peut synthétiser un ensemble d'effecteurs qui présentent un caractère immunomodulateur. La grande majorité des écosanoïdes proviennent de l'acide arachidonique, libéré de sa position C-2 des phospholipides membranaires, sous l'action de la phospholipase A 2. Il existe deux voies principales de synthèse des écosanoïdes, la voie directe donnant naissance aux leucotriènes et la voie cyclique donnant naissance aux prostaglandines et aux thromboxanes (figure 2).

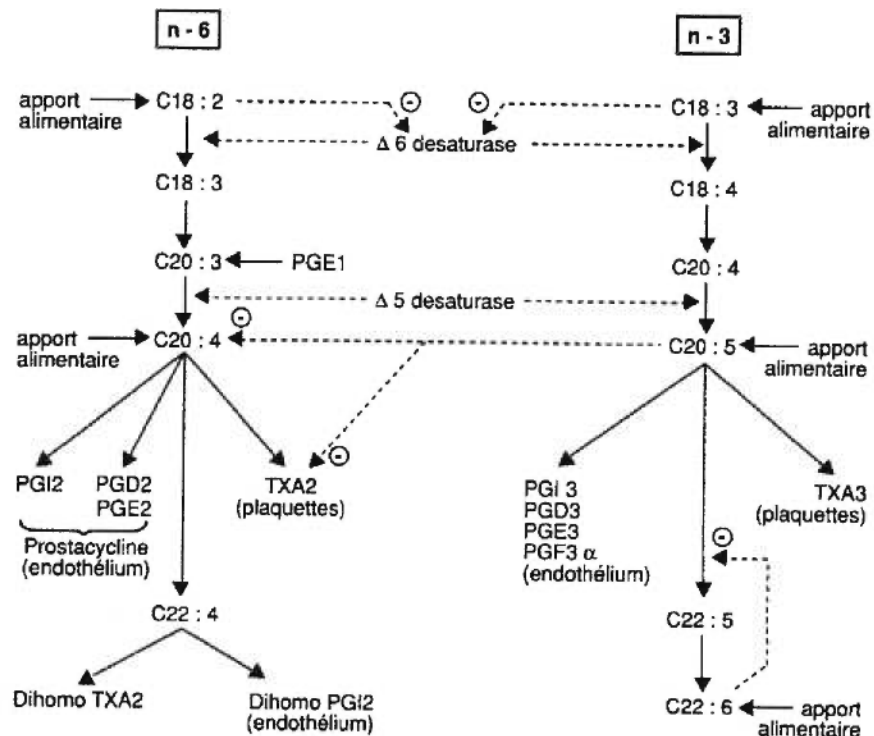


**Figure 2** : Voie de synthèse des écosanoïdes produits sous l'action de la cyclooxygénase et de la 5-lipoxygénase. (D'après Spiller G.A.

1996 [7])

Classiquement, sous l'action d'une dioxygénase (ou 5 lipoxygénase), deux atomes d'oxygène seront incorporés à l'acide arachidonique et créeront un groupement hydroperoxyl. A la suite d'une série de déshydratation successive, on voit apparaître les leucotriènes A<sub>4</sub> puis B<sub>4</sub> (figure 2). Ces derniers possèdent un pouvoir inflammatoire puissant et provoquent le recrutement et l'activation des monocytes et des macrophages[3]. Toutes les cellules de l'organisme, à l'exception des érythrocytes, synthétisent des écosanoïdes. Si les acides gras de la famille n-6 permettent la génération d'écosanoïdes de nature pro-inflammatoire (LTA<sub>4</sub>,LTB<sub>4</sub>), ceux de la famille n-3 produisent, en contrepartie, des écosanoïdes plutôt anti-inflammatoires (LTA<sub>5</sub>, LTB<sub>5</sub>). Arrive alors, une compétition entre les acides gras de la famille n-6 et n-3 pour l'enzyme delta-6-desaturase qui détermine, selon leur concentration, le type d' écosanoïdes produit (figure 3).

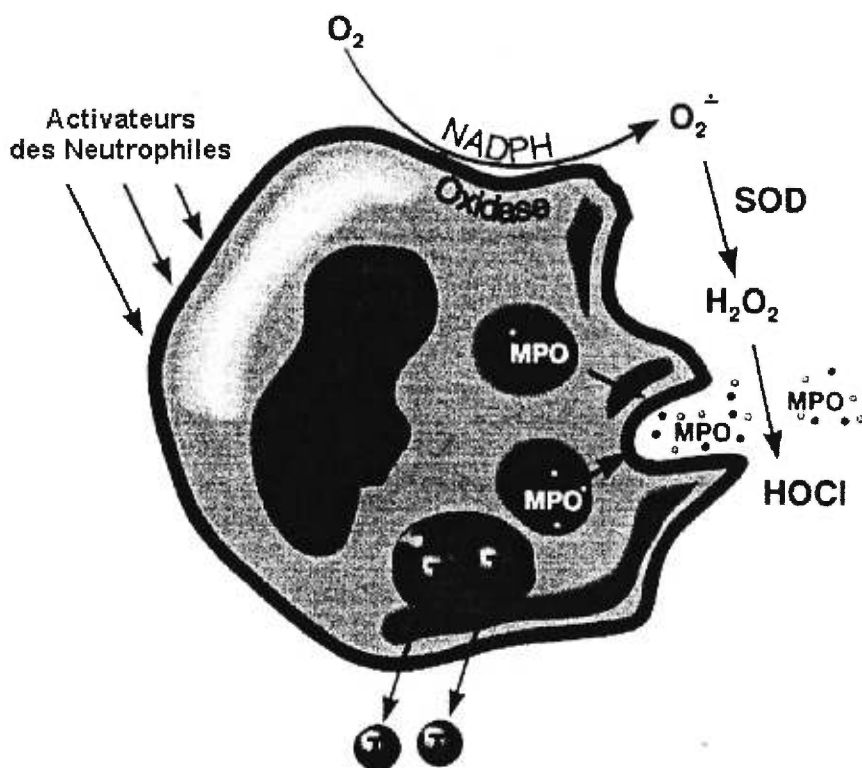
Puisque les acides gras n-3 sont plus sensibles à la peroxydation [4], il nous est permis de conclure qu'un stress oxydatif résultera en une augmentation des écosanoïdes à caractère pro-inflammatoire. Selon certains auteurs, ces réactions peuvent être derrière l'étiologie de l'arthrite rhumatoïde.



**Figure 3** : Compétition des acides gras des familles n-6 et n-3 pour la delta 6-désaturase réalisant un bilan pro-inflammatoire ou anti-inflammatoire des écosanoïdes. (D'après Duvelder , 1991 [73])

Un autre exemple de lipoperoxydation enzymatique biologique provient de la production d'anions superoxydes, de radicaux hydroxyles et d'hypochlorites par les cellules phagocytes (polymorphonucléaires et monocytes). En réponse à la présence de bactéries ou de virus, le phagocyte 'activé' entame la phagocytose en produisant une flambée oxydative basée sur l'anion superoxyde ( $O_2^{\cdot -}$ ) (figure 4).





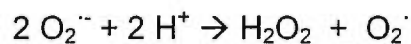
**Figure 4** : Illustration de la flambée oxydative du monocyte activé menant à la production d'anions superoxydes et d'hypochlorites.

La production d'anions superoxydes survient grâce à une flavoenzyme de la membrane plasmique du phagocyte, la NADPH oxydase. L'apport massif d'anions superoxydes est non seulement capable de détruire les bactéries et les virus, mais aussi d'endommager sérieusement les structures cellulaires avoisinantes.

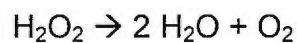
En effet,  $O_2^{\cdot -}$  favorise la dépolymérisation de l'acide hyaluronique, la dénaturation de l'ADN et du collagène, l'inactivation de certains enzymes et l'oxydation des acides gras polyinsaturés des structures lipidiques voisines [3] .

La granulomateuse chronique illustre bien la nécessité pour l'organisme de disposer d'un mode de production d'espèces réactives à caractère pro-oxydant. En effet, des mutations du gène encodant pour la NADPH-oxydase, on en connaît quatre formes différentes à ce jour, rendent l'enzyme non fonctionnelle et empêchent la production normale d'anions superoxydes. Classiquement, les individus porteurs de cette mutation sont davantage sujets aux infections fongiques et bactériennes [5, 6] . Les épisodes d'infections ont un caractère chronique et récurrent.

Le potentiel pro-oxydant de l'anion superoxyde est étroitement lié à l'action successive de la superoxyde dismutase et à celle de la catalase. La première catalyse la dismutation du  $O_2^{\cdot -}$  en peroxyde d'hydrogène ( $H_2O_2$ ) et en  $O_2$  selon l'équation :



C'est à ce niveau que la catalase agit normalement pour réduire le peroxyde d'hydrogène en eau :



Toutefois, la présence d'ions ferreux dans l'environnement moléculaire du  $\text{H}_2\text{O}_2$  permet aussi de réaliser la réaction dite de Fenton :

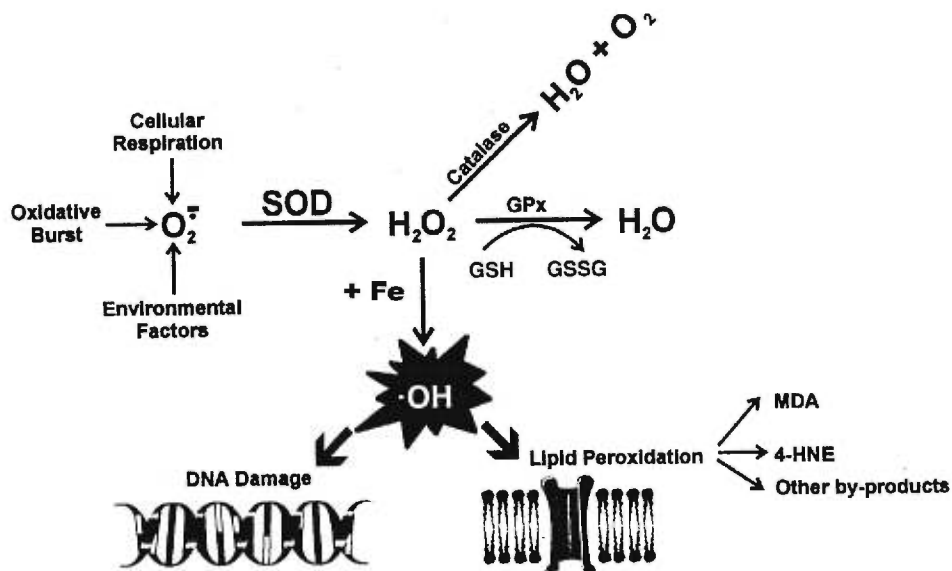


On voit alors apparaître le radical hydroxyl ( $\cdot\text{OH}$ ). Même si ce dernier est moins réactif que l'anion superoxyde, il possède une demi-vie relativement plus longue. En conséquence, cela lui laisse plus de temps pour engendrer une lipoperoxydation (tableau 1 et figure 5). Nous verrons, plus loin, que l'importance du fer dans cette situation est déterminante et préside probablement à bon nombre de lipoperoxydations non enzymatiques[3, 7].

Une dernière espèce réactive, produite par réaction enzymatique du macrophage, nous est fournie grâce à la formation d'hypochlorite ( $\text{ClO}^-$ ) (figure 4). En réagissant avec le  $\text{Cl}_2$  produit par la myéloperoxydase des macrophages, l'oxygène produit l'hypochlorite ( $\text{ClO}^-$ ) dont le pouvoir bactéricide surpasse de loin ceux du chlore et du superoxyde. Toutes ces actions, visant la défense du système immunitaire, peuvent, malgré tout, laisser fuir des espèces réactives ou des radicaux libres et ainsi engendrer une lipoperoxydation spontanée non enzymatique.

**Tableau 1** : Demi-vie de quelques radicaux libres *in vitro* mesurés à 37 degrés Celcius.

Radical	Demi-vie
Radical Hydroxyle	$10^{-9}$ sec
Radical Alkoxy	$10^{-6}$ sec
Radical Peroxyle	7 sec
Radical Organique : (carbon-centered radical)	$10^{-8}$ sec
Peroxyde Hydrogène	minutes
Anion Superoxide	$10^{-5}$ sec
Oxygène Singulet	$10^{-6}$ sec
Radical Semiquinone	Jours



**Figure 5 :** Figure illustrant le potentiel pro-oxydant du fer, lors de la dismutation de l'anion superoxyde (par la superoxyde dismutase) et de la réduction du peroxyde d'hydrogène (par la catalase). La production du radical hydroxyl survient lorsque le fer à l'état libre réagit avec le peroxyde d'hydrogène. Cette réaction est connue sous le nom de réaction de Fenton.

### 1.3 ASPECTS NÉFASTES DE LA LIPOPEROXYDATION

Quel que soit l'événement déclencheur, la séquence par laquelle la lipoperoxydation spontanée survient peut se résumer en trois étapes distinctes : 1) la phase d'initiation ; 2) la phase de propagation et 3) la phase de terminaison (figure 6).

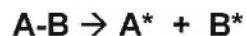
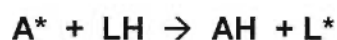
Durant la phase d'initiation, on assiste à l'apparition des radicaux libres. Ces derniers possèdent un potentiel réactif très grand et vont rapidement ( $10^{-9}$  à  $10^{-5}$  secondes) arracher un ou des électrons aux molécules avoisinantes qui les retiennent plus faiblement [3] (tableau 1).

Il s'ensuit une réorganisation intramoléculaire qui initie la phase de propagation. Avec ces réarrangements à l'intérieur de la molécule, visant à stabiliser le déséquilibre électronique, on voit apparaître des cassures dans la molécule cible. Il s'agit probablement de radicaux libres, puisqu'ils se comportent de la même façon que les oxydants de la phase d'initiation. Effectivement, ils sont capables d'arracher à leur tour des électrons aux molécules « donneuses ».

En cours de route, des composés plus stables et terminaux de réaction se forment, ce sont le malondialdéhyde (MDA), diverses formes d'hydroperoxydes organiques (R-OOH) et le 4-hydroxynonanal (4-HNE).

Certains d'entre eux, le MDA et le 4-HNE notamment, servent comme marqueurs de peroxydation [8]. Cependant, on remarque que même ces produits terminaux de peroxydation peuvent réagir avec d'autres molécules et former des complexes chimiques modificateurs de la fonction et/ou de la structure. Ces nouveaux complexes chimiques déterminent le devenir métabolique des molécules cibles. Enfin, les hydroperoxydes, ainsi formés, génèrent d'autres radicaux libres avec l'intervention des métaux de transition dont le fer est le plus dominant et le plus fréquent (réaction de Fenton) [9].

Ultimement, le système de propagation doit être arrêté. Cette étape de terminaison est atteinte lorsque deux radicaux libres se rencontrent et partagent de façon covalente leurs électrons ou lorsque des composés ou systèmes antioxydants entrent en jeu. A cette fin, la stratégie communément utilisée par la cellule consiste à former un composé stabilisé électroniquement. Cette nouvelle structure électronique a un pouvoir réactif faible, qui devra être régénéré sous sa forme antioxydante ou éliminé par l'organisme [3]. C'est le cas notamment du tocophérol, de l'ascorbate et du système de glutathion.

**INITIATION :****PROPAGATION :****TERMINAISON :**

**Figure 6** : Séquence classique par laquelle procède une lipoperoxydation non enzymatique.

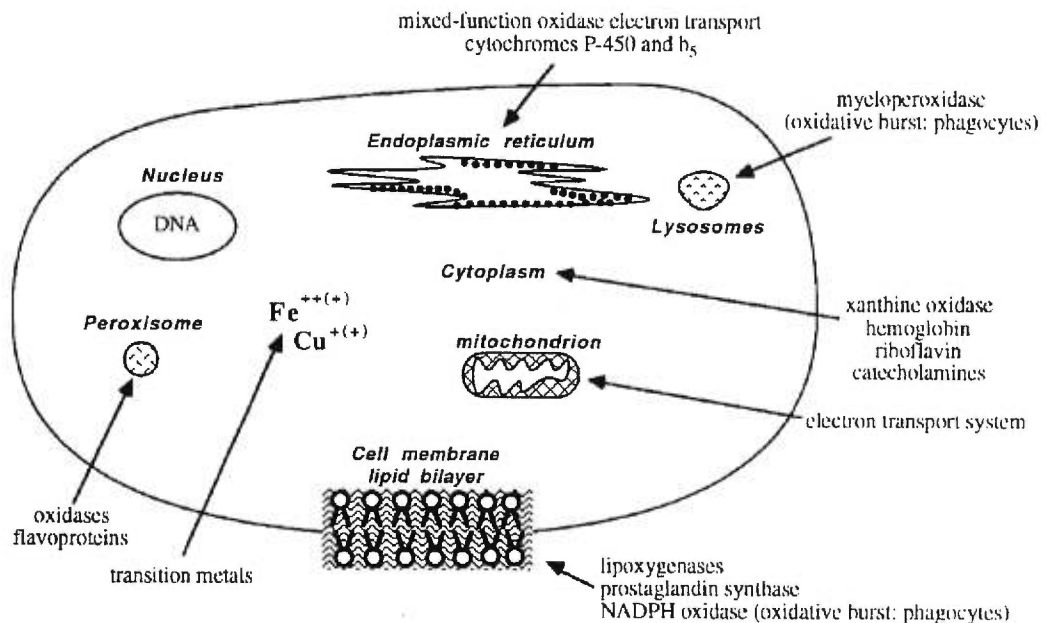
**1.4 LES RESPONSABLES DE LA PEROXYDATION**

Au niveau cellulaire, il existe plusieurs sources potentielles de radicaux libres (figure 7). Que ce soit au niveau des mitochondries, des membranes, du réticulum endoplasmique ou tout simplement du cytosol, plusieurs événements physiologiques peuvent présider à une flambée oxydative qui aboutit à la libération de radicaux libres [7, 10].



Les mécanismes impliqués dans la génération des radicaux libres varient selon le site initial de production. Elle implique, dans plusieurs cas, la coordination ou l'intervention du fer. Au niveau de la mitochondrie, par exemple, la respiration entraîne une fuite d'espèces réactives oxygénées. On ne sait précisément quel pourcentage de l'oxygène total, consommé par la mitochondrie, aboutit à la libération d'espèce oxygénée réactive. Quelques chercheurs, cependant, avancent que la proportion dépend du stade de respiration mitochondriale et qu'elle peut atteindre un maximum de 2% [11]. Il demeure important de savoir qu'une partie de l'oxygène consommé par la mitochondrie peut servir à former des espèces oxygénées réactives aboutissant à une lipoperoxydation [10].

Ce système, comme plusieurs autres, affecte les structures et les constituants mitochondriaux et entraîne un relargage d'une partie du fer normalement lié. L'apport en fer, catalytiquement actif, permet la réaction de Fenton et l'amorce d'une lipoperoxydation spontanée [7].



**Figure 7** : Sources intracellulaires potentielles pouvant conduire à la lipoperoxydation non-enzymatique. (D'après Kehrer,JP. 1994 [7])

Au niveau du réticulum endoplasmique, on découvre une famille d'oxydases aux fonctions mixtes. Ces enzymes interviennent essentiellement dans l'oxydation d'une variété de xénobiotiques et de substances endogènes. Grâce à l'action du NADPH, ces enzymes transforment des composés réduits en composés oxydés. Bien qu'en

situation basale l'implication de cette famille d'enzymes dans la lipoperoxydation spontanée soit négligeable, il demeure que la présence de certains xénobiotiques ou dans le cas d'une perturbation de leur régulation, plusieurs radicaux libres peuvent être libérés [3, 12].

Dans le cytosol, on retrouve aussi plusieurs oxydases solubles. Ces enzymes, surtout la xanthine oxydase (XO), la dopamine- $\beta$ -hydroxylase, l'acide D-amino oxydase, l'urate oxydase et les « fatty acyl coA oxydase », catalysent l'oxydation de substrats endogènes et exogènes. Elles sont ainsi susceptibles de générer des espèces oxygénées réactives[10].

Si les études actuelles n'ont pas, jusqu'à maintenant, déterminé la contribution réelle de l'acide D-amino oxydase, l'urate oxydase et du «fatty acyl coA oxydase » à la génération d'un stress[10]. La xanthine oxydase quant à elle, a fait l'objet de nombreuses études. Elle est capable de réduire directement l'oxygène en anion superoxyde et en peroxyde d'hydrogène et même générer le radical hydroxyl. Compte tenu de sa distribution dans plusieurs types cellulaires elle pourrait contribuer directement au dommage tissulaire par peroxydation, notamment durant les événements d'ischémie-reperfusion cardiaques et intestinaux. Retenons que cette flavoprotéine utilise le FAD et nécessite

absolument le fer dans son état ferreux et le molybdène pour permettre la génération d'anions superoxydes.

Par ailleurs, les métaux de transition semblent avoir intracellulairement, un très grand potentiel à produire les radicaux libres. Cofacteurs enzymatiques essentiels, les métaux de transition, en particulier le cuivre et le fer, facilitent le transfert d'électrons aux macromolécules tel que l'ADN, les protéines et les lipides [10, 13]. Leur propension à catalyser la dégradation des peroxydes organiques en font des éléments pro-oxydants idéaux qui conduisent à la lipoperoxydation spontanée.

Ce potentiel pro-oxydant explique probablement l'absence de fer à l'état libre dans l'organisme humain. Le fer est plutôt associé à des protéines chélatrices qui possèdent des fonctions de transport et de stockage. Le cas de la transferrine (protéine de transport du fer dans le plasma) illustre bien l'effort de l'organisme à contrôler les niveaux de fer libre.

Ainsi, chez un être humain en bonne santé, la capacité de la transferrine à fixer le fer n'est jamais atteinte et se situe généralement au tiers de son potentiel maximal [7]. Nonobstant l'étroite surveillance dont le fer fait l'objet in vivo, certains tissus, faiblement pourvus en éléments chélateurs présentent une sensibilité plus élevée à l'introduction de métaux de transition libre tel que le fer.

C'est à titre d'exemple le cas du cerveau où l'on retrouve énormément de fer lié, mais paradoxalement peu ou pas de protéines chélatrices dans le liquide encéphalo-rachidien. Ainsi, la libération du fer lié n'est pas forcément compensée par une chélation immédiate. Elle laisse donc plus de temps au fer pro-oxydant pour amorcer une lipoperoxydation et des dommages neuraux importants. Cette vulnérabilité du cerveau à affronter la libération de fer catalytiquement actif, participerait à plusieurs désordres neurologiques telles que les maladies de Parkinson et d'Alzeihmer[14-21].

## **1.5 DÉFENSES ANTIOXYDANTES**

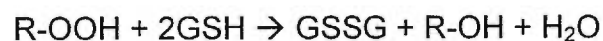
En réponse à toutes ces sources de production de radicaux libres, l'évolution a doté l'organisme de modes de défense. On peut distinguer deux niveaux d'intervention antioxydante, à savoir la défense enzymatique et la défense non enzymatique. Dans le deuxième cas, on distingue à la fois, les sources endogènes et exogènes.

### **1.5.1 Les défenses antioxydantes enzymatiques :**

Ce sont véritablement les systèmes de défense de première ligne. L'enjeu repose sur l'élimination rapide des éléments réactifs (anion superoxyde, peroxyde d'hydrogène et organique) susceptibles d'amorcer une peroxydation (tableau 2). Contre l'agression de l'anion superoxyde, les cellules eukaryotes disposent des enzymes de la

famille des superoxydes dismutases. Cette famille de métalloprotéines, dont la forme CuZnSOD est la plus commune, se retrouve distribuée de façon hétérogène dans l'ensemble des cellules[3]. La vitesse à laquelle ces enzymes transforment l'anion superoxyde en peroxyde d'hydrogène est de l'ordre de  $10^4$  fois plus rapide que la dismutation spontanée observée au pH physiologique in vitro [11] .

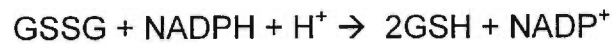
Pour éliminer le produit de la SOD, c'est à dire le  $H_2O_2$ , l'organisme compte sur l'action subséquente de la catalase[3]. Cette haemoprotéine, également bien répandue dans plusieurs types de cellules eukaryotes (au sein des peroxysomes), doit agir rapidement pour empêcher l'action peroxydative potentielle du fer libre. Les peroxydes organiques, quant à eux, devront être éliminés grâce à la glutathion peroxydase. Cette enzyme, nécessite le glutathion comme cofacteur enzymatique dans la réduction des peroxydes organiques libres et du peroxyde d'hydrogène selon les réactions ;



Plusieurs tissus humains possèdent une activité GSH-peroxydase retrouvée dans le cytoplasme des cellules et dans les mitochondries[3].

Il faut remarquer ici que l'action de cette enzyme entraîne une production de glutathion de forme oxydée qui devra être réduit à son tour en GSH pour servir comme cofacteur enzymatique. L'enzyme qui

réalise cette conversion, se nomme la glutathion reductase (GSH-reductase). C'est une protéine cytosolique dont la distribution tissulaire semble suivre celle de la GSH-péroxydase et qui utilise le NADPH pour reconvertir le GSSG en GSH selon la réaction ;



D'autres systèmes enzymatiques, même s'ils constituent une deuxième ligne de défense valent d'être mentionnés. Plusieurs glutathion transférases (GSH-transférase) présentent une activité de GSH-péroxydase. Notons aussi qu'elles sont spécifiques aux peroxydes organiques et ne peuvent pas réduire le peroxyde d'hydrogène.

Il existe aussi plusieurs types d'oxydoreductases (thioltransferase, GSSG reductase, thioredoxine, thioredoxine reductase) dont l'action consiste à réduire les groupements thiols oxydés des protéines et qui contribuent de cette façon à la défense antioxydante globale de la cellule[3].

D'autres enzymes de réparation et d'élimination contribuent, aussi d'une certaine façon, à la défense antioxydante. S'inscrivent dans cette classe : les enzymes protéolytiques, dont la macroxyprotéinase qui sert à éliminer les protéines modifiées non fonctionnelles ; les DNA

polymérase et DNA ligases qui assurent la réparation de l'ADN, les endonucléases et les glycosylases[10, 22].

**Tableau 2** : Système et molécules à caractères antioxydants chez l'être humain.

<b><u>Molécules (endogènes)</u></b>	<b><u>Système enzymatique</u></b>
• Glutathion	• Superoxyde dismutase
• Albumine	• Catalase
• Urate	• GSH-Peroxydase
• Transférine	• GSH-Transferase
• Lactoferine	• Oxydoreductase
• Ferritine	• Enzymes protéolytiques
• Haemosiderine	• DNA polymérase
• Céruloplasmine	• DNA ligase
• Taurine / hypotaurine	• Endonuclease
• Bilirubine	• Glycosylase
• Riboflavine	
<b><u>Molécules (exogènes)</u></b>	
• Tocophérol	
• $\beta$ -carotène / caroténoïdes	
• Ascorbate	
• Vitamine D	
• Vitamine K	



Finalement, on peut considérer que l'apoptose représente une forme de défense antioxydante contre les dommages peroxydatifs. En effet, lorsque les dommages cellulaires sont trop grands et que la cellule est irrémédiablement endommagée, le phénomène d'apoptose, défini comme la mort programmée, peut être perçu comme une défense ultime de la cellule pour protéger le tissu d'origine d'une propagation radicalaire[23]. Il est intéressant de remarquer qu'une partie des événements conduisant à l'apoptose implique la peroxydation. A ce moment précis, une intervention antioxydante peut renverser le processus[24-28].

En fait des données récentes nous indiquent que la peroxydation agit à deux niveaux dans l'apoptose : premièrement, en tant que signal facultatif durant la phase d'induction, et deuxièmement, comme conséquence du changement de perméabilité des mitochondries qui préside à la destruction finale de la cellule[23].

### **1.5.2 Les défenses antioxydantes non-enzymatiques**

Les systèmes non enzymatiques représentent une seconde défense contre la peroxydation, lorsque les enzymes antioxydants n'ont pu arrêter le flot peroxydatif. Ici encore, le rôle des métaux de transition dans l'établissement d'un stress oxydatif n'est pas négligeable.

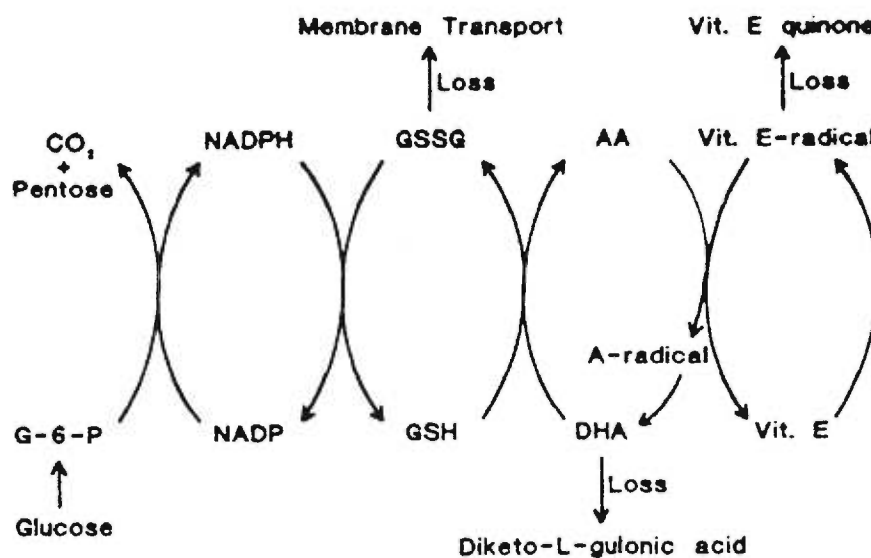
Plusieurs molécules liguantes et / ou transporteuses de métaux peuvent contribuer à diminuer l'efficacité des métaux de transition dans la génération d'un stress oxydant. Dans le cas du fer, ce sont la transferrine et la lactoferine qui servent aux transports du fer dans le plasma et de la ferritine, comme liguant assurant la réserve de fer et réduisant son caractère prooxydant. Dans le cas du cuivre, la céruloplasmine et l'albumine favorisent le transport plasmatique du métal et constituent des protéines à contribution antioxydante.

L'action des antioxydants endogènes et exogènes représente la dernière forme de défense contre l'agression des radicaux libres. Des petites molécules endogènes comme le glutathion, l'urate, la taurine, l'hypotaurine et la bilirubine constituent des exemples d'antioxydants d'origine endogène. Outre sa contribution en tant que cofacteur enzymatique, le glutathion peut aussi réagir directement avec les radicaux libres oxygénés pour former un radical thiyl stable. Par la suite, ce radical sera transformé en glutathion oxydé et entrera dans le cycle de conversion du GSSG en GSH[10].

S'il existe une abondante littérature traitant des méfaits de la peroxydation, il est probablement juste de dire qu'il existe tout autant d'indications sur les effets positifs des antioxydants exogènes. En effet, le tocophérol, le  $\beta$ -carotène et l'ascorbate retiennent beaucoup

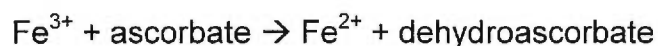
l'attention à cause de leur potentiel thérapeutique et prophylactique. Cependant, leur efficacité en tant qu'adjuvant curatif des désordres impliquant la peroxydation est influencée par plusieurs variables et un consensus reste encore à être trouvé dans de nombreux domaines.

Néanmoins, le mécanisme, soutenant leur potentiel antioxydant, est dans la plupart des cas bien établi. L' $\alpha$ -tocophérol agit davantage comme « briseur de propagation » lorsqu'il réagit avec les radicaux libres oxygénés [10, 12]. La réaction implique l'abandon d'un ion hydrogène de la part du tocophérol, qui devient ainsi un radical tocophéryl, stabilisé et très peu réactif. Par la suite, l'ascorbate, assisté du glutathion, permet de régénérer le tocophérol d'origine (figure 8).



**Figure 8** : Cycles de régénération du tocophérol grâce à l'ascorbate

L'action de l'ascorbate en interaction avec le GSH ne se limite pas qu'à la régénération du tocophérol. En réalité, l'ascorbate est considéré, par plusieurs auteurs, comme l'antioxydant par excellence du compartiment plasmatique[29]. Il réagit directement avec les radicaux libres oxygénés pour former le radical dehydroascorbate qui sera par la suite régénéré par le GSH (figure 8). Pourtant, ce système n'est pas parfait en présence de fer ferrique libre, car l'ascorbate catalyse la conversion du fer ferrique en fer ferreux[30] :



Rappelons que cette forme de fer libre entre directement dans la réaction de Fenton pour générer les radicaux hydroxyles :



Ce système est extrêmement efficace et représente l'un des meilleurs modèles d'initiation de la peroxydation, utilisés *in vitro*[30]. La question à savoir si la coexistence du fer libre et de l'ascorbate *in vivo* possède un potentiel pro-oxydant ou non, demeure entière. Même si la concentration d'ascorbate influence *in vitro* énormément sa capacité à réduire le fer libre à l'état d'ion ferreux, il semble que le plasma résiste fortement à la génération d'ions hydroxyl en présence d'ascorbate[31]. Il est probable *qu'in vivo* le plasma contient d'autres facteurs

antioxydants, protéiques ou autres, qui limitent la réaction de Fenton tout en permettant la réduction du fer ferrique sous sa forme ferreux. Ici encore, on peut supposer que l'organisme s'est doté de moyens propres à neutraliser le caractère pro-oxydant du fer et ce même en présence d'une quantité élevée d'ascorbate plasmatique.

Il est raisonnable de penser que la défense antioxydante du compartiment plasmatique est mise sur le compte de l'ascorbate et que la défense en milieu lipidique est assumée par le tocophérol et le  $\beta$ -carotène. Tout comme la vitamine E, les caroténoïdes peuvent agir comme briseurs de chaîne de propagation en capturant les radicaux libres oxygénés. Il semble que l'action du  $\beta$ -carotène soit maximale à pression réduite d'oxygène ; en situation de haute pression d'oxygène, les doubles liaisons du  $\beta$ -carotène sont vulnérables à l'attaque radicalaire. Néanmoins, on croit que dans cette situation, le tocophérol protège les doubles liaisons du  $\beta$ -carotène[32-35].

Il y a maintenant un intérêt grandissant pour le caractère antioxydant intrinsèque de certains médicaments. En effet, quelques molécules, utilisées dans le traitement de diverses maladies humaines, pourraient avoir une capacité antioxydante *in vivo*[12]. Ces qualités sont directement reliées à ces propres molécules ou à leurs métabolites.

Bien entendu, la contribution de ces molécules exogènes, comme moyen thérapeutique et à visée antioxydante, est fonction de leur concentration et de leur distribution dans l'organisme humain[12]. A titre d'exemple, mentionnons l'aspirine, le probucol, certains anti-inflammatoires non stéroïdiens (ibuprofen et naproxen), ainsi que certains médicaments utilisés dans le traitement de pathologies cardiovasculaires (captopril et lovastatin). Il demeure que ces molécules xénobiotiques sont actuellement utilisées pour leur potentiel thérapeutique et non pour renforcer la défense antioxydante de l'organisme humain, même si leurs actions sont parfois à caractère antioxydante. Par contre, les mécanismes doivent encore être précisés et il est, sans doute, trop tôt pour les inclure dans la prise quotidienne en prophylaxie antioxydante.

## **1.6 EXEMPLE DE L'ATHÉROSCLÉROSE**

Le foie occupe une place considérablement importante en tant que cible et comme protecteur de la lipoperoxydation. En effet, il contrôle étroitement les niveaux circulants de lipides, dont plusieurs sont d'excellents substrats de peroxydation (acides gras libres, triglycérides, phospholipides, cholestérol libre et estérifié). D'autre part, le foie reçoit, en période postprandiale, les antioxydants liposolubles qui sont représentés par le tocophérol, les caroténoïdes, le rétinol, la vitamine D

et la vitamine K[36]. Le foie «enfouit » ces molécules dans les lipoprotéines et les achemine ainsi aux tissus périphériques. De plus, le foie possède la capacité de conjuguer plusieurs xénobiotiques dont certains à caractère pro-oxydant et ainsi de diminuer la pression oxydante exercée sur le système en général.

Il est juste de dire que le foie est étroitement impliqué dans les niveaux de stress oxydatif de tout l'organisme. Ce rôle du foie, bien qu'ignoré par plusieurs, pourrait bien s'avérer crucial dans notre compréhension des phénomènes peroxydatifs associés aux pathologies humaines. Si on reconnaît que la peroxydation est impliquée d'une façon quelconque, dans certaines pathologies humaines (tableau 3), sa contribution réelle est plus difficile à établir. Pourtant dans certaines pathologies comme l'athérosclérose, l'arthrite rhumatoïde, le diabète et les maladies Inflammatoires de l'intestin, la peroxydation fait étroitement partie du processus.

L'athérosclérose représente sans doute le meilleur modèle pour illustrer l'implication des radicaux libres. Non seulement, les radicaux libres peuvent avoir des répercussions sur les lipides et les lipoprotéines dans l'intima des vaisseaux, mais ils peuvent jouer un rôle déterminant dans la violation de l'homéostasie du cholestérol et des lipides en circulation.

**Tableau 3** : Pathologies humaines et lésions pour lesquelles une contribution des radicaux libres est suggérée (répertorié selon les systèmes physiologiques).

<p><b><u>Pulmonaire</u></b></p> <ul style="list-style-type: none"> <li>• Lésions hyperoxiques normobariques</li> <li>• Dysplasie bronchopulmonaire</li> <li>• Inhalation d'oxydants : SO<sub>2</sub>, Nox, O<sub>3</sub></li> <li>• Amiantose</li> <li>• Composés chimiques : paraquat, bleomycine</li> <li>• Syndrome adulte de détresse respiratoire</li> <li>• Emphysème</li> <li>• Tabagisme</li> <li>• Fibrose pulmonaire idiopathique</li> </ul>	<p><b><u>Oculaire</u></b></p> <ul style="list-style-type: none"> <li>• Retinopathie du prématuré</li> <li>• Retinopathie photonique</li> <li>• Cataracte</li> </ul>
<p><b><u>Cardio-vasculaire</u></b></p> <ul style="list-style-type: none"> <li>• Ischémie / reperfusion</li> <li>• Composés chimiques : éthanol, doxorubicine</li> <li>• Athérosclérose</li> <li>• Maladie de Keshan (déficit en sélénium)</li> <li>• Hémochromatose</li> </ul>	<p><b><u>Téguments</u></b></p> <ul style="list-style-type: none"> <li>• Radiation ionisante et solaire</li> <li>• Lésion thermique</li> <li>• Composés chimiques : photosensibilisants (tétracycline)</li> <li>• Dermatitis</li> <li>• Porphyrie</li> </ul>
<p><b><u>Rénale</u></b></p> <ul style="list-style-type: none"> <li>• Néphrose auto-immune (inflammation)</li> <li>• Composés chimiques : aminoglycosides, métaux lourds</li> </ul>	<p><b><u>Musculaire</u></b></p> <ul style="list-style-type: none"> <li>• Dystrophie musculaire</li> <li>• Sclérose multiple</li> <li>• Exercice</li> </ul>
<p><b><u>Hépatique</u></b></p> <ul style="list-style-type: none"> <li>• Fer</li> <li>• Ischémie / reperfusion</li> <li>• Composés chimiques : hydrocarbures halogénés, quinone, acétaminophène, éthanol, cuivre</li> <li>• Endotoxines</li> </ul>	<p><b><u>Divers</u></b></p> <ul style="list-style-type: none"> <li>• Diabète</li> <li>• SIDA</li> <li>• Vieillesse</li> <li>• Exposition à la radioactivité</li> <li>• Composés chimiques : alloxan (diabète), surcharge en fer</li> <li>• Radiosensibilisant</li> <li>• Arthrite rhumatoïde</li> <li>• Inflammation</li> <li>• Trauma tissulaire</li> <li>• Ischémie / reperfusion</li> <li>• Pré-éclampsie</li> <li>• Endométriose</li> </ul>
<p><b><u>Tractus gastro-intestinal</u></b></p> <ul style="list-style-type: none"> <li>• Ischémie / reperfusion</li> <li>• Composés chimiques : anti-inflammatoire non stéroïdiens</li> </ul>	<p><b><u>Neurologique</u></b></p> <ul style="list-style-type: none"> <li>• Parkinson</li> <li>• Alzheimer</li> <li>• Sclérose ammiotrophique latérale</li> <li>• Neuropathie par l'aluminium</li> <li>• Ischémie / reperfusion</li> <li>• Syndrome de Down</li> <li>• Maladie de Battens</li> <li>• Dyskinésie tardive</li> <li>• Dommages traumatiques</li> <li>• Xéno-dommages neuronaux</li> </ul>
<p><b><u>Plasmatique</u></b></p> <ul style="list-style-type: none"> <li>• Composés chimiques : phénylhydrazine, primaquine et composés apparentés, sulfonamides, plomb</li> <li>• photooxydation protoporphyrine</li> <li>• malaria</li> <li>• anémie</li> </ul>	



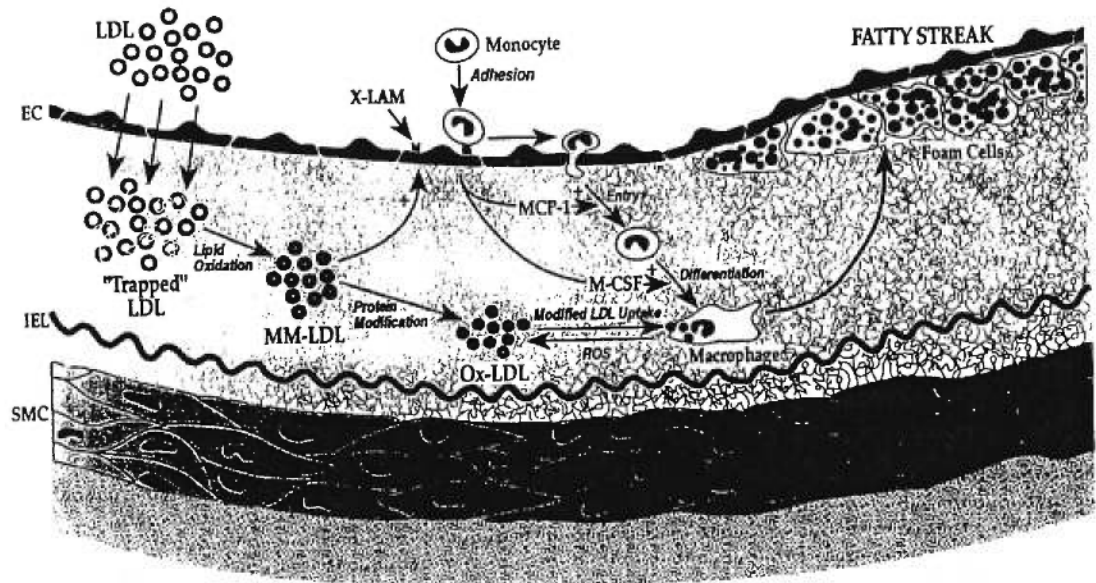
En situation normale, les lipoprotéines que l'on retrouve dans la circulation sanguine, présentent des concentrations de cholestérol et de triglycérides, propres à subvenir aux besoins de la périphérie. Les surplus sont retournés vers le foie sous la forme de résidus de chylomicrons ou de VLDL, de LDL et de HDL2.

On pense que ce retour permet au foie de «juger » des concentrations de cholestérol et de triglycérides en circulation et d'ajuster ainsi sa synthèse endogène aux besoins de la périphérie. Il arrive que ce mécanisme de contrôle se dérègle et entraîne une accumulation anormale des lipoprotéines LDL en circulation[37].

Cette accumulation favorise la migration des LDL de la lumière du vaisseau sanguin vers l'intima, à travers l'endothélium [38] (figure 9). Arrivé dans cet espace, les LDL subissent une première modification oxydative de leur constituant lipidique par les monocytes et les cellules endothéliales présentes[5]. On les dit alors minimalement modifiées (de l'anglais *minimally modified*). L'occupation massive de cet espace par les LDL minimalement modifiées, entraîne le recrutement des monocytes aux sites de migrations, leur transformation en macrophage et leur activation[5, 39]. C'est à ce niveau que les lipoprotéines vont subir une seconde attaque peroxydante. Ici, la peroxydation s'étend

jusqu'aux constituants protéiques, ce qui leur vaut le qualificatif de «fortement oxydées »[37]. Ce stress oxydatif permet, plus particulièrement, la modification oxydative de l'apoprotéine B100 du LDL, le rendant méconnaissable à son récepteur naturel ; le récepteur B/E. De telles lipoprotéines sont alors reconnues par les macrophages grâce à des récepteurs dits «scavengers» ou éboueurs[40]. Ces récepteurs, retrouvés à la surface des monocytes activés, ne sont pas sensibles au type de régulation observée dans d'autres tissus. En fait, l'activité d'internalisation de ces récepteurs n'est pas soumise à des mécanismes de régulation négative.

Il s'ensuit que le macrophage s'empare des LDL modifiées de façon incontrôlée et procède à l'estérification et l'accumulation démesurée du cholestérol. Ce phénomène produit alors, une accumulation massive d'esters de cholestérol dans le cytoplasme. A la longue, le macrophage se transforme en cellule spumeuse, c'est à dire fortement saturée d'esters de cholestérol et incapable de s'en départir.



**Figure 9 :** Représentation schématique des événements prenant place dans l'athérogenèse.

(D'après Berliner JA, 1996 [5])

Avec le temps, cette lésion précoce (strie lipidique) peut évoluer vers une lésion plus complexe. La plaque athéromateuse augmente alors et la lumière du vaisseau se rétrécit. A l'intérieur de l'endothélium, d'autres événements vont apparaître (libération de facteurs pro-inflammatoires et proliférateurs, recrutement des leucocytes, prolifération des cellules musculaires lisses)[41, 42] et augmenter les niveaux de stress oxydant

qui propulse plus loin encore, dans le vaisseau, l'évolution de l'athérosclérose. Alors, s'amorce une séquence d'événements irréversibles passant de la calcification de la plaque fibreuse pour aboutir à la formation d'un thrombus[37].

Dans ce contexte de stress oxydatif et de modifications des constituants lipoprotéiniques, le foie s'avère un élément clé dans l'épuration des lipoprotéines circulantes en trop grande concentration. En situation normale, les excès tissulaires de cholestérol sont pris en charge par les HDL et acheminés vers le foie. A cet égard, les HDL sont les principaux transporteurs de peroxyde dans la circulation ; les hydroperoxydes d'esters de cholestérol sont la forme prédominante de peroxydes transportés par le HDL[43].

De plus, il a été démontré que les cellules hépatocytaires HepG2 lient préférentiellement les HDL contenant ces peroxydes[44, 45]. Cette surveillance hépatique via les HDL contribue, probablement, à maintenir les peroxydes à des niveaux très faibles dans la circulation. Ce phénomène devient encore plus important lorsque l'on considère que l'intestin permet l'absorption oxystérols et de divers hydroperoxydes qui sont déversés dans la circulation sanguine via les chylomicrons et les VLDL[44, 46-49].

L'apport entéral, en pro-oxydants et en composés peroxydés, aurait donc pour effet d'augmenter les niveaux de stress oxydatif de l'organisme, tandis que le foie pourrait veiller à leur épuration. Dans certaines situations, les HDL peuvent subir une modification de leurs constituants suite à une peroxydation, ce qui pourrait affecter leur capacité à effluer le cholestérol de divers types de cellules[50]. Dans d'autres cas, le cholestérol, capturé par le foie à partir de HDL modifiées, n'est pas bien métabolisé. On assiste alors à une augmentation de la mise en réserve du cholestérol et à une diminution de sa conversion en acides biliaires[51-53].

### **1.7 SENSIBILITÉ DU FOIE AU STRESS OXYDATIF**

Toutefois, le foie lui-même n'est pas à l'abri d'une lipoperoxydation et de ses effets pervers. Plusieurs composés possèdent la capacité d'engendrer un déséquilibre de l'équilibre oxydant / antioxydant hépatique. Historiquement, la peroxydation au niveau du foie, a été étudiée en focussant sur le stress oxydatif, engendré par les haloalkanes tel le tétrachlorure de carbone (CCL<sub>4</sub>) et ses métabolites chez le rat[54]. Même si ces composés produisent un foyer de peroxydation, nous savons, aujourd'hui, que les lésions hépatiques (nécrose cellulaire et stéatose hépatique) peuvent être produites, sans l'intervention d'un stress oxydant[54]. Ce sont généralement les

métabolites secondaires, générés par l'activation des enzymes du cytochrome p450 qui initient la lipoperoxydation.

Brièvement, la séquence d'événements se déroule ainsi : clivage des haloalkanes par les enzymes du système p450, apparition subséquente du radical trichloromethyl ( $\text{CCl}_3^*$ ) qui réagit avec l'oxygène moléculaire et produit le radical trichloromethylperoxyl ( $\text{CCl}_3\text{O}_2^*$ ). Les deux formes de radicaux libres interagissent et même se lient, de façon covalente, à des macromolécules de la cellule ( $\text{CCl}_3^*$ ), ou bien ils propagent la peroxydation en s'attaquant aux constituants lipidiques de la membrane ( $\text{CCl}_3\text{O}_2^*$ )[54].

Le plus grand avantage de ce modèle, sur d'autres, réside dans l'extraordinaire flambée oxydative qui l'accompagne, même lors de l'administration du  $\text{CCl}_4$  en aiguë[54]. Néanmoins, l'ampleur des manifestations hépatiques observées, relève davantage de la toxicologie que de la peroxydation. De plus, il faut remarquer le caractère xénobiotique du  $\text{CCl}_4$  pour l'organisme humain, l'exposition n'étant que le fruit d'expositions industrielles ou environnementales fortuites.

Outre les haloalkanes, d'autres composés ont été étudiés pour leur capacité à engendrer une lipoperoxydation hépatique. Ce sont par

exemple : le menadione, le paraquat, le diquat, l'éthanol et l'acétaminophène.

Les trois premiers agents procèdent de façon similaire au niveau du foie, dans la génération d'un stress oxydatif. Ces agents sont appelés des composés «rédox actif» (de l'anglais redox-active compounds). Il a été clairement démontré qu'en subissant une réduction, ces molécules deviennent des radicaux libres qui libèrent des anions superoxydes lorsqu'ils sont réoxydés par l'oxygène moléculaire[54].

La cascade classique entraîne la dismutation de l'anion superoxyde en peroxyde d'hydrogène et la détoxyfication de ce dernier par la glutathion peroxydase. Le résultat, après plusieurs de ces cycles, est une déplétion sévère en glutathion cellulaire et en NADPH, ce qui provoque une augmentation du stress oxydatif dans l'hépatocyte.

L'éthanol, qu'il soit administré au foie, de façon chronique ou aiguë, entraîne des dommages cellulaires tant au niveau du cytosol que des peroxysomes et des microsomes. L'éthanol entraîne une lipoperoxydation hépatique en activant le système de monooxygénase-p450 hépatique (cytochrome P450 2E1). Cette activation produit un radical libre hydroxyethyl et une déplétion subséquente en glutathion

réduit. Ici encore, le résultat est une augmentation du stress oxydatif [54].

Quant à l'acétaminophène, elle est bien connue pour ses effets hépatotoxiques dont un certain nombre pourrait être causé par une peroxydation. En effet, les hépatocytes en culture, exposés à l'acétaminophène, subissent une mort cellulaire qui est toujours précédée par une augmentation des concentrations de marqueurs de peroxydation. De plus, la supplémentation au préalable en tocophérol peut prévenir la peroxydation et la mort cellulaire[55-57]. La toxicité de l'acétaminophène provient du N-acetyl-p-benzoquinone imine (NAPQI) qui augmente les niveaux de stress oxydatif et la déplétion en glutathion. D'ailleurs, l'acétaminophène *per se* possède des propriétés antioxydantes. Cette caractéristique apparaît quand on dépasse la capacité de l'hépatocyte à détoxifier. L'acétaminophène s'accumule alors, sans être métabolisée en NAPQI, ce qui confère ainsi une protection antioxydante à la cellule[58].

La liste complète des pro-oxydants dépasse ces quelques exemples. Elle inclut des xénobiotiques tels que le tert-butyl hydroperoxide, le cumène hydroperoxyde, l'halotane, l'acrolein, le bromobenzene, le nitrofurantoin, mais aussi des événements telles que l'ischémie / reperfusion du foie, l'irradiation et la chimiothérapie, etc. (tableau 4).



**Tableau 4** : Composés et événements susceptibles d'induire une peroxydation hépatique in vivo.

<b><u>ÉLÉMENTS</u></b>	<b><u>MOLÉCULES BIOLOGIQUES</u></b>
Fer	Transférine
Cuivre	Céruloplasmine
Aluminium	Myeloperoxidase
Cadmium	Dopamine $\beta$ -hydroxylase
Plomb	Acide D-amino oxydase
	Urate oxydase
<b><u>COMPOSÉS SIMPLES</u></b>	Acyl CoA oxydase
Anion superoxyde	NADPH oxydase
Radical hydroxyl et peroxyde	Xantine oxydase
Peroxyde d'hydrogène	Oxydases aux fonctions mixtes
Éthanol	Acides gras polyinsaturés
Hypochlorite	Tocophérol
	$\beta$ -carotène
<b><u>COMPOSÉS ORGANIQUES COMPLEXES</u></b>	Oxystérol
	<b><u>ÉVÉNEMENTS CELLULAIRES</u></b>
Hydroperoxydes organiques	Radiation ionisante
Haloalkanes	Rayonnement ultraviolet
Paracetamol	Dommages tissulaires : chimiques, mécanique ou ischémique
Nitrofurantoin	
Menadione	
Paraquat / diquat	
Cocaïne	

## 1.8 CONTEXTE HÉPATIQUE DU FER

Les modèles de peroxydation hépatique impliquant le fer sont de loin les plus utilisés et les mieux étudiés. Ceci est justifié par le rôle du foie dans l'homéostasie du fer et la synergie de ce dernier lors de l'induction peroxydative par d'autres composés. L'halotane[54], l'acétaminophène[3, 59], l'éthanol[60-64], le  $\text{CCl}_4$  [65] et les hydroperoxydes organiques[66-68] sont tous des exemples de xénobiotiques pro-oxydants où le fer aggrave et/ou est nécessaire pour provoquer une flambée oxydative.

Dans ces cas, le fer pro-oxydant peut agir en tant que cofacteur ou résulter de l'agression primaire par le xénobiotique. Il existe une formidable quantité d'articles qui s'intéressent au pouvoir pro-oxydant du fer. Dans la plupart des modèles, on utilise la surcharge en fer pour créer un déséquilibre en faveur des pro-oxydants cellulaires et ainsi induire un stress oxydatif. Nous l'avons mentionné auparavant, lorsque le fer est sous forme libre et catalytiquement accessible, il permet la réaction de Fenton. La plupart des modèles de surcharge en fer s'appuient sur cette propriété.

Il faut comprendre que la libération de fer sous sa forme libre survient lorsque des processus cellulaires anormaux sont en cours. La dissociation du fer de ses chélateurs et transporteurs implique souvent

un contexte de peroxydation. En effet, bien qu'on accepte l'idée d'une production de radical hydroxyl via la réaction de Fenton, il nous faut expliquer les sources de fer libre catalytiquement disponibles *in vivo*. Normalement, le fer subit une surveillance cellulaire très étroite afin qu'il ne se retrouve libre dans la cellule ou en circulation.

Pour y arriver, l'organisme dispose de protéines de transport (transferrine, lactoferrine), de protéines de stockage (ferritine, haemosidérine) et enfin de protéines où il est incorporé dans leurs fonctions (hémoglobine, myoglobine)[1]. Des 4 grammes de fer contenus en moyenne dans l'organisme humain, environ 65% s'associe à l'hémoglobine (~ 2.5 g) et la myoglobine (~ 200 mg). Le reste se retrouve, mis en réserve et lié à la ferritine (~ 4 mg), surtout au niveau du foie, mais aussi dans la moelle osseuse et la rate. Finalement, il doit y avoir un pool de fer libre en transit entre les différents compartiments, qui compte pour le reste du fer. Ce pool varie, dans son contenu en fer, et est difficile à évaluer de façon précise. Enfin, notons que l'on excrète environ 0.6 mg de fer quotidiennement[1, 69].

Le consensus veut que le fer puisse être délocalisé de ces chélateurs et ou transporteurs et impliqué dans une réaction de Fenton. Cependant, au pH physiologique il est peu probable que la transferrine ou la lactoferrine libère le fer. Par contre, en situation d'ischémie / reperfusion

où le pH peut atteindre des valeurs  $< 5.6$ , la libération du fer est envisageable[70]. La mobilisation du fer de la ferritine peut être induite par l'anion superoxyde et l'ascorbate[70, 71]. Des résultats similaires peuvent être obtenus par les systèmes générant l'anion superoxyde, comme celui de la xantine / xantine oxydase ou encore via les cellules polymorphonucléaires[72]. Le peroxyde d'hydrogène est aussi capable de mobiliser le fer des protéines hémiques.

Finalement, l'haemosiderrine représente une forme de fer lié, plus difficilement mobilisable que la ferritine. On a suggéré que cette situation représente peut-être une forme de protection de la cellule contre la mobilisation du fer de la ferritine[70].

En conclusion, on peut affirmer que de façon générale, tout dommage tissulaire du genre mécanique, chimique, ischémique ou toute atteinte permettant la rupture cellulaire avec le relâchement de son contenu permet la libération de fer libre qui peut-être mis à profit dans les événements peroxydatifs[70].

### **1.9 FONCTIONS HÉPATIQUES RELIÉES AU CHOLESTÉROL**

Le foie occupe une place de toute première importance dans la physiologie normale. Celui-ci assiste à la détoxification et à l'élimination d'agents toxiques pour l'organisme. D'autre part, le foie synthétise

plusieurs des protéines et enzymes que l'on retrouve en circulation, et participe activement à pourvoir aux besoins énergétiques de l'organisme. Bien que nous l'ayons souligné auparavant, il convient d'insister, à nouveau, sur le rôle important du foie, dans le métabolisme du cholestérol. Cette molécule assure des fonctions vitales dans le corps humain.

En effet, le cholestérol participe, dans le maintien de l'intégrité membranaire, et sert de précurseur pour l'élaboration d'hormones stéroïdiennes et des acides biliaires. La synthèse et l'utilisation du cholestérol doivent être étroitement régulées afin d'éviter l'accumulation excessive et le dépôt anormal dans les tissus. C'est à l'égard de ces événements pouvant mener à l'athérosclérose, que le «grand public » est appelé à exercer un contrôle sévère de la consommation du cholestérol. En fait, la majorité des niveaux circulants de cholestérol en période de jeûne est d'origine hépatique[73]. Ce contexte métabolique illustre bien les difficultés cliniques que l'on rencontre à faire diminuer les niveaux de cholestérol par une intervention nutritionnelle.

En réalité, le foie représente l'unité centrale, visée par les thérapies pharmacologiques qui sont assistées par des interventions nutritionnelles. Très souvent, l'approche médicamenteuse vise à bloquer ou à diminuer les voies hépatiques de synthèse du cholestérol afin

d'obtenir une diminution des niveaux circulants. Il est juste de dire que seul le foie peut réaliser un circuit complet d'événements métaboliques menant à la synthèse, la transformation et l'élimination du cholestérol hors de l'organisme.

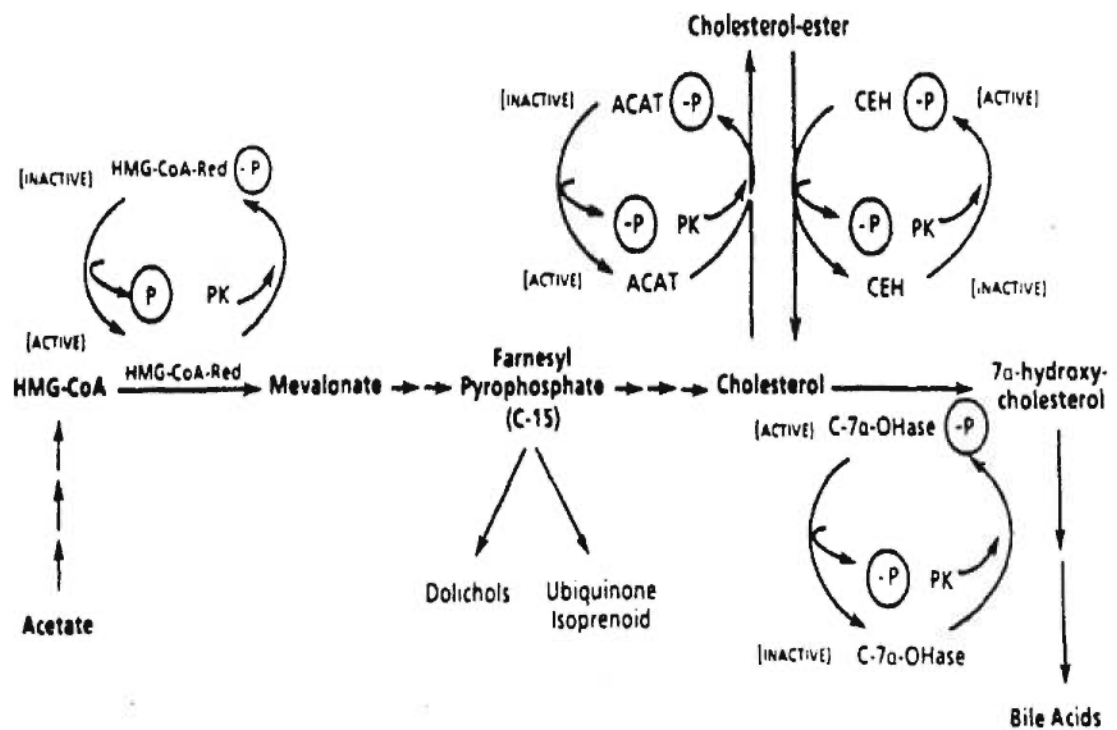
Le foie est le seul organe capable d'éliminer l'excès de cholestérol de l'organisme en le convertissant en acides biliaires et en le déversant dans la bile. Le circuit qu'emprunte les lipoprotéines d'origine hépatique en circulation, permet de «relier » le foie biochimiquement aux tissus périphériques. Classiquement, le foie produit des VLDL et des LDL qu'il largue dans la circulation. Sous l'action de la lipoprotéine lipase et de la lipase hépatique, les particules vont déposer une certaine quantité de leur contenu lipidique dans les tissus périphériques et changer de densité pour atteindre la forme de LDL dense[73].

A ce stade, ces lipoprotéines continueront de déverser leur cholestérol de la périphérie vers les tissus grâce à leur reconnaissance par le récepteur B/E, particulièrement par le foie. Les LDL sont alors internalisées et catabolisées. Leur captation augmente les niveaux intracellulaires hépatiques en cholestérol, ce qui entraîne l'inactivation de l'HMG CoA reductase. Le nouveau cholestérol est alors drainé vers la cholestérol 7- $\alpha$ -hydroxylase pour être converti en acides biliaires[74].

L'excès de cholestérol libre pourra subir l'action de l'acyl cholestérol acyl transférase (ACAT) pour être stocké sous forme d'esters de cholestérol. Lorsque la bile est déversée dans la lumière intestinale pour participer à l'absorption des nutriments liposolubles, on assiste à la réabsorption des acides biliaires via la veine porte en direction du foie. Ce circuit a reçu le nom de cycle enterohépatique. Le retour des acides biliaires au foie entraîne l'inactivation de la cholestérol 7- $\alpha$ -hydroxylase et de l'HMG CoA reductase[73, 74].

Comme on peut le constater, la régulation étroite de ce système assure l'homéostasie du cholestérol à travers l'organisme. La figure 10 illustre cette étroite collaboration des trois enzymes clés dans l'homéostasie du cholestérol : HMG CoA reductase, ACAT et 7- $\alpha$ -hydroxylase.

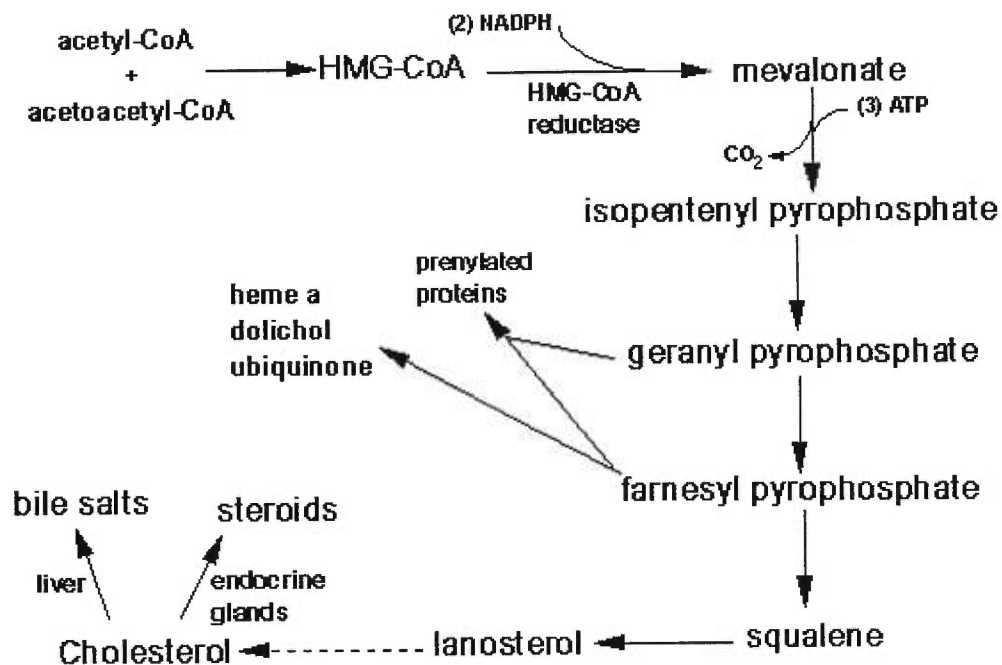
La synthèse du cholestérol à partir de l'acétyl-CoA comprend plus de 20 étapes enzymatiques mais peut se résumer en 5 étapes distinctes (figure 11) : 1) Formation de 3-hydroxy-3-méthylglutaryl-CoA à partir des acétyl coA, 2) transformation de l'HMG-CoA en mévalonate sous le contrôle de l'HMG CoA reductase, 3) Conversion du mévalonate en isopentenyl pyrophosphate (IPP) avec perte de CO<sub>2</sub>, 4) Production de squalène à partir de l'IPP et 5) conversion du squalène en cholestérol.



**Figure 10** : Illustration des interrelations de l'HMG-CoA réductase, de l'ACAT et de la cholestérol 7 $\alpha$ -hydroxylase dans le tissu hépatique.

(D'après Vlahcevic, 1991 [74])



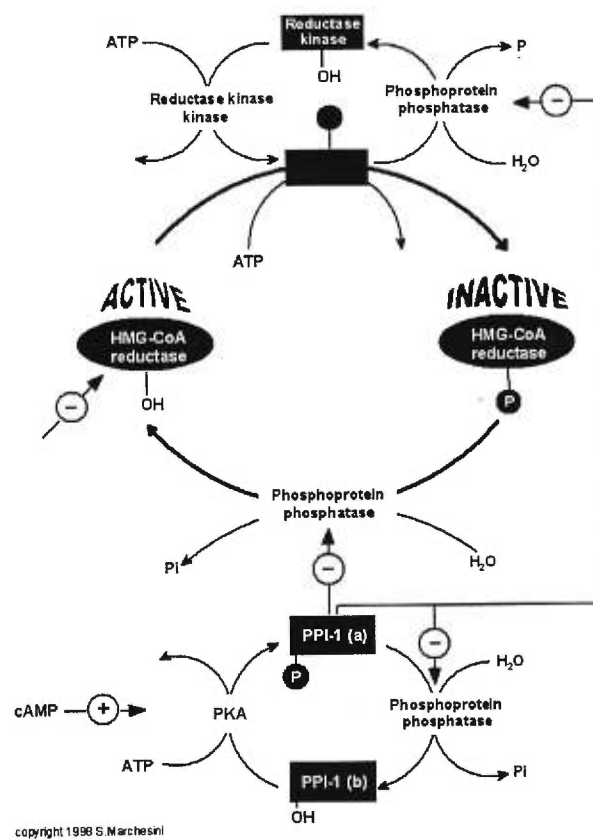


copyright 1996 M.W.King

**Figure 11** : Résumé de la voie de synthèse du cholestérol à partir de l'acétate sous le contrôle de l'HMG CoA reductase.

L'étape limitante de cette synthèse de novo se situe au niveau de l'HMG CoA reductase (étape 2). L'HMG CoA reductase peut être régulée par modification covalente directe ou par contrôle intracellulaire du niveau total de la protéine[75, 76]. Cette enzyme microsomiale est sous forme active lorsque non phosphorylée et perd sa capacité enzymatique lorsque phosphorylée (figure 12). Ceci permet un contrôle rapide des niveaux d'activité.

A plus long terme, le contrôle de l'activité de l'HMG CoA reductase a lieu au niveau de la synthèse et de la dégradation de l'enzyme[77]. Ainsi, lorsque les niveaux cellulaires de cholestérol sont faibles ou élevés, on assiste respectivement à l'augmentation de la synthèse de l'enzyme ou à l'augmentation de sa dégradation.



**Figure 12 :** Mécanismes de régulation post-transcriptionnelle de l'HMG CoA reductase .

Si l'on comprend assez bien le fonctionnement de l'HMG CoA reductase et celui de ses régulations multiples, on ne peut en dire autant de l'ACAT. Certaines études suggèrent qu'elle a pour principale fonction de prévenir l'accumulation «toxique » de cholestérol libre dans les membranes cellulaires et de promouvoir l'assemblage des chylomicrons (intestin) et des VLDL (foie)[78, 79].

Nous savons que cette enzyme est attachée au réticulum endoplasmique rugueux et que les niveaux d'activité sont fortement influencés par les concentrations cellulaires de cholestérol et d'oxysterol (25-hydroxycholestérol) originant des LDL[80]. En quelque sorte, l'ACAT préférerait utiliser le cholestérol sous forme libre d'origine exogène plutôt que d'estérifier du cholestérol d'origine endogène. La représentation que l'on se fait actuellement de ce système, implique que l'ACAT «travaillerait » sur le cholestérol qui atteint le réticulum endoplasmique, ce qui constituerait une étape intermédiaire avant de bifurquer vers la membrane plasmique ou l'assemblage des lipoprotéines[80].

On considère que l'ACAT est aussi régulé par le degré de phosphorylation. Mais des études sont encore nécessaires pour identifier les protéines responsables de ce processus et déterminer son ampleur sur l'activité de l'enzyme[81]. Les données, actuellement

disponibles, nous indiquent qu'il y aurait en fait une «famille » de gènes ACAT fonctionnels chez l'humain (trois gènes différents à ce jour ont été identifiés et ont reçu les noms de ACAT 1, 2 et 3). Les formes 1 et 2 présentent l'activité essentielle d'estérification du cholestérol. Par ailleurs, on ignore tout de leurs coordinations dans divers organes[82, 83].

Si l'unité enzymatique de production du cholestérol hépatique est représentée par l'HMG CoA reductase et l'unité de stockage par l'ACAT, l'unité responsable de l'élimination du cholestérol revient à la voie de synthèse des acides biliaires dont la cholestérol 7- $\alpha$ -hydroxylase représente l'étape limitante. Cette enzyme du réticulum endoplasmique catalyse la conversion du cholestérol en 7- $\alpha$ -hydroxycholestérol [74](figure 13).

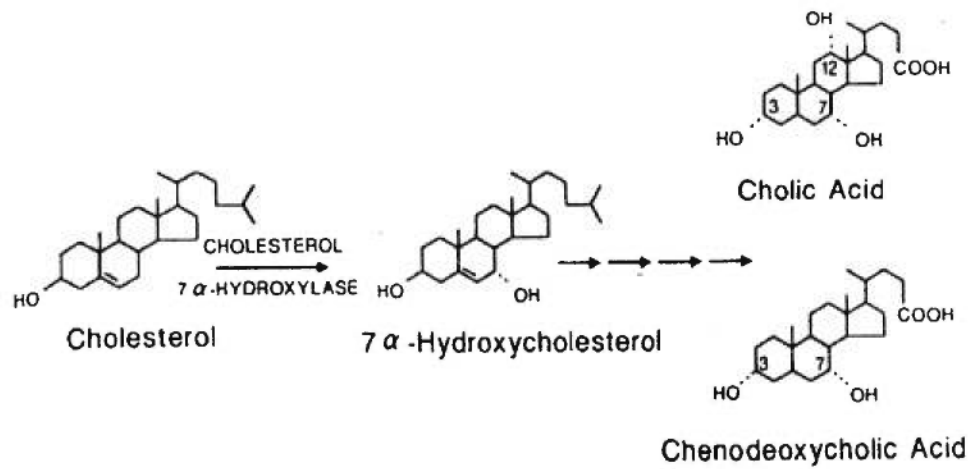
L'enzyme ne peut convertir que le cholestérol, car les oxystérols et les stérols acides sont convertis par l'oxystérol 7- $\alpha$ -hydroxylase mitochondriale[84]. La cholestérol 7- $\alpha$ -hydroxylase serait elle aussi contrôlée par des mécanismes de phosphorylation (active) / déphosphorylation (inactive)[85, 86].

La régulation de l'activité de la cholestérol 7 $\alpha$ -hydroxylase se fait aussi au niveau de sa transcription. Ainsi, lorsque les acides biliaires sont réabsorbés dans le petit intestin puis acheminés au foie, un signal est acheminé au gène de la cholestérol 7 $\alpha$ -hydroxylase qui entraîne la diminution de la transcription du gène. Le cholestérol diététique chez le rat et la souris augmente la transcription du gène alors que, chez l'humain, les primates, le lapin et le hamster, il inhibe la synthèse de l'ARNm[84].

On croit que l'activation de la transcription du gène, chez le rat et la souris, provient d'une séquence promotrice, absente chez l'être humain, sensible aux oxystérols et induisant la transcription du gène (séquence LXR)[84]. Des données récentes indiquent que l'oxystérol 7 $\alpha$ -hydroxylase serait, en quelque sorte, une voie d'appoint à la synthèse d'acides biliaires 7 $\alpha$ -hydroxylées lorsque la voie classique de la cholestérol 7 $\alpha$ -hydroxylase est compromise[84].

On suggère donc que, les stérols neutres sont convertis en cholate par la cholestérol 7 $\alpha$ -hydroxylase et que, les stérols acides sont convertis en chenodéoxycholate par l'oxystérol 7 $\alpha$ -hydroxylase. Nous ne sommes qu'au début de ces investigations et l'enjeu des futures recherches sera d'identifier la contribution réelle des deux voies métaboliques

d'hydroxylation en position  $7\alpha$  dans le métabolisme du cholestérol et des acides biliaires.



**Figure 13** : Schématisation de la voie de synthèse des acides biliaires neutres sous le contrôle de la cholestérol  $7\alpha$ -hydroxylase.

## II. HYPOTHÈSE

Il est maintenant clair que l'on ne peut considérer le rôle de la peroxydation en centrant seulement notre attention sur un organe ou un mécanisme isolé. Les produits de peroxydation, qu'ils soient d'origine endogène ou exogène, «voyagent » dans l'organisme humain et risquent, par conséquent, d'interagir avec d'autres systèmes ou organes, notamment le foie. Ce dernier préside à plusieurs processus fondamentaux dans la physiologie du corps humain. Les fonctions de synthèse et de sécrétion protéiques ainsi que l'approvisionnement en facteurs liposolubles, en particulier les antioxydants, lui confèrent un rôle majeur dans le contrôle du stress oxydatif.

Cependant, étant le foyer central de l'homéostasie du cholestérol et d'autres types de lipides, il devient la cible de choix d'atteinte peroxydative. Pourtant, on ne connaît ni la sensibilité des voies de régulations du cholestérol au stress oxydatif ni les répercussions qu'une telle atteinte pourrait avoir sur l'homéostasie périphérique et hépatique du cholestérol.

C'est dans ce cadre que s'inscrivent les objectifs de nos études. Des stratégies expérimentales sont élaborées pour répondre à la question

que tient l'effet de la lipoperoxydation sur le carrefour métabolique du cholestérol.

En premier lieu, les modèles *in vivo* consistent à diminuer les niveaux de défenses antioxydantes, en utilisant des diètes riches en oxydants. Dans un deuxième temps, des modèles *in vitro* sont employés pour se rapprocher le plus possible des mécanismes impliqués. L'approche nutritionnelle aussi séduisante soit elle *in vivo*, ne permet pas de cibler le foie. Le caractère pro-oxydant du fer est exploité autant dans les modèles *in vivo* que dans les modèles *in vitro*. La surcharge en fer, chronique ou aiguë, offre la possibilité d'étudier la place que tient le stress oxydatif dans l'homéostasie du cholestérol.



### III. ARTICLES ORIGINAUX

- Sylvain Brunet, France Guertin, Louise Thibault, Victor Gavino, Edgard Delvin and Emile Levy. Iron-salicylate complex induces peroxidation, alters hepatic lipid profile and affects plasma lipoprotein composition. *Atherosclerosis* : 129, 159-168, 1997
- Brunet S, Thibault L, Lepage G, Seidman EG, Dubé N and Levy E. Modulation of endoplasmic reticulum-bound cholesterol regulatory enzymes by iron/ascorbate-mediated lipid peroxidation. (*Free radicals Biology and Medicine*, sous presse).
- Sylvain Brunet, Louise Thibault, Edgar Delvin, Wagner Yotov, Moïse Bendayan and Emile Levy. Dietary iron overload and induced lipid peroxidation are associated with impaired plasma lipid transport and hepatic sterol metabolism in rats. *Hepatology*, 29 : 1809-1817, 1999
- Sylvain Brunet, Isabelle Suc, Nadia Dubé, Louise Thibault and Emile Levy. Lipoprotein metabolism alterations in iron-loaded HepG2 cells. (*Biochemical & Biophysical Research Communications*., 4 novembre 1999).



## Iron-salicylate complex induces peroxidation, alters hepatic lipid profile and affects plasma lipoprotein composition

Sylvain Brunet <sup>a</sup>, France Guertin <sup>a</sup>, Louise Thibault <sup>a</sup>, Victor Gavino <sup>a</sup>, Edgard Delvin <sup>b</sup>,  
Emile Levy <sup>a,\*</sup>

<sup>a</sup> Department of Nutrition, Pediatric Research Center, Hôpital Sainte-Justine, Université de Montréal, 3175 Côte Sainte-Catherine, Montréal, Québec H3T 1C5, Canada

<sup>b</sup> Department of Biochemistry, Université de Montréal, Québec, Canada

Received 23 October 1995; received in revised form 29 October 1996; accepted 30 October 1996

### Abstract

Iron overload, with its associated toxic effects, has serious health consequences and results in damage to the liver, heart and other organs. Salicylate may be used as the lipophilic carrier, transporting more iron into hepatocytes. In this study, we examined the effect of the combined administration of these compounds on plasma lipid profile and lipoprotein composition, as well as on hepatic lipid concentration. Male Sprague-Dawley rats were injected i.p. with Fe (15 mg/kg weight). This injection was repeated 24 h later with a gavage of sodium salicylate (700 mg/kg). Control rats received 0.9% NaCl only. The peroxidation indices TBARS ( $P < 0.001$ ) and conjugated dienes ( $P < 0.05$ ) significantly increased in the blood (50 and 122%, respectively) and liver (333 and 101%, respectively) of Fe-salicylate-treated rats. Concomitantly, blood and liver arachidonic acid content was diminished by iron treatment. In parallel, the plasma lipid profile was markedly affected in Fe-salicylate treated-rats. Lower plasma concentrations of total cholesterol (25%,  $P < 0.0001$ ) cholesteryl ester, (34%,  $P < 0.001$ ) and high-density lipoprotein-cholesterol (50%,  $P < 0.001$ ) were observed. Lipoprotein composition analysis revealed enrichment of free cholesterol and depletion of cholesterol ester in very low-density, intermediate-density, low-density and high-density (HDL<sub>2</sub>, HDL<sub>3</sub>) lipoproteins. Furthermore, SDS-polyacrylamide gel electrophoresis revealed several alterations in the apolipoprotein distribution of these lipoproteins. The activity of lecithin:cholesterol acyltransferase was unchanged and could not account for the reduction of cholesterol esterification. As for the plasma, the liver exhibited a significant ( $P < 0.001$ ) decrease in total cholesterol ( $2.42 \pm 0.07$  versus  $1.89 \pm 0.06$  mg/g liver), essentially due to a reduction in cholesteryl ester ( $0.93 \pm 0.07$  versus  $0.51 \pm 0.03$  mg/g liver,  $P < 0.001$ ). Again, the activity of ACAT (dpm/mg microsomal protein) was not lower ( $12\,700 \pm 1250$ ) than that of controls ( $9650 \pm 1080$ ). Thus, the iron-salicylate was able to induce peroxidation and to profoundly affect the intravascular and intrahepatic lipid, and plasma lipoprotein metabolism. Additional work is needed to elucidate the mechanisms involved in the underlying lipid and lipoprotein abnormalities. © 1997 Elsevier Science Ireland Ltd.

**Keywords:** Peroxidation; Iron; Salicylate; Lipoproteins; LCAT; ACAT

### 1. Introduction

Iron can initiate lipid peroxidation by catalyzing the generation of free radicals, resulting in damage to both cellular organelles and the cell membrane [1,2]. Im-

pairment of hepatic oxidative metabolism and electron transport chain in mitochondria, selective reductions of hemoproteins in microsomes and increased fragility of lysosomes are among the reported functional organelle defects [3–6]. Evidence has been provided for iron-induced lipid peroxidation in both in vitro systems and in vivo in different iron overload models [6–8]. For example, lipid peroxidation develops rapidly when isolated

\* Corresponding author. Tel.: +1 514 3454626; fax: +1 514 3454999.

hepatocytes in suspension are incubated with iron salts [9,10]. Furthermore, the formation of iron-induced lipid peroxidation affects several enzymes [11,12].

The impact of lipoprotein peroxidation has traditionally been studied through *in vitro* modification of low-density and high-density lipoproteins by chemical processes such as acetylation, methylation and conjugation with malondialdehyde [13–16]. Despite the iron-induced lipid peroxidation, there is surprisingly no thorough examination of lipid profile as well as lipoprotein composition and metabolism in relation to acute iron overload. Fasting plasma lipoproteins essentially derive from the liver. Since liver dysfunction occurs following iron overload and the liver is the major recipient of iron excess [17], lipoprotein processing may be affected. In the present study, we examined whether lipid profile and lipoprotein composition are impaired in rats by acute iron treatment. To this end, we chose a well-known combination of iron–salicylate to increase iron delivery to the liver, salicylate being an efficient transporter of iron into hepatocytes and kupfer cells [18].

## 2. Material and methods

### 2.1. Animal treatment

The experiments were performed on male Sprague-Dawley rats (250–300 g) obtained from the Charles River Breeding Company (Montreal, Canada). Rats were allowed free access to water and food. Room temperature was maintained at 22°C with a 12 h light cycle. After 2–3 days of acclimatization, half of the rats received a single *i.p.* injection of FeCl<sub>3</sub> (15 mg/kg weight in a final volume of 300  $\mu$ l saline). This injection was repeated 24 h later together with a gavage of sodium salicylate (700 mg/kg weight in a final volume of 1 ml saline). Control rats were treated in the same manner but with 0.9% NaCl or salicylate alone.

### 2.2. Blood collection and preparation

Twenty-four hours after the last injection, rats were fasted overnight before receiving pentobarbital (48 mg/kg body wt *i.p.*). Blood was collected in an EDTA-containing tube (1 mg/ml) and separated immediately by low speed centrifugation (26 000 rpm, 25 min, 4°C). For determination of the thiobarbituric acid reactive substance (TBARS) values, a fraction of plasma was immediately frozen in liquid nitrogen and stored at –80°C until analysis.

### 2.3. Isolation of lipoprotein

Lipoproteins were isolated from fresh plasma by

conventional discontinuous density gradient ultracentrifugation as previously described [19]. Briefly, very low-density (VLDL), intermediate density (IDL) and low-density lipoproteins (LDL) were isolated at  $d = 1.006$ , 1.019 and 1.063 g/ml respectively, at 40 000 rpm at 5°C for 18 h. The separation of high-density lipoprotein (HDL) subpopulations was performed at 40 000 rpm for 48 h at 5°C at the following densities: 1.125 g/ml for HDL<sub>2</sub> and 1.21 g/ml for HDL<sub>3</sub>. Each fraction was dialyzed intensively against phosphate buffered saline (PBS) with 0.001 M EDTA, pH 7.0 at 4°C.

### 2.4. Plasma lipid and lipoprotein analysis

Plasma concentrations of total cholesterol (TC), free cholesterol (FC) and triglycerides (TG) were measured enzymatically using commercial kits (Boehringer Mannheim, Montreal, Canada) as previously described [20]. Cholesteryl ester was calculated as the difference between total and free cholesterol. The analysis of fatty acid composition was carried out as described previously [23]. HDL-cholesterol (HDL-C) was measured after precipitation of VLDL and LDL with phosphotungstic acid [20]. Lipoprotein-protein was quantified according to Lowry et al. [21] with bovine serum albumin as a standard. Phospholipids (PL) were determined by the Bartlett method [22]. Apolipoprotein content of plasma lipoproteins was qualitatively assayed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [19,20]. Gels were stained with Coomassie blue for 1 h and destained in 7% acetic acid. The bands of apolipoproteins were identified by comparison with the mobility of apolipoprotein and molecular weight standards.

### 2.5. Determination of hepatic lipid

In order to determine hepatic lipid content and lipid peroxidation, the livers were promptly removed, rinsed with PBS containing 3 mM EDTA, blotted dry and weighed. A sample was homogenised in 0.9% NaCl containing 3 mM EDTA to prevent spurious lipid peroxidation and to chelate any ionic iron that may have been released during tissue preparation. Aliquots of homogenates were lipid-extracted with chloroform-methanol (2:1, v.v) as previously described [23]. TG, FC and CE were determined by enzymatic methods, using the Boehringer Mannheim kits as described previously [29].

### 2.6. Thiobarbituric reactive substance and conjugated diene assessment

TBARS were determined from liver and plasma specimens frozen in liquid nitrogen and stored at –80°C as described by Buege et al. [24]. The color complex was

measured by spectrophotometer at 530 nm. TBARS were quantified using a standard curve of MDA equivalents, generated by acid hydrolysis of 1,1,3,3-tetraethoxypropane. Conjugated dienes were assayed in the cellular lipid extract of hepatocytes and erythrocytes and calculated from the optical density at 234 nm [6].

### 2.7. Lecithin:cholesterol acyltransferase (LCAT) activity

LCAT activity in plasma was determined by conversion of [ $^3\text{H}$ ]cholesterol to [ $^3\text{H}$ ]cholesterol esters (CE) as reported previously [25]. LCAT activity was expressed as  $\mu\text{mol CE/ml plasma/h}$ .

### 2.8. Acylcoenzyme A:cholesterol acyltransferase (ACAT) activity

In order to determine ACAT activity, rat liver microsomes were isolated. Briefly, the homogenate was centrifuged at  $20\,000 \times g$  for 15 min. The supernatant was centrifuged at  $100\,000 \times g$  for 60 min, and the resulting microsomal fraction was suspended in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, to give a final concentration of 10% (w/v). Thereafter, the microsomal preparation (0.1–0.2 mg protein) was mixed with 1 mg of defatted bovine serum albumin in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA. The mixture was preincubated for 5 min at  $37^\circ\text{C}$ , and the reaction was started by the addition of 25 nmol, 1.45  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]oleoyl coenzyme A (55 mCi/mmol, Amersham, Montreal, Canada). This assay was essentially performed as described by Einarsson et al. [26]. Results were expressed as dpm/mg protein.

### 2.9. Other methods

The measurement of total bilirubin in serum was achieved by adding diazotized sulfanilic acid to produce azobilirubin. Following 10 min incubation, the reaction was stopped by the addition of an ascorbic acid solution, and the color developed with alkaline tartrate.

Table 1  
Assessment of lipid peroxidation and liver toxicity

Index	Control	Salicylate	Fe-salicylate treated
CD in erythrocyte (nmol/mg cell protein)	69 $\pm$ 7	76 $\pm$ 9	153 $\pm$ 13*
CD in liver (nmol/mg tissue protein)	112 $\pm$ 11	123 $\pm$ 10	225 $\pm$ 22*
Bilirubin ( $\mu\text{mol/l}$ )	1.0 $\pm$ 0.4	0.2 $\pm$ 0.5	0.7 $\pm$ 0.5
ALT (I.U./l)	29.0 $\pm$ 6.2	40.2 $\pm$ 7.8	46.5 $\pm$ 22.0
AST (I.U./l)	62.7 $\pm$ 7.7	71.7 $\pm$ 10.7	137.7 $\pm$ 77.9

Conjugated diene (CD) were assayed in the lipid extract of erythrocytes and liver homogenates. Liver toxicity was assessed by examining serum bilirubin, alanine ALT and AST.  $X \pm \text{S.E.M.}$  for  $n = 4/\text{group}$ .

\*  $P < 0.05$ .

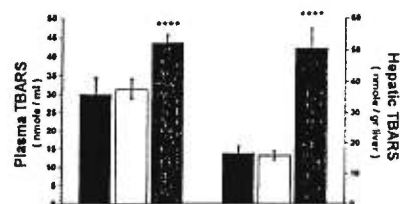


Fig. 1. TBARS concentration in the plasma and liver of control (■), salicylate (□) and Fe-salicylate treated (■) rats. All values are mean  $\pm$  S.D. for  $n = 10/\text{group}$ . \*\*\*\*  $P < 0.001$ .

The absorbance was measured at 600 nm. The enzymes alanine aminotransferase and aspartate aminotransferase were measured by standard enzymatic kits (Beckman, Montreal, Quebec).

### 2.10. Statistical analysis

All values were expressed as the mean  $\pm$  S.E.. Statistical differences were assessed by using Student's two-tailed  $t$ -test.

## 3. Results

### 3.1. Thiobarbituric acid reactivity, conjugated dienes and liver function

In order to examine whether treatment with iron and salicylate was able to induce peroxidation, TBARS were measured in plasma and liver. As shown in Fig. 1, a significant increase in the peroxidative index represented by TBARS was observed. It is noteworthy that the TBARS production in the plasma was 50% higher ( $P < 0.001$ ) compared to controls, whereas it was much higher in the liver (333%,  $P < 0.001$ ). However, salicylate alone could not stimulate plasma and liver peroxidation. A similar trend was noticed with conjugated dienes. The production of these components was signifi-

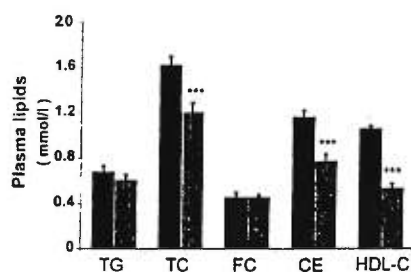


Fig. 2. Plasma lipids and lipoproteins in control (■) and Fe-salicylate (■) treated-rats. Values are mean  $\pm$  S.D. for  $n = 20$ /group. TG, triglyceride; TC, total cholesterol; FC, free cholesterol; CE, cholesterol ester; HDL-C, HDL-cholesterol. \*\*\*  $P < 0.005$

cantly higher in the plasma and liver from Fe-salicylate-treated rats than from control rats (Table 1). However, no significant alterations in plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), or bilirubin were found during the Fe-salicylate treatment (Table 1).

### 3.2. Plasma lipid profile

The plasma lipid profile was affected in the animals treated with Fe-salicylate. As shown in Fig. 2, a significant ( $P < 0.0001$ ) decrease of total cholesterol (25%) characterized this group of rats. The fall in cholesterol was likely due to the diminution of CE ( $P < 0.001$ ), since free cholesterol was not affected. Given that HDL is the main carrier in rats, its cholesterol content was measured in order to explore if it could account for the plasma cholesterol reduction. A 50% decline of the HDL cholesterol value ( $P < 0.0001$ ) was noted and was associated with the decrease in plasma cholesterol.

The changes in plasma fatty acid composition is shown in Table 3. The profile displayed both a substantial reduction in the relative content of the  $n-3$  and  $n-6$  families and a concomitant rise of saturated fatty acids. It is important to note that arachidonic acid, a target for peroxidation, was diminished by iron treatment. Since TBARS are derived largely from 20:4, we measured the plasma TBARS:20:4 ratio, which was increased in iron-treated animals (3.35) in comparison with controls (1.56). These data confirmed the presence of peroxidation in rats treated with iron. Similarly, the ratio of plasma CD:20:4 ratio was higher (320%) than the control value.

### 3.3. Lipid and apolipoprotein composition of plasma lipoproteins

Plasma lipoproteins of control and Fe-salicylate treated rats were isolated by sequential ultracentrifugation at 1.006 g/ml, 1.019 g/ml, 1.063 g/ml, 1.150 g/ml and 1.21 g/ml, which correspond to VLDL, IDL, LDL,

HDL<sub>2</sub> and HDL<sub>3</sub> density, respectively (Table 2). Most of these classes were free cholesterol-enriched and poorer in cholesteryl ester in Fe-salicylate treated rats compared with controls. In addition, TG content was also higher in LDL and HDL<sub>2</sub> and PL<sub>s</sub> were increased in VLDL, IDL and HDL<sub>3</sub>. As expected from these changes in lipoprotein components, differences were also noted in ratios of TG + CE:FC + PL + PR, TG:CE, CE:PL and CE:PR. A general trend of increase was observed in TG:CE ratio, and decrease in CE:PL and CE:PR ratios of Fe-salicylate-treated rats. It should be noted that the mass ratio of core constituents (TG + CE) to surface constituents (FC + PR + PL) is indicative of the size of spherical lipoprotein particles. In general, lighter and larger populations are relatively enriched with core components as compared with denser and smaller particles. The calculated value of these ratios indicates that VLDL and HDL<sub>3</sub> are smaller in Fe-salicylate-treated rats.

The SDS-PAGE showed an apolipoprotein distribution in isolated lipoproteins of Fe-salicylate-treated rats, which differed from that obtained from control rats (Fig. 3). More specifically, electrophoresis revealed a relative diminution in apo A-I, apo A-II and apo A-IV in VLDL of rats treated with Fe-salicylate. Moreover, a different pattern of apo E isoforms characterized the VLDL of iron-treated rats. With regard to LDL-apolipoproteins, a marked apo B100 increase and apo B48 decrease were apparent in iron-treated rats (Fig. 3).

In the HDL<sub>2</sub> fraction, SDS-PAGE analysis revealed an enrichment of apo B100 and apo B48 in Fe-salicylate-treated rats. Furthermore, these abnormalities were associated with a strong decrease of apo A-I, apo A-II, apo A-III and apo E isoforms (Fig. 4a). Finally, HDL<sub>3</sub> apolipoprotein electrophoresis also revealed a decline in apo A-IV, concomitant with a rise in apo A-II when compared with the control pattern. Apo E isoform expression was also altered in the HDL<sub>3</sub> fraction (Fig. 4(b)).

### 3.4. Lipid composition in the liver

The liver lipid pattern was profoundly affected by the treatment of Fe-salicylate. As shown in Fig. 5, the liver exhibited a significant ( $P < 0.001$ ) drop in total cholesterol (22%) essentially due to a reduction in esterified cholesterol (45%,  $P < 0.001$ ). Moreover, liver triglycerides were dramatically and significantly decreased ( $P < 0.005$ ) (52%) in the Fe-salicylate-treated group. The fatty acid composition of liver homogenate was also modified by iron administration (Table 4). In particular, the experimental group displayed an impoverishment in 20:4  $n-6$ . When TBARS and CD were expressed relatively to 20:4, the ratios were 3.41 and 3.48 in iron-treated animals versus 0.86 and 10.45, in

Table 2  
Chemical composition of lipoproteins

Lipoproteins	Composition (mg/100 mg of lipoproteins)						Weight ratios (mg/100 mg of lipoproteins)					
	TG	CE	FC	PL	PR*	TG:CE	CE:PL	CE:PR	(TG+CE): (FC+PL+PR)			
<b>VLDL</b>												
Control	53.88 ± 1.80	4.63 ± 0.21	3.53 ± 0.11	8.87 ± 0.22	29.11 ± 1.99	11.74 ± 0.58	0.53 ± 0.03	0.16 ± 0.02	1.43 ± 0.10			
Fe-salicylate treated	47.84 ± 2.09	2.91 ± 0.61	4.36 ± 0.20***	10.43 ± 0.28***	34.26 ± 1.50	20.68 ± 4.68	0.28 ± 0.06*	0.09 ± 0.02*	1.04 ± 0.08*			
<b>IDL</b>												
Control	38.49 ± 1.48	12.02 ± 1.19	4.81 ± 0.09	15.62 ± 0.62	29.09 ± 1.78	3.38 ± 0.36	0.79 ± 0.11	0.43 ± 0.05	1.03 ± 0.07			
Fe-salicylate treated	39.32 ± 2.77	6.11 ± 0.46****	5.63 ± 0.23**	21.35 ± 0.64****	27.50 ± 1.94	6.73 ± 0.88**	0.29 ± 0.02****	0.22 ± 0.01****	0.85 ± 0.09			
<b>LDL</b>												
Control	13.06 ± 0.46	31.40 ± 1.19	7.97 ± 0.12	23.66 ± 0.36	23.91 ± 0.87	0.42 ± 0.03	1.33 ± 0.06	1.33 ± 0.09	0.80 ± 0.03			
Fe-salicylate treated	30.23 ± 2.38****	14.94 ± 2.69****	6.11 ± 0.65*	20.34 ± 1.79	28.39 ± 2.52	2.65 ± 0.73*	0.77 ± 0.16**	0.57 ± 0.14***	0.82 ± 0.01			
<b>HDL 2</b>												
Control	1.46 ± 0.19	34.56 ± 1.69	4.69 ± 0.33	26.74 ± 0.65	32.56 ± 1.30	0.32 ± 0.01	0.29 ± 0.01	4.18 ± 0.22	0.57 ± 0.04			
Fe-salicylate treated	9.33 ± 1.98****	27.19 ± 1.06****	6.62 ± 0.35****	27.11 ± 1.29	29.74 ± 2.05	0.35 ± 0.08	1.01 ± 0.03****	0.95 ± 0.10****	0.58 ± 0.04			
<b>HDL 3</b>												
Control	0.528 ± 0.002	26.22 ± 0.45	2.16 ± 0.13	25.84 ± 0.46	45.25 ± 0.66	0.020 ± 0.001	1.02 ± 0.02	0.58 ± 0.02	0.37 ± 0.01			
Fe-salicylate treated	0.709 ± 0.080	22.22 ± 0.77****	2.80 ± 0.20*	30.19 ± 1.16**	44.08 ± 2.13	0.032 ± 0.004*	0.74 ± 0.01****	0.51 ± 0.04	0.30 ± 0.01****			

Values are means ± S.E.M. and differences between untreated ( $n = 6$ ) and treated ( $n = 6$ ) rats are significant at a level of at least: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$  and \*\*\*\*  $P < 0.001$ .  
\* PR, protein.

controls, respectively. Again, these measures are indicative of increased peroxidation in iron-treated rats.

### 3.5. LCAT and ACAT enzymatic activity

In view of the evident diminution of cholesteryl ester in the plasma and liver, the two enzymes responsible for the esterification process were assessed. Our data showed no substantial differences in plasma LCAT activity or microsomal ACAT activity between the two groups of rats (Fig. 6).

## 4. Discussion

In vitro studies have well shown that iron stimulates lipid peroxidation [27]. The data of this study demonstrate increased levels of peroxidative indices concomitant with significant alterations in plasma lipid profile, lipoprotein composition and liver lipid concentrations in Fe-salicylate-treated rats.

The presence of high TBARS levels in the plasma and liver points to the induction of peroxidation following Fe-salicylate administration. However, the reliability and specificity of this peroxidation marker has been questioned [28]. We, therefore, confirmed lipid peroxidation by the measurement of conjugated dienes, which with the simultaneous decrease of polyunsaturated fatty acids, constitutes a valuable evidence for lipid peroxidation [29]. Thus, the data presented suggest that acute iron administration induces lipid peroxidation in vivo.

In order to assess whether acute hepatic toxicity accounts for the observed lipid and lipoprotein changes, we monitored AST, ALT and bilirubin. Our

Table 3  
Total plasma fatty acid composition of control and iron-treated rats

Fatty acid	Control (mol%)	Fe-salicylate treated (mol%)
14:0	0.61 ± 0.11	0.44 ± 0.17
16:0	23.11 ± 0.50	29.27 ± 0.40**
16:1 n-7	1.57 ± 0.58	1.64 ± 0.31
18:0	11.30 ± 1.32	16.91 ± 1.01*
18:1 n-9	9.25 ± 0.95	10.89 ± 1.33
18:2 n-6	22.40 ± 1.38	20.03 ± 1.11
18:3 n-6	0.12 ± 0.04	ND
18:3 n-3	1.06 ± 0.06	0.68 ± 0.04**
20:2 n-6	0.70 ± 0.67	0.12 ± 0.10*
20:3 n6	0.40 ± 0.05	ND
20:4 n-6	19.19 ± 1.22	13.12 ± 1.52*
20:5 n-3	1.07 ± 0.36	0.31 ± 0.22**
22:5 n3	0.72 ± 0.02	0.56 ± 0.05
22:6 n-3	7.55 ± 0.94	3.91 ± 0.73*
24:1 n-9	0.28 ± 0.04	0.54 ± 0.12*

Data are mean ± S.E.M. (n-3). Fatty acids contributing less than 0.1% of the total have been omitted from the Table. Fe-salicylate vs. controls: \* P < 0.05, \*\* P < 0.001.

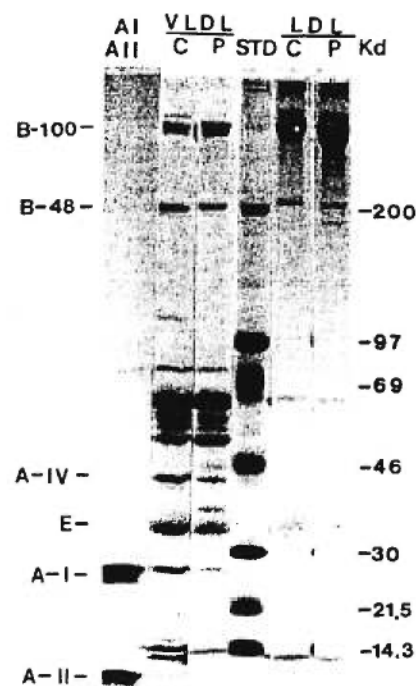


Fig. 3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis showing the apolipoprotein composition of VLDL and LDL from Fe-salicylate treated (P) and control (C) rats. Molecular weight standards (STD) were used as reference proteins. STD were phosphorlyase B (97 400), BSA (66 200), ovalbumin (46 000), carbonic anhydrase (30 000), Trypsin inhibitor (21 500) hysozyme (14 300).

results failed to support a strong association between the documented circulating and hepatic lipid alterations and liver toxicity found in chronic iron overload [30].

The presence of high TBARS and CD levels in the plasma and liver points to the induction of peroxidation in parallel with the occurrence of lipid and lipoprotein perturbations in Fe-salicylate-treated rats. The lipid alterations caused by iron loading included a decrease in cholesteryl ester levels, both in the plasma and liver. We therefore assessed the activity of two enzymes converting free cholesterol into the esterified form, i.e. LCAT in the plasma and ACAT in the liver. LCAT plays a central role in the metabolism of plasma lipoproteins [31]. It catalyzes the transfer of an acyl chain from the sn-2 position of phosphatidylcholine to the 3-hydroxyl group of cholesterol in HDL forming lysophosphatidylcholine and cholesteryl ester, respectively. Thus, LCAT maintains the unesterified cholesterol gradient, which permits cholesterol efflux of net cholesterol from peripheral cells to HDL partic-

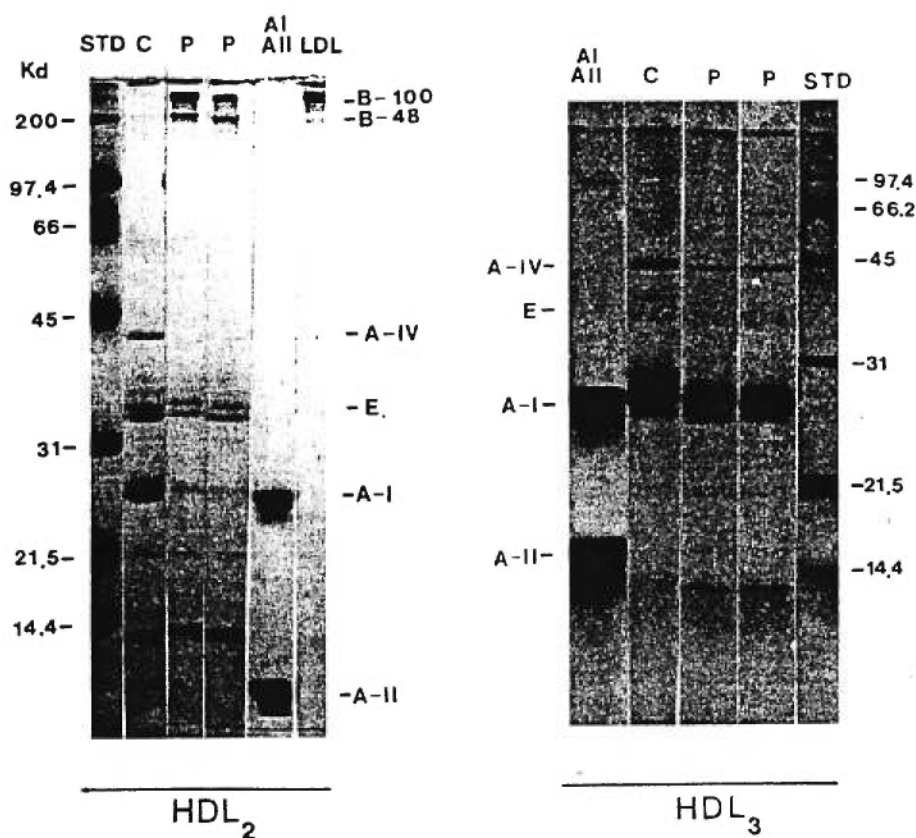


Fig. 4. Apolipoprotein patterns of HDL<sub>2</sub> and HDL<sub>3</sub> fractions in Fe-salicylate (P) and control (C) rats, on 15% polyacrylamide gel containing 0.1% SDS. The location of apolipoprotein species of HDL<sub>2</sub> (A) and HDL<sub>3</sub> (B) were identified by comparison with molecular weight standards.

les. In our studies both HDL fractions, HDL<sub>3</sub> and HDL<sub>2</sub> were depleted in CE and enriched in free cholesterol. As LCAT can be down-regulated by oxidant stress [32], it was reasonable to determine whether the observed changes in CE were corrected with LCAT inhibition in iron-treated animals. However, alterations

in LCAT were not apparent with iron treatment. At this time, we have no clear explanation for the CE/FC changes in iron-treated animals. In a recent study, Chen and Loo incubated plasma with a source of peroxy radicals [32]. They noted that the reduction of LCAT activity did not appear to correlate closely with TBARS formation. They concluded that LCAT activity can be down-regulated by oxidant stress, but not necessarily

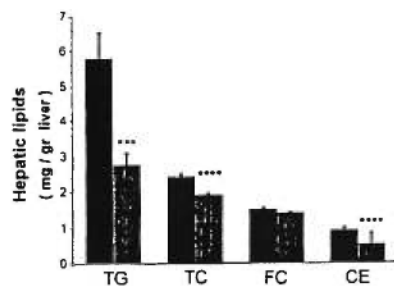


Fig. 5. Lipid composition in the liver of control (■) and Fe-salicylate treated rats (■). Values are mean  $\pm$  S.D. for  $n = 10$ /group. \*\*\*  $P < 0.005$ , \*\*\*\*  $P < 0.001$ .

Table 4  
Fatty acid composition of liver homogenate

Fatty acid	Control (mol%)	Fe-salicylate treated (mol%)
16:0	22.03 $\pm$ 0.40	24.66 $\pm$ 0.91
16:1 $n-7$	0.48 $\pm$ 0.17	0.73 $\pm$ 0.40
18:0	19.91 $\pm$ 0.59	24.32 $\pm$ 0.92*
18:1 $n-7$	7.77 $\pm$ 0.42	9.28 $\pm$ 0.78
18:2 $n-6$	23.32 $\pm$ 0.78	22.02 $\pm$ 0.88
20:4 $n-6$	19.81 $\pm$ 0.88	14.63 $\pm$ 0.91**
22:6 $n-3$	6.65 $\pm$ 0.61	4.02 $\pm$ 0.44*

Values are means  $\pm$  S.E.M. for 3 animals group.  
\*  $P < 0.05$ , \*\*  $P < 0.01$ .



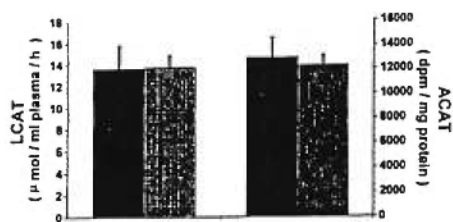


Fig. 6. LCAT and ACAT activity in control (■) and Fe-salicylate-treated rats (▨). Values are mean  $\pm$  S.D. for at least  $n = 5$ /group.

by lipid peroxidation. McCall et al. have reached a similar conclusion, suggesting that LCAT activity reduction in plasma treated with cigarette smoke seems to precede lipid peroxidation [33]. Similarly, ACAT was not modified by Fe-salicylate treatment. It is important to mention that Musanti and Ghiselli [27] noticed a reduction in HDL-oxidized cholesteryl ester in macrophages, without an alteration of ACAT activity. At this time, we are not able to determine whether alterations in plasma and liver cholesteryl ester values are the results of cholesterol or fatty acid peroxidation.

Relatively little attention has been paid to *in vivo* HDL modification or oxidation caused by free radical mediated processes. Most of the available studies have focused on HDL behavior after *in vitro* free radical mediated oxidation [16]. In general, the modification or oxidation of isolated HDL has been carried out in test tubes by different oxidizing systems before studying its metabolic fate. In contrast to this approach, we postulated that HDL concentration and composition could be markedly affected by *in vivo* Fe-salicylate catalyzed lipid peroxidation. In our experiments, acute Fe-salicylate overload resulted in abnormal HDL composition. Bowry et al. found that 85% of detectable hydroperoxides of cholesteryl esters were present in HDL, with the LDL being virtually peroxide free. Also, HDL lipids became more readily oxidized than those in LDL when plasma was exposed to a mild flux of radicals [34]. Furthermore, HDL CE hydroperoxides were found to be rapidly removed by human-derived HepG<sub>2</sub> hepatoma cells, and with more efficient uptake than that of unoxidized CE [35]. The authors of this report suggested that such an efficient selective uptake of HDL hydroperoxides, together with their rapid detoxification, could represent an important antioxidant defense aimed at preventing the accumulation of toxic levels of lipid hydroperoxides in the circulation and extravascular space. Alternatively, lipid oxidation can result in oxysterol formation. For example, *in vitro* biological oxidation of LDL and HDL have been noted to alter the amount of CE [36,37]. In our study, we observed changes in apolipoprotein composition of various lipoprotein classes. Previous investigations have reported the susceptibility of apolipo-

proteins to peroxidation [38,39]. The potential formation of Schiff-base complexes between the lipid ester core aldehydes and the amino groups of proteins suggests marked effects upon protein association with lipid membranes [40]. Further studies are needed to clearly demonstrate whether iron overload reduces the hepatic synthesis of apolipoproteins or affects their composition in lipoproteins via their interactions with membranes.

Considerable progress has been made in recent years in the study of many enzymes in relation to lipid peroxidation. Several enzymes including glyoxalase,  $\beta$ -amylase, choline oxidase, succinic oxidase, cytochrome oxidase, trypsin, papain, urease [41], ribonuclease A, lysozyme, carboxypeptidase A, lactate dehydrogenase, creatine kinase [42], Ca<sup>2+</sup>-ATPase [43], glucose-6-phosphatase and UDP-glucuronyltransferase [44] were inactivated by lipid peroxidation. Since triglycerides were dramatically decreased in the liver, we hypothesize that their synthesis might be altered by lipid peroxidation in the pathological condition involving free radicals mediated by Fe-salicylate administration. This view supports the conclusions that glycerol-3-phosphate acyltransferase, the rate-limiting enzyme in the biosynthesis of glycerolipids [45], lost its activity in a peroxidative environment [46]. Inactivation was evident with lipid peroxidation induced by iron in their system.

In previous experiments, the combination of iron and salicylate was found to stimulate lipid peroxidation [18]. Salicylate was suggested to serve as a lipophilic carrier, transporting more iron into hepatocytes and Kupffer cells [18]. In our experiments, salicylate alone was unable to stimulate the formation of MDA in the liver and blood circulation. However, salicylate effects may be determined by salicylate-metal complexes formed rather than by salicylate itself [18].

Salicylic acid is frequently utilized by humans in its acetylated form as aspirin. Aspirin is rapidly converted to salicylate by esterases in the gastrointestinal tract and in the liver [47]. Given that hepatic iron is often increased in various conditions, the administration of salicylate can be particularly toxic.

In conclusion, the present rat model provides clear evidence that acute Fe-salicylate loading causes marked abnormalities in the concentration and composition of plasma lipids and lipoproteins in association with lipid peroxidation. Further studies are needed to explore the mechanisms involved in this experimental condition.

#### Acknowledgements

The authors thank Danielle St-Cyr Huot for help in preparation of the manuscript. This work was sup-

ported in part by a research grant (MT-10583) from the Medical Research Council of Canada and from NSERC, and constituted part of a PhD thesis of BS. This work was presented in part at the Annual Meeting of FASEB, and the Xth International Symposium on Atherosclerosis, Montreal, October 9–14, 1994.

## References

- [1] Wills ED. Lipid peroxide formation in microsomes. *Biochem J* 1969;113:315.
- [2] Gutteridge JMC. The protective action of superoxide dismutase on metal-iron catalyzed peroxidation of phospholipids. *Biochem Biophys Res Commun* 1977;77:379.
- [3] LeSage GD, Kost LJ, Barham SS, LaRusso NF. Biliary excretion of iron from hepatocyte lysosomes in the rat. A major excretory pathway in experimental iron overload. *J Clin Invest* 1986;77:90.
- [4] Myers BM, Prendergast FG, Holman R, Kunts SM, LaRusso NF. Alterations in the structure, physicochemical properties, and pH of hepatocyte lysosomes in experimental iron overload. *J Clin Invest* 1991;88:1207.
- [5] Bacon BR, O'Neill R, Park CH. Iron-induced peroxidative injury to isolated not hepatic mitochondria. *J Free Radic Biol Med* 1986;2:339.
- [6] Halliwell B, Gutteridge JM. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 1984;219:1.
- [7] Girotti AW. Mechanisms of lipid peroxidation. *J Free Radic Biol Med* 1985;1:87.
- [8] Goldberg L, Martin LE, Batchelor A. Biochemical changes in the tissues of animals injected with iron. 3. Lipid peroxidation. *Biochem J* 1961;83:291.
- [9] Högborg J, Orrenius S, O'Brien PJ. Further studies on lipid-peroxide formation in isolated hepatocytes. *Eur J Biochem* 1985;59:449.
- [10] Högborg J, Moldeus P, Arborgh B, O'Brien PJ, Orrenius S. The consequences of lipid peroxidation in isolated hepatocytes. *Eur J Biochem* 1975;59:457.
- [11] Ramasarma T, Paton B, Goldfarb S. Inactivation of 3-hydroxy-3-methylglutaryl CoA reductase by  $Fe^{++}$  and a cytosolic protein. *Biochem Biophys Res Commun* 1981;100:170.
- [12] Thomas PD, Poznansky MJ. Lipid peroxidation inactivates rat liver microsomal glycerol-3-phosphate acyl transferase. *J Biol Chem* 1990;265:2684.
- [13] Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest* 1991;88:1785.
- [14] Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci USA* 1979;76:333.
- [15] Mahley RW, Innerarity TL, Weisgraber KB, Oh SY. Altered metabolism (in vivo and in vitro) of plasma lipoproteins after selective chemical modification of lysine residues of the apoproteins. *J Clin Invest* 1979;64:743.
- [16] Guertin F, Brunet S, Gavino V, Tuchweber B, Levy E. Malondialdehyde-modified high-density lipoprotein cholesterol: plasma removal, tissue distribution and biliary sterol secretion in rats. *Biochim Biophys Acta* 1994;1214:137.
- [17] Bacon BR, Britton RS. The pathology of hepatic iron overload: a free radical-mediated process. *Hepatology* 1990;11:127.
- [18] Gunther T, Grossrau R, Hollriegel V, Vormann J. Effect of Fe, salicylate and Zn on metallothionein and lipid peroxidation in vivo. *J Trace Elem Electrolytes Health Dis* 1991;5:95.
- [19] Levy E, Lepage G, Bendayan M et al. Relationship of decreased hepatic lipase activity and lipoprotein abnormalities to essential fatty acid deficiency in cystic fibrosis patients. *J Lipid Res* 1989;30:1197.
- [20] Levy E, Thibault LA, Roy CC, Bendayan M, Lepage G, Letarte J. Circulating lipids and lipoproteins in glycogen storage disease type I with nocturnal intragastric feeding. *J Lipid Res* 1988;29:215.
- [21] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265.
- [22] Bartlett GR. Phosphorous assay in column chromatography. *J Biol Chem* 1959;234:466.
- [23] Levy E, Garofalo C, Thibault L et al. Intraluminal and intracellular phases of fat absorption are impaired in essential fatty acid deficiency. *Am J Physiol* 1992;262:G319.
- [24] Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978;52:302.
- [25] Levy E, Thibault L, Turgeon J et al. Beneficial effects of fish-oil supplements on lipids, lipoproteins, and lipoprotein lipase in patients with glycogen storage disease type I. *Am J Clin Nutr* 1993;57:922.
- [26] Einarsson K, Benthin L, Ewerth S, Hellers G, Stahlberg D, Angelin B. Studies on acyl-coenzyme A: cholesterol acyltransferase activity in human liver microsomes. *J Lipid Res* 1989;30:739.
- [27] Fuhrman B, Oiknine J, Aviram M. Iron induces lipid peroxidation in cultured macrophages, increases their ability to oxidatively modified LDL, and affects their secretory properties. *Atherosclerosis* 1994;111:65.
- [28] Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic Biol Med* 1990;9:515.
- [29] Recknagel RO, Glende EA. Spectrophotometric detection of lipid conjugated dienes. *Methods Enzymol* 1984;105:331.
- [30] Sharma BK, Bacon BR, Britton RS et al. Prevention of hepatocyte injury and lipid peroxidation by iron chelators and  $\alpha$ -tocopherol in isolated iron-loaded rat hepatocytes. *Hepatology* 1990;12:31.
- [31] Glomset JA. The plasma lecithin:cholesterol acyltransferase reaction. *J Lipid Res* 1968;9:155.
- [32] Chen C, Loo G. Effect of peroxy radicals on lecithin/cholesterol acyltransferase activity in human plasma. *Lipids* 1995;30:627.
- [33] McCall MR, Van den Berg JJM, Kuypers FA et al. Modification of LCAT activity and HDL structure: new links between cigarette smoke and coronary heart disease risk. *Arterioscler Thromb* 1994;14:248.
- [34] Bowry VW, Stanley KK, Stocker R. High density lipoprotein is the major carrier of lipid hydroperoxides in human blood from fasting donors. *Proc Natl Acad Sci USA* 1992;89:10316.
- [35] Sattler W, Stocker R. Greater selective uptake by Hep G2 cells of high-density lipoprotein cholesteryl ester hydroperoxides than of unoxidized cholesteryl esters. *Biochem J* 1993;294:771.
- [36] Musanti R, Giselli G. Interaction of oxidized HDLs with J774-A1 macrophages causes intracellular accumulation of unesterified cholesterol. *Arterioscler Thromb* 1993;13:1334.
- [37] Zhang HF, Basra HJ, Stembrecher UP. Effects of oxidatively modified LDL on cholesterol esterification in cultured macrophages. *J Lipid Res* 1990;31:1361.
- [38] Anantharamaiah GM, Hughes TA, Iqbal M et al. Effect of oxidation on the properties of apolipoproteins A-I and A-II. *J Lipid Res* 1988;29:309.
- [39] Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 1989;320:915.

- [40] Esterbauer H, Gebicki HJ, Puhl H, Jürgens G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic Biol Med* 1992;13:341.
- [41] Wills ED. Effect of unsaturated fatty acids and their peroxides on enzymes. *Biochem Pharma* 1961;7:7.
- [42] Chio KS, Tappel AL. Inactivation of ribonuclease and other enzymes by peroxidizing lipids and by malondialdehyde. *Biochemistry* 1969;8:2827.
- [43] Tverdislova IL, Ritov VB. Lipid peroxidation as a major factor in the modification of the catalytic function of Ca-ATPase of the sarcoplasmic reticulum in hypercholesterolemia. *Biul Eksp Biol Med* 1987;103:415.
- [44] Horton AA, Fairhurst S. Lipid peroxidation and mechanisms of toxicity. *Crit Rev Toxicol* 1987;18:27.
- [45] Bell RM, Coleman RA. Enzymes of glycerolipid synthesis in eukaryotes. *Ann Rev Biochem* 1980;49:459.
- [46] Thomas PD, Poznansky MJ. Lipid peroxidation inactivates rat liver microsomal glycerol-3-phosphate acyl transferase. Effect of iron and copper salts and carbon tetrachloride. *J Biol Chem* 1990;265:2684.
- [47] Levy G. Pharmacokinetics of salicylate elimination in man. *J Pharm Sci* 1965;54:959.


**MODULATION OF ENDOPLASMIC RETICULUM-BOUND  
CHOLESTEROL REGULATORY ENZYMES BY  
IRON/ASCORBATE-MEDIATED LIPID PEROXIDATION**

**Brunet S, Thibault L, Lepage G, Seidman EG, Dubé N, Levy E**

Centre de Recherche, Hôpital Sainte-Justine and  
Département de Nutrition, Université de Montréal  
Montréal, Québec, Canada

**Running title:** Sterol enzymes and lipid peroxidation

**Address for correspondence:**

Dr. Emile Levy  
Division of Gastroenterology  
Research Center  
Hôpital Sainte-Justine  
3175 Côte Ste-Catherine Road  
Montreal (Quebec) H3T 1C5  
Canada  
Tel.: (514) 345-4626  
Fax: (514) 345-4999  


### ABSTRACT

Mammalian sterol regulatory enzymes are integral membrane proteins of the endoplasmic reticulum. They play a critical role in liver cholesterol homeostasis and the maintenance of overall cholesterol balance in different species. Since lipid peroxidation has been implicated in hepatic dysfunction and atherosclerosis, we hypothesized that its occurrence could alter the composition and properties of the bilayer lipid environment, and thereby affect the functions of these membrane proteins. Preincubation of rat liver microsomes with iron (Fe)/ascorbate (50  $\mu$ M/200  $\mu$ M), known to induce peroxidation, resulted in a significant inhibition of: (1) the rate-limiting enzyme in cholesterol biosynthesis, HMG-CoA reductase (46%,  $p < 0.01$ ), (2) the crucial enzyme controlling the conversion of cholesterol in bile acids, cholesterol 7 $\alpha$ -hydroxylase (48%,  $p < 0.001$ ) and the central enzyme for cholesterol esterification: Acyl-CoA:cholesterol acyltransferase (ACAT, 80%,  $p < 0.000$ ). The disturbances in these key enzymes took place concomitantly with the high production of malondialdehyde (350%,  $p < 0.007$ ) and the loss of polyunsaturated fatty acids (36.19 $\pm$ 1.06% vs. 44.24 $\pm$ 0.41 in controls,  $p < 0.0008$ ). While  $\alpha$ -tocopherol simultaneously neutralized lipid peroxidation, preserved microsomal fatty acid status and restored ACAT activity, it was not effective in preventing Fe/ascorbate-induced inactivation of both HMG-CoA reductase (44%,  $p < 0.01$ ) and cholesterol 7 $\alpha$ -hydroxylase (71%,  $p < 0.0001$ ). These results indicate that Fe/ascorbate alters the activity of the rate-determining steps in liver cholesterol metabolism, either directly or via lipid peroxidation, capable of modifying their membrane environment. The present data also suggest that the three regulatory enzymes respond differently when exposed to Fe/ascorbate or antioxidants, which may be due to dissimilar mechanisms.

**Key words:** HMG-CoA reductase, cholesterol 7 $\alpha$ -hydroxylase, acyl-CoA:cholesterol acyltransferase, membrane fluidity, fatty acid composition, microsomes, peroxidation

## INTRODUCTION

No doubt remains as to the central role of the liver in cholesterol homeostasis (1). The liver maintains cholesterol balance across the whole body and regulates the levels of circulating lipoprotein-cholesterol (2). It represents the most efficient organ for the elimination of cholesterol from the body via its massive LDL receptor activity, uptake and degradation of various types of lipoproteins, bile acid production, and biliary cholesterol secretion (1,2). Conversely, it contributes to circulating cholesterol by delivering very-low density lipoproteins and high-density lipoproteins (3,4).

Lipid peroxidation is involved in free radical-mediated tissue injury. It can cause hepatic dysfunction by damaging various enzymes or altering their activity (5). Specific inactivation of mitochondrial NAD-dependent aldehyde dehydrogenase and succinate dehydrogenase was induced by lipid peroxidation products (6,7). Peroxides also reduced the endoplasmic reticulum's ability to carry out hydroxylation reactions, including the hydroxylation of alanine and the oxidative demethylation of aminopyrine (8). Oxygen-radical generating systems affected other enzymes, such as glucose-6 phosphatase, while certain remained insensitive, i.e., NAD-NADP glycohydrolase and adenosine triphosphatase (8).

The impact of lipid peroxidation on liver sterol enzymes received little attention, despite their link in hepatic cholesterol homeostasis. HMG-CoA reductase has been demonstrated to be the rate-limiting enzyme in cholesterol biosynthesis (9). Cholesterol 7 $\alpha$ -hydroxylase catalyzes the conversion of cholesterol into bile acids (10), while acylcholesterol acyltransferase (ACAT) is responsible for the acylation of cholesterol into cholesterol esters, thereby strongly influencing the excretion of cholesterol from the liver (11). The most important targets of oxygen-radical

generating systems are membrane lipids (12,13), and the interaction of reactive oxygen species with polyunsaturated fatty acids may seriously affect the aforementioned membrane-bound enzymes of the endoplasmic reticulum. The purpose of the present study was to test the hypothesis that lipid peroxidation modifies the activity of these regulatory cholesterol enzymes. To this end, hepatic microsomal fractions were incubated with iron/ascorbate, a widely used oxygen-radical generating system. Attempts were also made to establish the mechanisms involved in enzyme activity modification, including membrane lipid composition.



## **METHODS**

**Preparation of Microsomes:** Male rats (225-250 g) were anesthetized with pentobarbital (48 mg/kg body weight). Their livers were removed and placed in ice-cold buffer (pH 7.4) containing 250 mM sucrose, 20 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM sodium fluoride, and 20 mM dithiothreitol. Livers were excised, rinsed, homogenized, and centrifuged for 15 min at 12,000 g at 4°C in order to prepare microsome fractions, a technique described earlier (14). The supernatant fraction was then centrifuged for 60 min at 100,000 g. The pellet was centrifuged for 60 min at 4°C. The washed microsomal pellets were quick-frozen and stored at -80°C for later use.

**Estimation of Lipid Peroxidation:** Microsomes (1.25 mg protein / ml) were incubated in 10 mM Tris-HCl buffer, pH 7.4, in a shaking water bath at 37°C for 30 min in the presence or absence of Fe<sup>2+</sup> (50 μM)/ascorbate (200 μM). The reaction was terminated by the addition of 3(2)-t-butyl-4-hydroxyanisole (BHA, 5 mM) in order to measure malondialdehyde (MDA), as an index of lipid peroxidation. The amount of free MDA formed during the reaction was determined by HPLC according to the modified method of Chirico (15). Proteins were first precipitated with a 10% NaWO<sub>4</sub> solution according to Lepage et al. (16). Protein free supernatant was then reacted with an isovolume of 0.5% TBA solution at 95°C for 30 min. After cooling to room temperature, pink chromogene was extracted with n-butanol and dried over a stream of nitrogen at 37°C. The dry extract was then resuspended in KH<sub>2</sub>PO<sub>4</sub>/Methanol (70:30) mobile phase before MDA detection by high-performance liquid chromatography (HPLC) (15).

**Analysis of Membrane Fatty Acid Composition:** To evaluate polyunsaturated fatty acid status, total lipid content of hepatic microsomes was extracted by the method of Folch et al (17). The analysis of fatty acid composition was carried out as described previously (18).

**Effect of Antioxidants:** In order to uncover whether lipid peroxidation was fully behind enzymatic changes, various antioxidants (catalase, reduced glutathion and  $\alpha$ -tocopherol) were added separately to the reaction mixture before incubation with  $\text{Fe}^{2+}$ /ascorbate (37°C for 30 min). Microsomal membranes were then used for enzyme estimation, MDA formation or other determinations.

**Assay of Microsomal HMG-CoA Reductase Activity:** Microsomal enzymatic activity was assayed as described previously (14). The reaction mixture contained 160 mmol/L potassium phosphate (pH 7.4), 300  $\mu\text{g}$  of microsomal protein, 20 mmol/L of glucose-6-phosphate, 2.5mmol/L of dithiothreitol and 1.2 units of glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of [3- $^{14}\text{C}$ ]-HMG-CoA (15,000 dpm/nmol). After incubation for 30 min at 37°C, the [ $^{14}\text{C}$ ]-mevalonate formed was converted into lactone, isolated by thin-layer chromatography, and counted using an internal standard to correct for incomplete recovery. HMG-CoA reductase activity was expressed as nanomoles of mevalonate synthesized per milligram of protein per minute.

**Microsomal ACAT Activity:** The standard ACAT determination was based as per our assay (14). To the mixture containing 75  $\mu\text{g}$  microsomal protein were added 5 $\mu\text{mol}$  [ $^{14}\text{C}$ ]oleoyl coenzyme A (specific activity  $\sim$  10,000 dpm/ $\mu\text{mol}$ ) to initiate the reaction in a buffer solution (pH 7.5) consisting of 0.1 mol/L of Tris-HCl, 0.25 mol/L of sucrose, and 1 mmol/L of ethylenediaminetetraacetic acid. After incubation for 10 min at 37°C, the reaction was stopped

by adding chloroform/methanol (2:1, vol/vol) followed by [<sup>3</sup>H]cholesteryl oleate as an internal standard to estimate recovery.

**Determination of Microsomal 7 $\alpha$ -Hydroxylase Activity:**

Microsomal cholesterol 7 $\alpha$ -hydroxylase activity was measured according to Hylemon et al (19). Briefly, 1 mg of microsomal protein was diluted in a potassium phosphate buffer, pH 7.4, containing 50 mM NaF, 5mM DTT, 1mM EDTA, 20% glycerol, 0.015% CHAPS and 0.25 mM desferal. The reaction was started by the addition of an NADPH regenerating system containing 1 mM NADP, 10 mM glucose-6-phosphate and 0.15 UI glucose-6-phosphate dehydrogenase. Following the 20-min reaction at 37°C, the endogenous 7 $\alpha$ -hydroxylcholesterol form was derivatized with a 0.1% cholesterol oxidase solution. Dried petroleum ether extract was resuspended in 70% acetonitrile / 30% methanol mobile phase, prior to HPLC chromatography, with UV detection at 240 nm, using 7- $\beta$  hydroxycholesterol as an internal standard. The 7 $\alpha$ -hydroxycholesterol product was expressed as pmoles/min/mg protein.

**Statistical Analysis:** All values were expressed as the mean  $\pm$  SEM. The data were evaluated by ANOVA, where appropriate, and the differences between the means were assessed using the Student's two-tailed t test.

## **RESULTS**

### **Effect of Fe<sup>2+</sup>/Ascorbate on Lipid Peroxidation**

The level of lipid peroxidation in microsomes was tested following the addition of Fe<sup>2+</sup>/ascorbate and by recording the formation of MDA. The time course of MDA generation was linear for at least 90 min (data not shown). Furthermore, the extent of lipid peroxidation was shown to be related to the increase in Fe<sup>2+</sup>/ascorbate concentrations (data not shown). As illustrated in Figure 1, the amount of MDA formed at 30 min under the stimulation of Fe<sup>2+</sup>/ascorbate (50 μM/200 μM) was 3.5-fold the control values (p<0.0066). However, the preincubation of microsomes with α-tocopherol, a radical scavenger, for 30 min led to a protection against Fe<sup>2+</sup>/ascorbate-mediated lipid peroxidation (Fig. 1). The effect of other antioxidants was also examined. Our findings clearly showed that catalase and reduced glutathion were all less effective than α-tocopherol in abolishing lipid peroxidation (data not shown).

### **Effect of Fe<sup>2+</sup>/Ascorbate on Microsomal Enzyme Activities**

The basal activity of HMG-CoA reductase, cholesterol 7α-hydroxylase and ACAT is shown in Figure 2. The activity of microsomal glycerol-3-phosphate acyltransferase (GPAT) was also assayed and used as a reference value, since it was reported to be very sensitive to oxidative stress (20). The incubation of suspensions of liver microsomal preparation with Fe<sup>2+</sup>/ascorbate caused marked inhibition of GPAT (55%, p<0.018), HMG-CoA reductase (46%, p<0.011), cholesterol 7α-hydroxylase (48%, p<0.001) and ACAT (80%, p<0.000). In order to study the effects of an antioxidant in microsomal defense against oxidative modification, we first

preincubated microsomes with  $\alpha$ -tocopherol (500  $\mu$ M) at concentrations that suppressed MDA formation (Fig. 3). While  $\alpha$ -tocopherol fully restored ACAT activity and almost fully GPAT activity (84%), in the presence of peroxidative stress, it was not effective in preventing the inactivation of HMG-CoA reductase and cholesterol 7 $\alpha$ -hydroxylase. With  $\alpha$ -tocopherol supplementation, there was no recovery of the latter enzyme, which still exhibited an activity of 56% and 29%, respectively, relative to controls. The lack of recovery was probably due to  $\alpha$ -tocopherol's own influence on HMG-CoA reductase and cholesterol 7 $\alpha$ -hydroxylase, since its addition alone caused an inactivation of 36% and 57%, respectively.

Pilot experiments were carried out in order to test the indirect effect of  $\text{Fe}^{2+}$ /ascorbate on the enzymatic assays. The omission of pre-incubation periods did not result in enzymatic changes (results not shown). Furthermore, persistent effects on enzyme activity were observed following the re-isolation and washing of microsomes that were first incubated with  $\text{Fe}^{2+}$ /ascorbate (results not shown).

#### **Effect of $\text{Fe}^{2+}$ /Ascorbate on Microsomal Fatty Acid Composition**

Since peroxidation can lead to PUFA depletion, as a result of an attack on membrane phospholipids, we determined fatty acid profiles under our different experimental conditions. There were only a few noticeable differences between the fatty acid composition of control and  $\alpha$ -tocopherol-treated microsomes, which were probably due to the slight peroxidation taking place in control microsomes during incubation. On the other hand, microsomes exposed to  $\text{Fe}^{2+}$ /ascorbate exhibited marked alterations compared with control and  $\alpha$ -tocopherol-treated microsomes. A significant decrease in eicosapentaenoate (20:5n-3), docosahexaenoate (22:6n-3), linolenate (18:3n-6), arachidonate (20:4n-6) and other n-3 and n-6 fatty acids was recorded in

microsomes exposed to Fe/ascorbate (Table 1). Conversely, these same microsomes showed the highest percentage of saturated and n-7 fatty acids (Table 1). As expected from these data, the tabulation of the percentage of total fatty acids belonging to individual families disclosed a decrease in n-3 and n-6 classes along with an increase in saturates, n-7 and n-9 categories (Table 2). Furthermore, PUFA/saturate and EFA/non-EFA ratios were lower, whereas trans fatty acids as well as commonly used indices for EFA deficiency,  $\text{fam } 7/18:2n-6$  ( $16:1n-7/18:2n-6$ ) were higher than control or  $\alpha$ -tocopherol-treated microsomes. However, the supply of  $\alpha$ -tocopherol prevented the loss of polyunsaturated fatty acids, resulting from  $\text{Fe}^{2+}$ /ascorbate exposure. Therefore, it is likely that unsaturated fatty acids of microsomes incubated with  $\text{Fe}^{2+}$ /ascorbate were sensitive to peroxidation.

#### **Post Peroxidation Protein Content**

We measured protein content before and after lipid peroxidation in order to determine whether the reduction in enzyme activity is due to protein degradation. Our data (not shown) did not reveal any variation between the microsomes isolated by ultracentrifugation prior to and post peroxidation.

## DISCUSSION

The liver is a key organ in the regulation of body cholesterol. It is the major site of both synthesis and net excretion of cholesterol, either directly as free cholesterol in the bile or after conversion into bile acids. In this study, we have assessed the effects of  $\text{Fe}^{2+}$ /ascorbate on the activity of the three rate-determining steps in hepatic cholesterol metabolism. In particular, we focused on the principal rate-limiting enzymes catalyzing *de novo* cholesterol synthesis, bile acid production and cholesterol esterification (Fig. 4). We proposed that such regulatory enzymes would be modified by iron-catalyzed free radical-mediated lipid peroxidation. Our findings showed that the  $\text{Fe}^{2+}$ /ascorbate system appeared to cause microsomal membrane peroxidation in view of the markedly increased MDA levels. Concomitantly, there was a significant loss of PUFA accompanied with a drop in membrane fluidity. The addition of  $\text{Fe}^{2+}$ /ascorbate to microsomes *in vitro* resulted in an extensive inhibition of HMG-CoA reductase, cholesterol 7 $\alpha$ -hydroxylase and ACAT activities. The antioxidant activity of  $\alpha$ -tocopherol, while restoring ACAT, could not completely prevent the inactivation of both HMG-CoA reductase and cholesterol 7 $\alpha$ -hydroxylase.

Iron-catalyzed generation of reactive oxygen species has been implicated in the pathogenesis of many disorders, including atherosclerosis (21), cancer (22), ischaemia reperfusion injury (23), inflammatory bowel disease (24) and conditions of iron overload. The experiments we report were designed to gain a better understanding of the interrelationship between lipid peroxidation and sterol regulatory enzymes. We have chosen  $\text{Fe}^{2+}$ /ascorbate as an inducer of lipid peroxidation. An extensive literature documents the ability of these combined molecules to initiate strong lipid peroxidation in native and reconstituted biological membrane

systems (12,20,26,27).  $\text{Fe}^{2+}$  may result in the formation of hydroxyl radical ( $\text{HO}\cdot$ ), ferryl radical ( $\text{Fe-OH}^{3+}$ ), perferryl ion, and an  $\text{Fe}^{2+}/\text{Fe}^{3+}$  complex containing  $\text{O}_2$  as a bridging ligand. Ascorbic acid can amplify the oxidative potential of iron by promoting metal-ion induced lipid peroxidation (28,29). Accordingly, in our investigation,  $\text{Fe}^{2+}$ /ascorbate was very effective in inducing lipid peroxidation in view of the recovered high MDA levels, a commonly used measure of lipid peroxidation. Supporting evidence comes from the loss of polyunsaturated fatty acids and the scavenger activity of  $\alpha$ -tocopherol. In this context,  $\alpha$ -tocopherol was selected as an antioxidant, since it is regarded as the major lipid-soluble antioxidant, stabilizing biomembranes and preventing oxidative attack on membrane lipids (30,31). It is also the most powerful vitamin compound ( $\alpha > \beta > \gamma > \delta$ ) (31). It should be noted that the addition of ascorbate alone to microsomes had no effect on lipid peroxidation and sterol regulatory enzyme activity. Besides, iron acted as a pro-oxidant and was able to reduce optimal enzyme activity, but these actions were slower and less efficient compared with the administration of iron/ascorbate together.

In the first series of experiments, the effects of  $\text{Fe}^{2+}$ /ascorbate on sterol regulatory enzymes was studied. Based on our data, the addition of this prooxidant combination caused an activity loss of HMG-CoA reductase, cholesterol  $7\alpha$ -hydroxylase and ACAT. The  $\text{Fe}^{2+}$ /ascorbate system could have decreased these enzymatic activities via different mechanisms, including a direct effect on the enzymes or indirect lipid peroxidation. From our experiments, it appeared that the physical state of the endoplasmic reticulum membrane was related to the enzyme derangements. This is consistent with 1) the high levels of MDA resulting from the peroxidative attack; and 2) the variation of fatty acid composition displaying an enrichment in saturated, n-7 and n-9 fatty acids along with impoverishment of PUFA. Since all the enzymes studied were membrane-bound proteins, the impairment of membrane integrity or membrane breakdown was



reasonably expected to disturb their functioning. Previous work from different laboratories showed that intactness and structural integrity of the biological membrane are prerequisites for the proper maintenance of cellular homeostasis and functions (32,33).

To what extent are sterol regulatory enzymes influenced by lipid peroxidation? This was assessed by the second series of experiments in which  $\alpha$ -tocopherol was administered to block iron-catalyzed free-radical-mediated lipid peroxidation. The incubation of microsomes with  $\alpha$ -tocopherol prevented lipid peroxidation and normalized fatty acid composition, thereby protecting the microsome membrane from peroxidative damage. However, it prevented ACAT inhibition in the presence of Fe/ascorbate. In fact, not only did  $\alpha$ -tocopherol not modify HMG-CoA reductase activity, but it also amplified cholesterol 7 $\alpha$ -hydroxylase inactivation. Thus, taken together, our studies point to distinct mechanisms governing the three sterol regulatory enzyme inactivation by Fe/ascorbate: 1) The supply of  $\alpha$ -tocopherol alone was sufficient to restore full ACAT activity, supporting the direct action of lipid peroxidation. In line with this assumption, several workers have reported the important role of phospholipid-fatty acid composition in the activity of microsomal ACAT (34-37). We, therefore, stipulate that  $\alpha$ -tocopherol's ability to avoid disrupting the phospholipid environment, preserves ACAT activity. 2) The treatment of microsomes with  $\alpha$ -tocopherol had a partial impact on HMG-CoA reductase. It is interesting to note the nature of the down regulation of this enzyme by Fe/ascorbate. A possible mechanism would involve an indirect effect on the physical properties of the membrane in which HMG-CoA reductase is embedded. The active site region of the enzyme resides on a soluble cytoplasmic fragment of approximately 53 kDa that is anchored to the endoplasmic reticulum via a membrane-spanning domain, possibly sensitive to fluidity alterations (38). In addition, the remaining inhibition in the presence of  $\alpha$ -tocopherol suggests yet one more

potential mechanism. The cysteine residues in the catalytic site of the enzyme may be directly sensitive to inactivation by sulfhydryl oxidation (39). 3) The inhibition of cholesterol 7 $\alpha$ -hydroxylase could not be reversed by  $\alpha$ -tocopherol treatment, which strongly suggests a direct interaction with Fe/ascorbate. Earlier studies have proposed that cholesterol 7 $\alpha$ -hydroxylase could be regulated post-translationally by mechanisms involving disulfide bonds in the enzyme structure (40). The loss of activity in our studies may be caused by the binding of iron/ascorbate to these specific sites. Accordingly, some proteins have been reported to be modified by iron and copper (41-44).

Cholesterol is essential for the cell growth and viability of eukaryotic cells, yet the unregulated cellular accumulation of cholesterol predisposes to atherosclerosis. Cellular cholesterol homeostasis remains crucial for the prevention of cardiovascular disease, and numerous studies have reported the beneficial effects of HMG-CoA reductase and ACAT inhibitors on hypercholesterolemia and atherosclerosis (45,46). It is intriguing that the exposure of liver microsomes to iron-catalyzed peroxidation in our study reduced the activity of the two aforementioned enzymes, since considerable evidence has accumulated implicating lipid peroxidation and oxidative modification of LDL in atherosclerotic lesion development. More studies are warranted to elaborate on the fate of the key regulatory enzymes of cholesterol during lipid peroxidation and to clarify their intimate link to atherosclerosis in the peroxidative condition.

In conclusion, it is tempting to propose that HMG-CoA reductase, cholesterol 7 $\alpha$ -hydroxylase and ACAT, each rate limiting for a major pathway in liver cholesterol metabolism, are regulated by different mechanisms mediated by Fe/ascorbate.

**ACKNOWLEDGMENTS**

This was supported by the Canadian Foundation for Ileitis and Colitis (CFIC) grant. The authors thank Mrs. Danielle St-Cyr Huot for valuable technical assistance.

## **LEGENDS**

**Figure 1:** Lipid peroxidation in microsomes challenged with Fe<sup>2+</sup>/ascorbate. Rat liver microsomes were incubated in Tris-HCl buffer (pH 7.4) containing 50 μM Fe<sup>2+</sup>/200 μM ascorbate for 30 min at 37°C. The production of MDA was assessed by HPLC as described in Methods. Results are expressed as nmol/mg protein. Data represent mean ± SE in 4-6 different microsomal preparations.

<sup>a</sup> p<0.005, vs. control or tocopherol; <sup>b</sup> p<0.01, vs. tocopherol + Fe/ascorbate.

**Figure 2:** Effect of Fe<sup>2+</sup>/ascorbate on the key sterol regulatory enzymes. Microsomes, prepared from normal rats, were preincubated with 50 μM Fe<sup>2+</sup>/200 μM ascorbate for 30 min, and assayed for HMG-CoA reductase, cholesterol 7α-hydroxylase and ACAT as described in Methods. Glycerol-3-phosphate acyltransferase activity was tested as a positive control in view of its known high sensitivity to lipid peroxidation (19).

<sup>a</sup> p<0.01; <sup>b</sup> p<0.002; <sup>c</sup> p<0.001; <sup>d</sup> p<0.0001.

**Figure 3:** Influence of α-tocopherol on Fe<sup>2+</sup>/ascorbate-mediated inactivation of HMG-CoA reductase, cholesterol 7α-hydroxylase and ACAT. The experimental protocol was the same as in Figure 2, except that the test preincubation mixture contained α-tocopherol (500 μM). The values are presented as the mean ± SE for n=4-6 microsomal preparations. The statistical significance between control and experimental data: <sup>a</sup> p<0.02; <sup>b</sup> p<0.01; <sup>c</sup> p<0.001; <sup>d</sup> p<0.0001.

**Figure 4:** Microsomal enzymes involved in liver cholesterol homeostasis.

**REFERENCES**

1. Marzolo, M.P.; Rigotti, A.; Nervi, F. Secretion of biliary lipids from the hepatocyte. *Hepatology* **12**:134S-142S, 1990.
2. Dietschy, J.M.; Turley, S.D.; Spady, D.K. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different abnormal species, including humans. *J. Lipid Res.* **34**:1637-1659, 1993.
3. Cooper, A.D. Role of the liver in the degradation of lipoproteins. *Gastroenterology* **88**:192-205, 1985.
4. Turley, S.D; Dietschy, J.M. The metabolism and excretion of cholesterol by the liver. In: Arias, I.M.; Jacoby, H.; Popper, D.; Schachter, D.; Schafritz, D.A., eds. *The liver: biology and pathology*. New York: Raven: 1988;617-641.
5. Kanazawa, K., Ashida, H. Target enzymes on hepatic dysfunction caused by dietary products of lipid peroxidation. *Arch. Biochem. Biophys.* **288**:71-78, 1991.
6. Kanazawa, K.; Ashida, H.; Minamoto, S.; Natake, M. The effect of orally administered secondary autoxidation products of linoleic acid on the activity of detoxifying enzymes in the rat liver. *Biochim. Biophys. Acta* **879**:36-43, 1986.
7. Kanazawa, K.; Ashida, H.; Inoue, N.; Natake, M. Succinate dehydrogenase and synthetic pathways of glucose 6-phosphate are also the markers of the toxicity of orally administered secondary autoxidation products of linoleic acid in rat liver. *J. Nutr. Sci. Vitaminol.* **34**:25-37, 1989.
8. Wills, E.D. Effects of lipid peroxidation on membrane-bound enzymes of the endoplasmic reticulum. *Biochem. J.* **123**:983-991, 1971.

9. Goldstein, J.L.; Brown, M.S. The low density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* **46**:897-930, 1977.
10. Mayant, N.G.; Mitropoulos, K.A. Cholesterol 7 $\alpha$ -hydroxylase. *J. Lipid Res.* **18**:135-153, 1977.
11. Suckling, K.E.; Stange, E.F. Role of acyl-CoA:cholesterol acyltransferase in cellular cholesterol metabolism. *J. Lipid Res.* **26**:647-671, 1985.
12. Dudeja, P.K.; Brasitus, T.A. Inactivation of rat small intestinal brush-border membrane alkaline phosphatase by oxygen free radicals. *Gastroenterology* **105**:357-366, 1993.
13. Sevanian, A.; Hochstein, P. Mechanisms and consequences of lipid peroxidation in biological systems. *Annu. Rev. Nutr.* **5**:365-390, 1985.
14. Levy, E.; Garofalo, C.; Rouleau, T.; Gavino, V.; Bendayan, M. Impact of essential fatty acid deficiency on hepatic sterol metabolism in rats. *Hepatology* **23**:848-857, 1996.
15. Chirico, S. High performance liquid chromatography-based thiobarbituric acid tests. *Methods in Enzymology* **223**:314-318, 1994.
16. Lepage, G.; Munoz, G.; Champagne, J.; Roy, C.C. Preparative steps necessary for the accurate measurement of malondialdehyde by high performance liquid chromatography. *Anal. Biochem.* **197**:277-283, 1991.
17. Folch, J.M.; Lees, M.; Sloane Stanley, G.H. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**:497-509; 1957.
18. Lepage, G.; Levy, E.; Ronco, N.; Smith, L.; Galéano, N.; Roy, C.C. Direct transesterification of plasma fatty acids for the diagnosis of essential fatty acid deficiency in cystic fibrosis. *J. Lipid Res.* **30**:1483-1490; 1989.

19. Hylemon, P.B.; Studer, E.S.; Pandak, W.M.; Heuman, D.M.; Vlahcevic, Z.R.; Chiang, J.Y.L. Simultaneous measurement of cholesterol 7 $\alpha$ -hydroxylase activity by reverse-phase high-performance liquid chromatography using both endogenous and exogenous [4-<sup>14</sup>C] cholesterol as substrate. *Anal. Biochem.* **182**:212-216; 1989.
20. Thomas, P.D.; Poznansky, M.J. Lipid peroxidation inactivates rat liver microsomal glycerol-3-phosphate acyl transferase. *J. Biol. Chem.* **265**:2684-2691; 1990.
21. Quinn, M.T.; Parthasarathy, S.; Fong, L.G.; Steinberg, D. Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc. Natl. Acad. Sci. USA* **84**:2959-2998; 1987.
22. Halliwell, B.; Butteridge, J.M.C. Role of free radicals and catalytic metal ions in human disease: An overview. *Meth. Enzym.* **186**:1-85; 1990.
23. Ferrari, R.; Alfieri, O.; Curello, S.; Ceconi, C.; Cargnoni A.; Marzollo, P.; Pardini, A.; Caradonna, E.; Visioli, O. Occurrence of oxidative stress during reperfusion of the human heart. *Circulation* **81**:201-211; 1990.
24. Parks, D.A. Oxygen radicals: mediators of gastrointestinal pathophysiology. *Gut* **30**:293-298, 1989.
25. Bacon, B.R.; Brown, K.E. Iron metabolism and disorders of iron overload. In: Kaplowitz, N., ed. *Liver and biliary diseases*. Baltimore: Williams and Wilkins; 1996:349-362.
26. Bidlack, W.R.; Tappel, A.L. Damage to microsomal membrane by lipid peroxidation. *Lipids* **8**:177-182; 1973.

27. Jourd'heuil, D.; Vaananen, P.; Meddings, J.B. Lipid peroxidation of the brush-border membrane: membrane physical properties and glucose transport. *Am. J. Physiol.* G1009-G1015; 1993.
28. Bachowski, G.J.; Thomas, J.P.; Girotti, A.W. Ascorbate-enhanced lipid peroxidation in photooxidized cell membranes: cholesterol product analysis as a probe of reaction mechanism. *Lipids* **23**:580-586, 1988.
29. Haenen, G.R.; Bast, A. Protection against lipid peroxidation by a microsomal glutathione-dependent labile factor. *FEBS Lett.* **159**:24-28; 1983.
30. Burton, G.W.; Joyce, A.; Ingold, K.U. Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes? *Arch. Biochem. Biophys.* **221**:281-290; 1983.
31. Burton, G.W.; Ingold, K.U. Vitamin E: Application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc. Chem. Res.* **19**:194-201; 1986.
32. Stubbs, C.D.; Smith, A.D. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochim. Biophys. Acta* **779**:89-137; 1984.
33. Shinitzky, M. In: Shinitzky, M., ed. *Physiology of Membrane Fluidity*. Boca Raton, FL, CRC: 1984:1-52.
34. Suckling, K.E.; Boyd, G.S.; Smellie, C.G. Properties of a solubilised and reconstituted preparation of acyl-CoA:cholesterol acyltransferase from rat liver. *Biochim. Biophys. Acta* **710**:154-163; 1982.



35. Mathur, S.N.; Spector, A.A. Effect of liposome composition on the activity of detergent-solubilized acylcoenzyme A:cholesterol acyltransferase. *J. Lipid Res.* **23**:692-701; 1982.
36. Chautan, M.; Termine, E.; Amirayan, N.; Leonardi, J.; Pauli, A.M.; Portugal, H.; Lafont, H. Compared effect of n-3 and n-6 dietary fatty acids on rat intestinal acyl-CoA:cholesterol acyltransferase activity. *Scand. J. Gastroenterol.* **24**:632-640; 1989.
37. Pal, S.; Davis, P.J. Effects of different types of polyunsaturated fatty acids on cholesterol esterification in human fibroblasts. *Biochem Int.* **25**:281-288; 1991.
38. Liscum, L.; Cummings, R.D.; Anderson, R.G.W.; DeMartino, G.N.; Goldstein, J.L.; Brown, M.S. 3-Hydroxy-3-methylglutaryl-CoA reductase: a transmembrane glycoprotein of the endoplasmic reticulum with N-linked "high-mannose" oligosaccharides. *Proc. Natl. Acad. Sci. USA* **80**:7165-6169; 1983.
39. Gilbert, H.F. Molecular and cellular aspects of thiol-disulfide exchange. *Adv. Enzymol.* **63**:69-172; 1990.
40. Danielsson, H.; Kalles, I.; Wikvall, K. Regulation of hydroxylations in biosynthesis of bile acids. Isolation of a protein from rat liver cytosol stimulating reconstituted cholesterol 7 $\alpha$ -hydroxylase activity. *J. Biol. Chem.* **259**:4258-4262; 1984.
41. Taborsky, G. Oxidative modification of proteins in the presence of ferrous ion and air. Effect of ionic constituents of the reaction medium on the nature of the oxidation products. *Biochemistry* **12**:1341-1348; 1973.
42. Samuni, A.; Chevion, M.; Czapski, G. Unusual copper-induced sensitization of the biological damage due to superoxide radicals. *J. Biol. Chem.* **256**:12632-12635; 1981.

43. Kim, K.; Kim, I.H.; Lee, K.-Y.; Rhee, S.G.; Stadtman, E.R. The isolation and purification of a specific "protector" protein which inhibits enzyme inactivation by a thiol/Fe(III)/O<sub>2</sub> mixed-function oxidation system. *J. Biol. Chem.* **263**:4704-4711; 1988.
44. Halliwell, B.; Gutteridge, J.M.C. In: *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford, 1985; 139-189.
45. Bocan, T.M.; Mueller, S.B.; Brown, E.Q.; Lee, P.; Bocan, M.J.; Rea, T.; Pape, M.E. HMG-CoA reductase and ACAT inhibitors act synergistically to lower plasma cholesterol and limit atherosclerotic lesion in the cholesterol-fed rabbit. *Atherosclerosis* **139**:21-30; 1998.
46. Hay, J.M.; Yu, W.M.; Ashraf, T. Pharmacoeconomics of lipids-lowering agents for primary and secondary prevention of coronary artery disease. *Pharmacoeconomics* **15**:47-74;1999.

**Table 1: Microsomal Fatty Acid Composition in Normal Conditions and Following Various Treatments**

Fatty acid	Control (C)	Fe/ascorbate (Fe/A)	$\alpha$ -tocopherol (T)	Fe/Ascorbate + Tocopherol	Statistical significance (P<)		
					Fe/A vs. C	T vs. C	Fe/A vs. T Fe/A+T vs. C
10:0	0.02±0.01	0.01±0.00	0.01±0.00	0.01±0.00	NS	NS	NS
12:0	0.32±0.08	0.29±0.02	0.26±0.02	0.19±0.02	NS	NS	NS
14:0	0.29±0.02	0.31±0.01	0.27±0.01	0.27±0.01	NS	NS	NS
15:0	0.13±0.01	0.14±0.00	0.18±0.00	0.12±0.00	NS	NS	NS
16:0	21.13±0.19	24.41±0.39	20.72±0.09	20.67±0.10	0.00	NS	NS
17:0	0.22±0.20	0.24±0.00	0.20±0.00	0.20±0.00	NS	NS	NS
18:0	20.53±0.80	23.58±0.46	20.01±0.03	19.90±0.02	0.00	0.00	0.00
20:0	0.07±0.00	0.08±0.00	0.07±0.00	0.08±0.00	0.00	0.01	NS
22:0	0.16±0.00	0.18±0.00	0.16±0.00	0.16±0.00	0.00	NS	NS
24:0	0.51±0.00	0.63±0.02	0.52±0.01	0.51±0.01	0.01	NS	NS
18:3(n-3)	0.03±0.00	0.02±0.00	0.15±0.01	0.16±0.01	NS	0.00	0.00
20:5(n-3)	0.02±0.00	0.01±0.00	0.01±0.00	0.02±0.00	0.00	0.02	NS
22:5(n-3)	0.23±0.01	0.18±0.01	0.24±0.00	0.24±0.00	0.00	NS	NS
22:6(n-3)	2.01±0.04	1.31±0.12	2.16±0.01	2.17±0.00	0.00	0.01	0.01
18:2(n-6)	10.37±0.08	10.47±0.08	10.93±0.06	11.03±0.04	NS	0.00	0.00
18:3(n-6)	0.31±0.00	0.27±0.00	0.30±0.00	0.30±0.00	0.00	0.02	0.00
20:2(n-6)	0.28±0.01	0.28±0.01	0.27±0.00	0.26±0.00	NS	NS	NS
20:3(n-6)	0.94±0.01	0.87±0.01	0.93±0.00	0.93±0.00	0.00	NS	NS
20:4(n-6)	28.95±0.29	21.82±0.95	28.96±0.14	29.01±0.17	0.00	NS	NS
22:4(n-6)	0.95±0.01	0.86±0.03	0.97±0.01	0.97±0.00	0.02	0.02	0.01
16:1(n-7)	1.19±0.01	1.33±0.03	1.16±0.01	1.16±0.00	0.01	0.00	0.00
18:1(n-7)	2.62±0.01	2.93±0.04	2.59±0.01	2.59±0.01	0.00	NS	NS
18:1(n-9)	7.70±0.04	8.57±0.12	7.93±0.03	8.00±0.04	0.00	0.00	0.00
20:1(n-9)	0.15±0.00	0.17±0.01	0.16±0.01	0.17±0.01	NS	NS	NS
20:3(n-9)	0.16±0.00	0.13±0.01	0.15±0.00	0.16±0.00	0.01	NS	NS
24:1(n-9)	0.20±0.01	0.23±0.01	0.19±0.01	0.18±0.01	0.03	NS	NS

Rat liver microsomes were prepared and submitted to fatty acid analysis. Total lipids were extracted, transesterified and injected into a 60-m capillary column of gas chromatograph. The data were evaluated by ANOVA and the difference between the means was analyzed by two tail T test. NS, non-significant.

Table 2: Microsomal Fatty Acid Families and Ratios

Fatty acid	Control	Fe/Ascorbate	$\alpha$ -Tocopherol	Fe/Ascorbate + Tocopherol
Saturated (%)	43.39 $\pm$ 0.39	49.90 $\pm$ 0.84 <sup>ab</sup>	42.34 $\pm$ 0.14 <sup>a</sup>	42.10 $\pm$ 0.15 <sup>a</sup>
PUFA (%)	44.24 $\pm$ 0.41	36.19 $\pm$ 1.06 <sup>ab</sup>	45.08 $\pm$ 0.17	45.26 $\pm$ 0.20
PUFA/Saturated <sup>c</sup>	1.02 $\pm$ 0.02	0.72 $\pm$ 0.03 <sup>ab</sup>	1.07 $\pm$ 0.01	1.07 $\pm$ 0.01 <sup>a</sup>
Total n-3 (%)	2.29 $\pm$ 0.04	1.52 $\pm$ 0.13 <sup>ab</sup>	2.57 $\pm$ 0.02 <sup>a</sup>	2.59 $\pm$ 0.01 <sup>a</sup>
Total n-6 (%)	41.80 $\pm$ 0.37	34.53 $\pm$ 0.92 <sup>ab</sup>	42.36 $\pm$ 0.17	42.50 $\pm$ 0.21
Total n-7 (%)	3.81 $\pm$ 0.02	4.25 $\pm$ 0.07 <sup>ab</sup>	3.75 $\pm$ 0.01 <sup>a</sup>	3.75 $\pm$ 0.01
Total n-9 (%)	8.20 $\pm$ 0.05	9.10 $\pm$ 0.12 <sup>ab</sup>	8.45 $\pm$ 0.04 <sup>a</sup>	8.50 $\pm$ 0.04 <sup>a</sup>
EFA/non-EFA <sup>d</sup>	0.789 $\pm$ 0.013	0.566 $\pm$ 0.026 <sup>ab</sup>	0.816 $\pm$ 0.006	0.821 $\pm$ 0.007
16:1(n-7)/18:2(n-6)	0.114 $\pm$ 0.001	0.126 $\pm$ 0.002 <sup>ab</sup>	0.106 $\pm$ 0.001 <sup>a</sup>	0.105 $\pm$ 0.000 <sup>a</sup>
Fam7/18:2(n-6)	0.368 $\pm$ 0.004	0.406 $\pm$ 0.004 <sup>ab</sup>	0.343 $\pm$ 0.003 <sup>a</sup>	0.340 $\pm$ 0.002 <sup>a</sup>
18:2(n-6)/20:4(n-6)	0.358 $\pm$ 0.001	0.484 $\pm$ 0.023 <sup>ab</sup>	0.377 $\pm$ 0.002 <sup>a</sup>	0.380 $\pm$ 0.001 <sup>a</sup>
Trans	0.104 $\pm$ 0.021	0.290 $\pm$ 0.045 <sup>ab</sup>	0.115 $\pm$ 0.001	0.118 $\pm$ 0.004

<sup>a</sup> Different from control.<sup>b</sup> Different from  $\alpha$ -tocopherol and Fe/ascorbate + Tocopherol<sup>c</sup> Double bound index<sup>d</sup> (n-6) + (n-3) / (n-7) + (n-9) + saturated

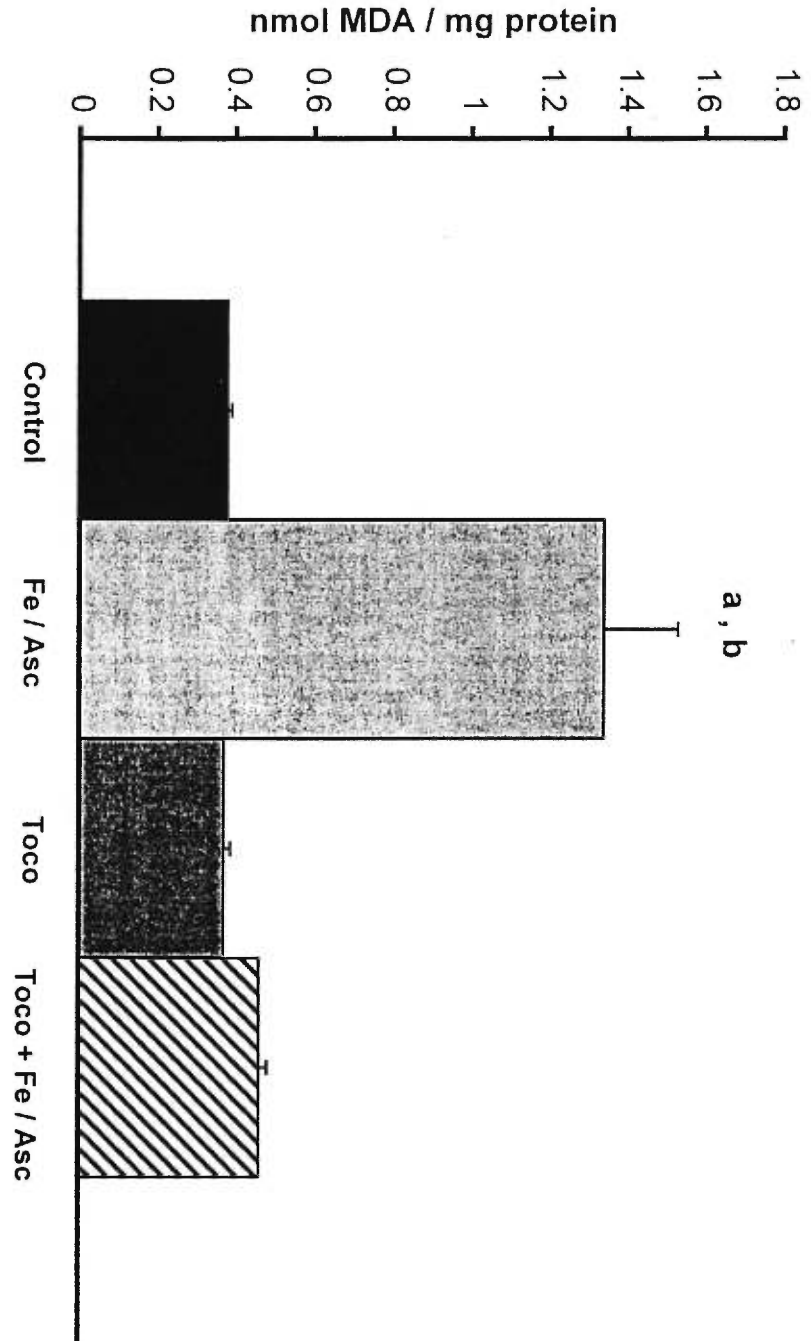


Figure 1

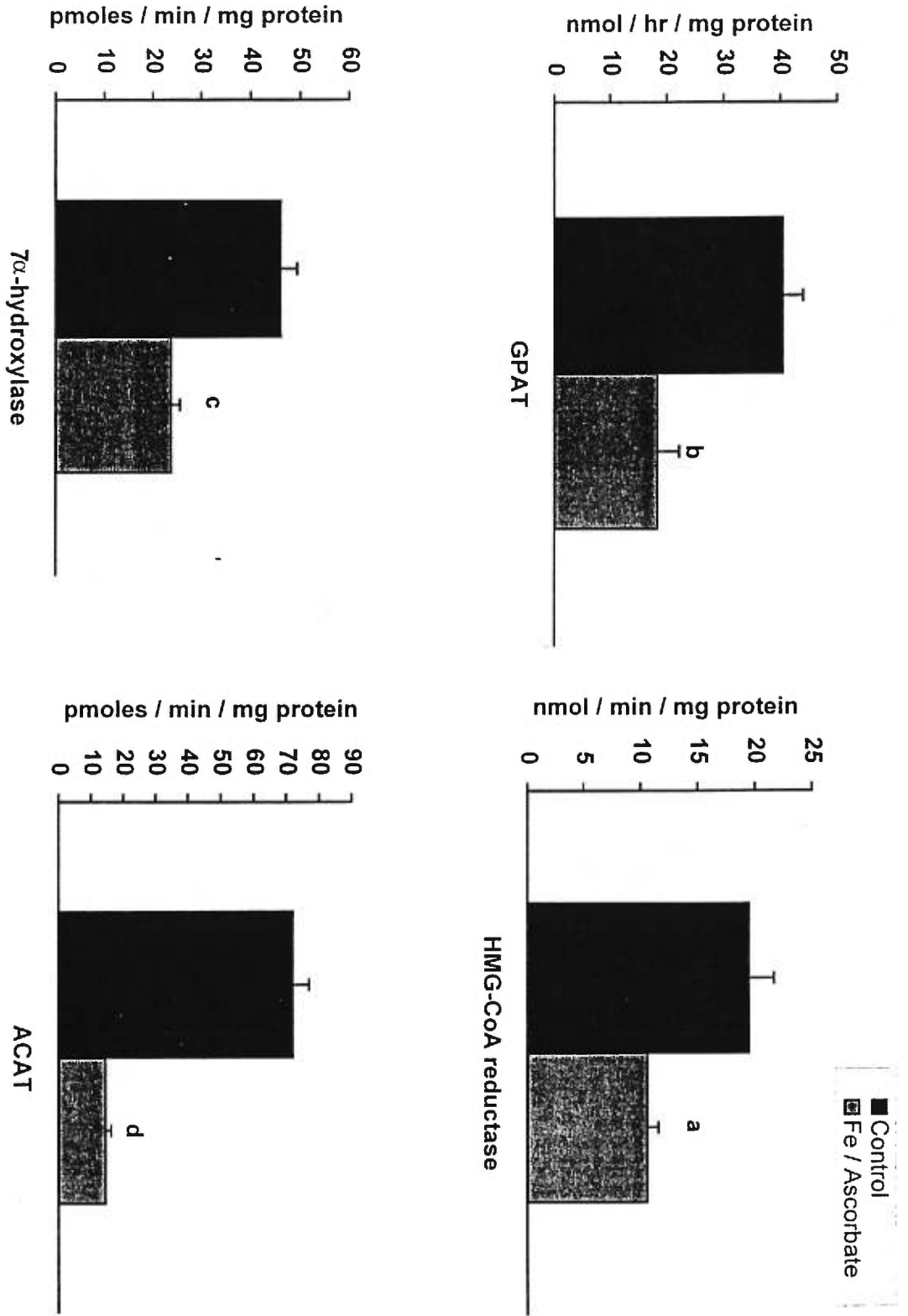


Figure 2

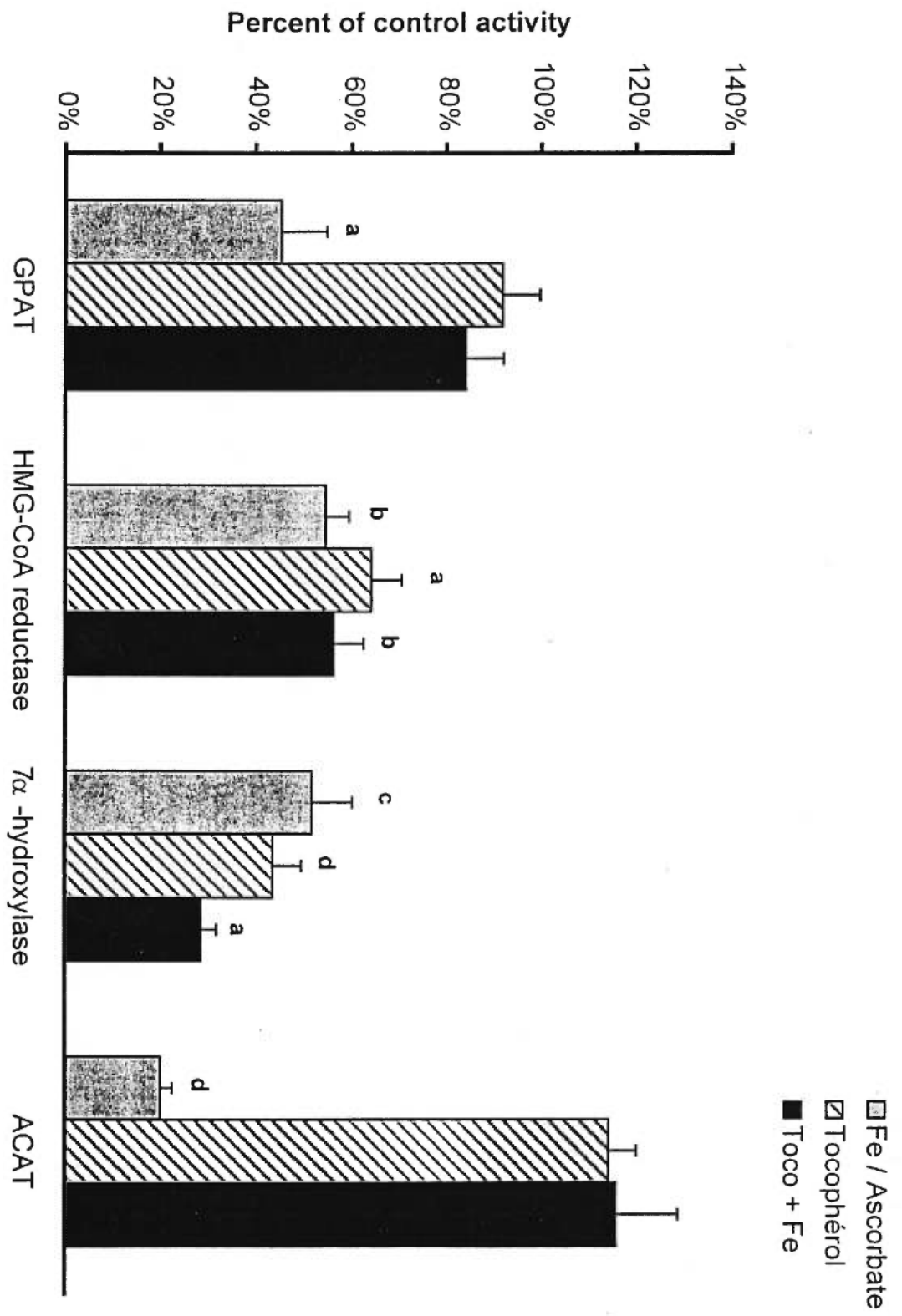


Figure 3

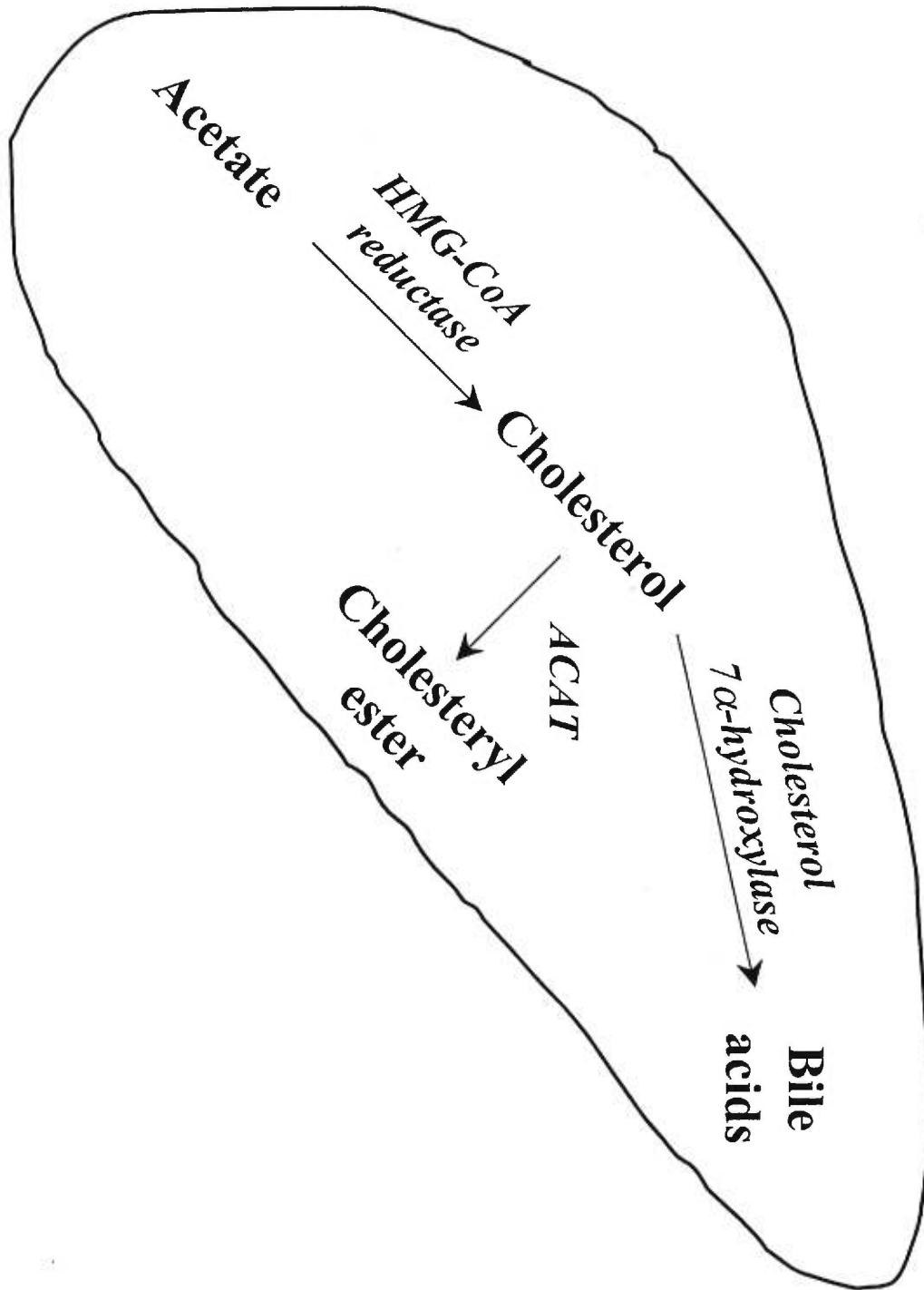


Figure 4



## Dietary Iron Overload and Induced Lipid Peroxidation Are Associated With Impaired Plasma Lipid Transport and Hepatic Sterol Metabolism in Rats

Sylvain Brunet,<sup>1</sup> Louise Thibault,<sup>1</sup> Edgard Delvin,<sup>2</sup> Wagner Yotov,<sup>1</sup> Moïse Bendayan,<sup>3</sup> and Emile Levy<sup>1</sup>

Although hemochromatosis is characterized by dramatic morphological and functional alterations in the liver, little is known about the effects of an excess of iron on lipid metabolism. Therefore, we determined the effect of chronic iron overload on plasma lipid profile and lipoprotein composition, as well as on hepatic cholesterol metabolism and biliary sterol output. Rats administered a diet enriched with 3% iron carbonyl for 12 weeks displayed a 30-fold increase in iron ( $P < .0001$ ) and a 5-fold rise in malondialdehyde ( $P < .001$ ) in the liver. When compared with pair-fed controls, iron-overload rats showed a significant increase in triglycerides ( $P < .005$ ), free cholesterol ( $P < .006$ ), cholesteryl ester ( $P < .007$ ), and high-density lipoprotein (HDL)-cholesterol ( $P < .003$ ). Triglyceride and cholesteryl ester enrichment, protein depletion, size increase, and apolipoprotein composition alterations characterized the very low density lipoprotein (VLDL) and HDL particles of iron-overload rats. Assessment of the activity of intracellular key enzymes for cholesterol homeostasis in these rats disclosed a reduction in 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (66%,  $P < .005$ ) and cholesterol 7 $\alpha$ -hydroxylase (58%,  $P < .0004$ ) with an increment of acyl-CoA: cholesterol acyltransferase (62%,  $P < .002$ ). The lack of optimal enzymatic activity may be a result of marked membrane lipid peroxidation that brings about fluidity drop ( $P < .029$ ) in microsomes of iron-overload rats ( $5.00 \pm 0.013$ ) versus controls ( $8.20 \pm 0.03$ ), reflected by polarization. A decline of the pool size of cholesterol and bile acids was noted in iron-overload rats during a 6-hour bile drainage. Our results show that experimental iron overload causes marked perturbations in plasma lipid transport and hepatobiliary sterol metabolism. Given the positive correlation of malondialdehyde with most of the altered parameters, iron-catalyzed lipid peroxidation may well be one of the involved mechanisms. (HEPATOLOGY 1999;29:1809-1817.)

Numerous pathological alterations have been reported in patients with primary or secondary iron overload.<sup>1,2</sup> Cirrhosis, skin pigmentation, impaired cardiac function, arthropathy, diabetes mellitus, cancer, and endocrinopathies are among the most toxic manifestations of each form of iron overload.<sup>3-5</sup> Although the mechanisms remain obscure, it has been suggested that iron-catalyzed free radical-mediated lipid peroxidation plays a significant role in these abnormal events.<sup>6,7</sup> Indeed, iron in similar fashion to other transition metals, can initiate peroxidation by producing highly reactive hydroxyl radicals from hydrogen peroxide via Fenton type reactions and by complexing with oxygen directly to yield reactive perferryl and ferryl ions.<sup>8,9</sup> Consequently, peroxidative decomposition of the polyunsaturated fatty acids occurs in membrane phospholipids, thereby affecting vital organelle integrity and cell function.<sup>10-12</sup>

Hepatocellular changes have specifically been noted with iron overload because the liver is the major recipient of the excess of iron.<sup>13</sup> For example, iron-induced lipid peroxidation in hepatic mitochondria and microsomes results in defective electron transport and reduced concentrations in cytochromes P-450 and b5, respectively.<sup>14,15</sup> Increased lipid peroxidation in lysosomal membranes is also evidenced and is associated with low membrane fluidity and a defect in lysosomal acidification.<sup>11</sup> Despite the aforementioned findings, the effects of iron-induced lipid peroxidation on hepatic cholesterol metabolism have not received much attention. However, the liver remains the major organ in the body capable of synthesizing and excreting cholesterol, as such, or after its conversion into bile acids.<sup>16,17</sup> It also constitutes the major site of lipogenesis and very low density (VLDL) production, with the ability to form a portion of high-density lipoproteins (HDL) and to take up and degrade other lipoprotein fractions.<sup>18-20</sup> It is endowed with microsomal enzymes, assuring cholesterol balance, e.g., HMG-CoA reductase (the regulatory key enzyme involved in *de novo* cholesterol synthesis), cholesterol 7 $\alpha$ -hydroxylase (the rate-limiting enzyme in the conversion of cholesterol into bile acids), and acyl-CoA: cholesterol acyltransferase (ACAT) (transforming free cholesterol into cholesterol esters, which influences the excretion of cholesterol from the liver).<sup>21,22</sup> Our hypothesis was that iron-catalyzed lipid peroxidation, membrane disruption and distortion change the activity of lipid-dependent enzymes. Given that the liver represents the source of biliary lipids and plasma lipoproteins, we expected qualitative and quantitative changes in these components.

Abbreviations: VLDL, very low density lipoproteins; HDL, high density lipoproteins; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ACAT, cholesterol acyltransferase; EDTA, ethylenediaminetetraacetic acid; MDA, malondialdehyde; LDL, low density lipoproteins; PBS, phosphate buffered saline; FC, free cholesterol; TG, triglycerides; CE, cholesteryl ester; PL, phospholipids; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel; TMA-DPH, (1 (4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate).

From the Departments of <sup>1</sup>Nutrition, <sup>2</sup>Biochemistry, and <sup>3</sup>Anatomy Centre de Recherche, Hôpital Sainte-Justine, Université de Montréal, Montréal, Québec, Canada. Received October 27, 1998; accepted March 1, 1999.

Address correspondence to: Dr. Emile Levy, CI-Nutrition Unit, Hôpital Sainte-Justine, 3175 Côte Ste-Catherine Road, Montreal (Quebec) H3T 1C5. E-mail: levye@ere.umontreal.ca; fax: (514) 345 4999.

Copyright © 1999 by the American Association for the Study of Liver Diseases. 0270-9139/99/2906-0026\$3.00/0

## MATERIALS AND METHODS

**Animal Treatment.** The experiments were performed on male weaning Sprague Dawley rats purchased from the Charles River Breeding Company (Montreal, Quebec, Canada). Rats were allowed free access to water and food. After 2 to 3 days of acclimatization, the rats were randomly divided into two groups: one was assigned to a 3% iron-enriched diet (AIN-76A), and the other was pair-fed with standard AIN-76A diet. The rats were maintained at 22°C with a 12-hour light cycle. Body weights and food intakes were recorded twice weekly to monitor growth. After 12 weeks on these diets, rats were fasted overnight before being used for the following studies.

**Blood Collection.** Rats were anesthetized by the intraperitoneal injection of pentobarbital (48 mg/kg body wt i.p.) (Abbott Laboratories, Montreal). Blood was collected in an EDTA-containing tube (1 mg/mL) and separated immediately by low speed centrifugation (2500 rpm, 25 min, 4°C). For the determination of malondialdehyde (MDA), a fraction of plasma was immediately frozen in liquid nitrogen and stored at -80°C until analysis.

**Lipoprotein Isolation.** Lipoproteins were isolated from fresh plasma by conventional discontinuous density gradient ultracentrifugation in a Beckman L5-65 preparative ultracentrifuge (Beckman Inc., Montreal) by using a Ti-50 rotor as previously described in detail.<sup>23</sup> Briefly, after preliminary centrifugation to remove chylomicrons, VLDL and low density (LDL) lipoproteins were isolated at a density of 1.006 and 1.063 g/mL, respectively, running at 40,000 rpm for 18 hours at 5°C. The separation of HDL (1.21 g/mL) was performed at 40,000 rpm for 48 hours at 5°C. Each fraction was dialyzed intensively against phosphate buffered saline (PBS) with 0.001 mol/L EDTA, pH 7.0 at 4°C.

**Plasma Lipid and Lipoprotein Analysis.** Plasma concentrations of total cholesterol, free cholesterol (FC), and triglycerides (TG) were measured enzymatically using commercial kits (Boehringer Mannheim, Montreal, Canada) as previously described.<sup>24</sup> Cholesteryl ester (CE) was calculated as the difference between total and free cholesterol  $\times 1.7$ . Lipoprotein-protein was quantified according to Lowry et al.<sup>25</sup> with bovine serum albumin as a standard. Phospholipids (PL) were determined by the Bartlett method.<sup>26</sup> Apolipoprotein content of plasma lipoproteins was qualitatively assayed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously.<sup>23,24</sup> Gels were stained with Coomassie blue for 1 hour and destained in 7% acetic acid. The bands of apolipoproteins were identified by comparison with the mobility of apolipoprotein and molecular weight standards. HDL-cholesterol (HDL-C) was measured after precipitation of VLDL and LDL with phosphotungstic acid.<sup>23,24</sup>

**Hepatic Lipids Determination.** To determine hepatic lipid content and lipid peroxidation, the livers were promptly removed, rinsed with PBS containing 3 mmol/L EDTA, blotted dry, and weighed. Special attention was paid to rapidly freeze aliquots in liquid nitrogen and keep them at -80°C until assayed. Then, a sample was homogenized in 0.9% NaCl containing 3 mmol/L EDTA to prevent spurious lipid peroxidation and to chelate any ionic iron that might have been released during tissue preparation. Aliquots of homogenates were lipid-extracted with chloroform-methanol (2:1, vol/vol), and their TG, FC, and CE were determined.

**Bile Sampling.** With the animals anesthetized under pentobarbital (48 mg/kg body wt i.p.), the common bile duct was cannulated with a PE-10 catheter, securely tied. The animals were restrained in individual cages and bile was collected at hourly intervals for 6 h to determine the biliary pool of lipids and bile acids.<sup>18,21,27</sup>

**MDA and Glutathione Assessment.** Free MDA levels were determined from frozen specimens of liver, hepatic microsomes, bile, and plasma according to a modified method of Chirico.<sup>28</sup> Proteins were first precipitated with a 10% NaWO<sub>4</sub> solution according to Lepage et al.<sup>29</sup> Protein free supernatant was then reacted with an isovolume of 0.5% thiobarbituric acid solution at 95°C for 30 minutes. After cooling to room temperature, pink chromogene was extracted with n-butanol and dried over a stream of nitrogen at 37°C. The dry

extract was then resuspended in KH<sub>2</sub>PO<sub>4</sub>/Methanol (70:30) mobile phase before MDA detection by high-performance liquid chromatography.<sup>28</sup> Reduced and oxidized glutathione was measured by Tietze's technique.<sup>30</sup>

**Iron Determination.** Nonheme iron quantity was measured in liver according to Torrance et al.<sup>31</sup> Briefly, liver was hydrolyzed overnight at 60°C before the addition of chromogen reagent. The absorbance was determined spectrophotometrically at 535 nm.

**Liver Microsome Preparation and Fluidity Measurement.** Experimental and control rats from each dietary group were treated identically. After their sacrifice, the microsomal fraction was prepared as described previously.<sup>21</sup> Fluidity of microsomal membranes was estimated by the incorporation of a fluorescent probe (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluene-sulfonate) (TMA-DPH) and measurement of polarization.<sup>32</sup> To a total of 100 to 200 µg microsomal protein in potassium phosphate buffer solution (pH 7.2) were added 5 µL TMA-DPH (1 mmol/L in acetonitrile) by vortexing for 10 seconds. Following an incubation of 30 minutes at 37°C and evaporation of acetonitrile, fluorescence was measured in a spectrofluorometer at 25°C with polarization filters parallel and perpendicular to the excitation beam. Excitation was at 360 nm and emission at 420 nm.

**Assay of Microsomal HMG-CoA Reductase Activity.** Microsomal enzymatic activity was assayed as described by Brown et al.<sup>33</sup> The reaction mixture contained 160 mmol/L potassium phosphate (pH 7.4), 300 µg of microsomal protein, 20 mmol/L of glucose-6-phosphate, 2.5 mmol/L of nicotinamide-adenine dinucleotide phosphate, 12.5 mmol/L of dithiothreitol, and 1.2 units of glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of [3-<sup>14</sup>C]HMG-CoA (15,000 dpm/nmol). After incubation for 30 minutes at 37°C, the [<sup>14</sup>C]mevalonate formed was converted into lactone, isolated by thin-layer chromatography<sup>12</sup> and counted using an internal standard to correct for incomplete recovery. HMG-CoA reductase activity was expressed in nanomoles of mevalonate synthesized per milligram of protein per minute.

**Cholesterol 7 $\alpha$ -Hydroxylase Activity.** Microsomal cholesterol 7 $\alpha$ -hydroxylase activity was measured according to Hylemon et al.<sup>34</sup> Briefly, 1 mg of microsomal protein was diluted in a potassium phosphate buffer, pH 7.4, containing 50 mmol/L NaF, 5 mmol/L dithiothreitol, 1 mmol/L EDTA, 20% glycerol, 0.015% 3-[(3-cholesterolpropyl)dimethylammonio]-1-propane sulfonate (CHAPS), and 0.25 mmol/L desferal. Reaction was started by the addition of an NADPH regenerating system containing 1 mmol/L NADP, 10 mmol/L glucose-6-phosphate and 0.15 UI glucose-6-phosphate dehydrogenase. Following the 20-minute reaction at 37°C, the endogenous 7 $\alpha$ -hydroxycholesterol form was derivatized with a 0.1% cholesterol oxidase solution. Dried petroleum ether extract was resuspended in 70% acetonitrile/30% methanol mobile phase, before high-performance liquid chromatography, with UV detection at 240 nm, using 7 $\beta$ -hydroxycholesterol as an internal standard. The 7 $\alpha$ -hydroxycholesterol product was expressed as pmol/min/mg protein.

TABLE 1. Characteristics of Iron Overload and Control Rats

Gross Characteristic	Control	Iron Overload	P<
Body weight (g)	268.60 $\pm$ 12.78*	285.00 $\pm$ 19.48	NS
Liver weight (g)	7.46 $\pm$ 0.30	13.54 $\pm$ 0.80	0.0001
Liver/body weight (%)	2.78 $\pm$ 0.13	4.77 $\pm$ 0.18	0.01
Total hepatic protein (mg/g)	98.35 $\pm$ 5.83	97.82 $\pm$ 2.98	NS
Iron content (µg/g liver)	162 $\pm$ 8	4812 $\pm$ 162	0.0000
Plasmatic albumin (g/l)	14.5 $\pm$ 0.71	14.5 $\pm$ 0.5	NS
ALT (U/L)	34.0 $\pm$ 11	42.0 $\pm$ 7.0	NS
AST (U/L)	57.0 $\pm$ 7.1	59.0 $\pm$ 10.0	NS
Plasmatic bilirubin (µmol/L)	1.5 $\pm$ 0.7	1.25 $\pm$ 0.43	NS

Abbreviation: NS, not significant.

\*Mean  $\pm$  SE, n = 10/group.

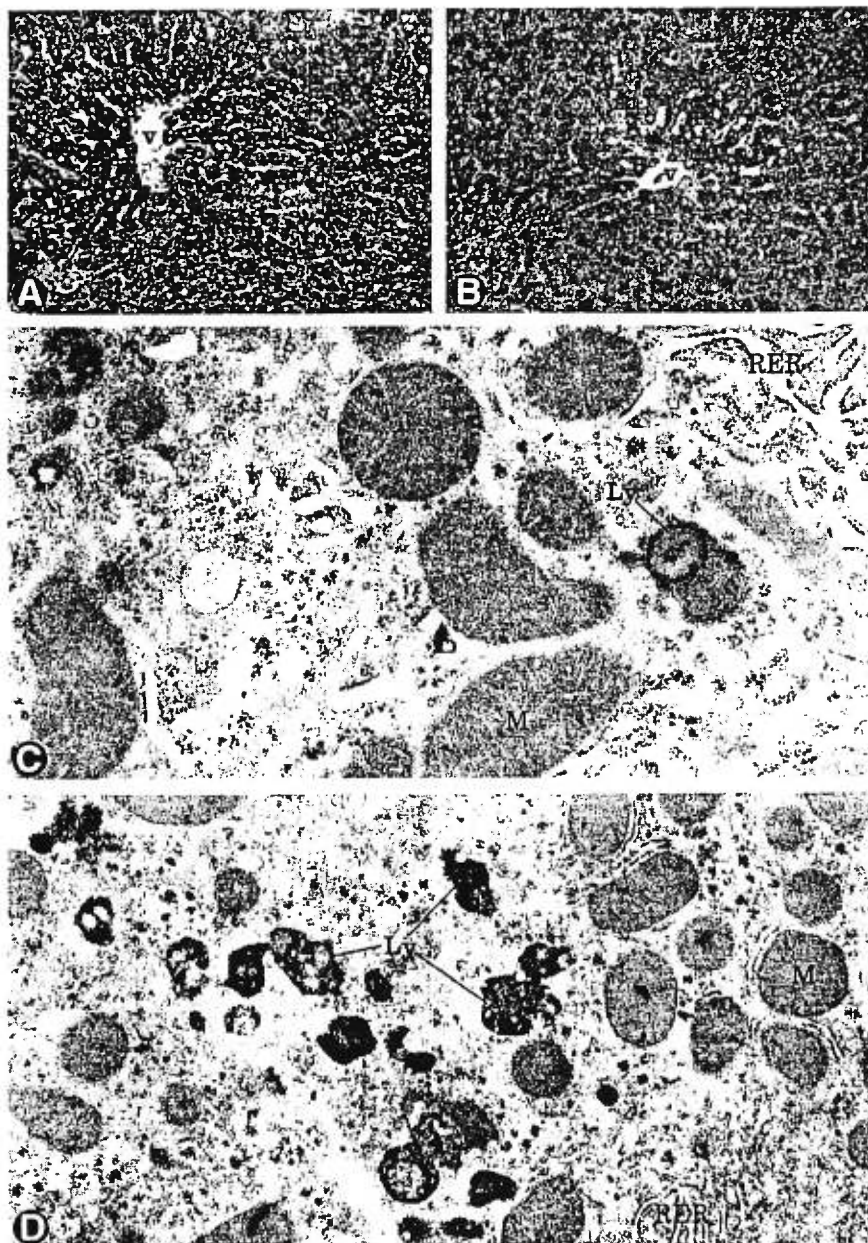


FIG. 1. Light (A, B) and electron (C, D) micrographs of liver tissues from control (A, C) and experimental (B, D) animals. In both cases, the tissues appear intact and display the classical characteristics. For the experimental case, however, a moderate proliferation of electron dense lysosomal structures (Lys) was observed. V, central vein; RER, rough endoplasmic reticulum; M, mitochondria. Original magnifications: A and B  $\times 150$ ; C  $\times 34,000$ ; D  $\times 20,000$ .

**Acylcoenzyme A: Cholesterol Acyltransferase (ACAT) Activity.** The determination of this enzyme was based on the assay described previously.<sup>21</sup> To the assay mixture containing 75  $\mu\text{g}$  microsomal protein were added 5  $\mu\text{mol}$  [<sup>14</sup>C] oleoyl coenzyme A (sp. act.  $\sim 10,000$  dpm/ $\mu\text{mol/L}$ ) to initiate the reaction in a buffer solution (pH 7.5) consisting of 0.1 mol/L of Tris-Cl, 0.25 mol/L of sucrose, and 1 mmol/L of EDTA acid. After the incubation for 10 min at 37°C, the reaction was stopped by adding chloroform/methanol (2:1, vol/vol) followed by [<sup>3</sup>H] cholesteryl oleate as an internal standard to estimate recovery.

**Electron Microscopy.** Liver tissue from control and experimental animals was sampled and fixed by immersion with 1% glutaralde-

hyde. The tissues were then postfixed with 1% osmium tetroxide and embedded in Epon according to standard techniques. Semithin sections were performed from different blocks for each sample, stained, and examined at the light microscope level. For electron microscopy, ultra-thin sections were counterstained and examined with a Phillips 410 electron microscope.

**Other Methods.** The measurement of total bilirubin in serum was achieved by adding diazotized sulfanilic acid to produce azobilirubin. Following a 10-minute incubation, the reaction was stopped by the addition of an ascorbic acid solution, and the color developed with alkaline tartrate. The absorbance was measured at 600 nm. The enzymes alanine aminotransferase and aspartate aminotransferase

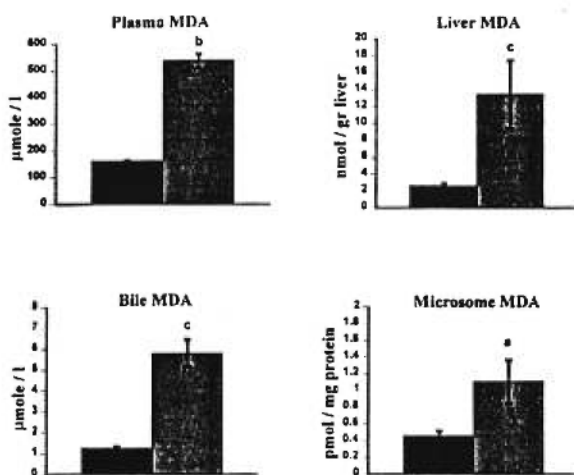


FIG. 2. Malondialdehyde levels in the plasma and hepatobiliary system. Rats were fed a 3% carbonyl iron diet and sacrificed after 12 weeks. MDA was determined in the plasma, liver, bile and hepatic microsomes. For needs of comparison, animals were pair-fed with Ain-76A. Values are means  $\pm$  SEM for  $n = 10$ /group. \* $P < .04$ , <sup>b</sup> $P < .004$ , <sup>c</sup> $P < .001$ .

were measured by standard enzymatic kits (Beckman, Montreal, Quebec).

**Statistical Analysis.** All values were expressed as the mean  $\pm$  SE. Statistical differences were assessed by Student's two-tailed *t*-test.

## RESULTS

**Animal and Tissue Characteristics.** Gross animal characteristics were evaluated after 12 weeks of feeding either with a regular diet or an iron-enriched diet. As shown in Table 1, no differences could be observed in animal body weight and liver protein content. Nevertheless, the feeding of 3% iron carbonyl induces a rise in liver weight of iron-overload rats. Indeed the chronic supplementation of rats with iron led to about a 30-fold elevation of total hepatic iron content over control pair-fed values. Despite iron overload, plasma levels of transaminases, albumin, and bilirubin were within normal range. Furthermore, light and electron microscopy examination of liver tissues revealed no major differences between control and experimental samples beside a moderate proliferation of lysosomal structures in the experimental tissues (Fig. 1). The other cellular components displayed their classical characteristics.

**Iron-Induced Lipid Peroxidation.** As expected from excessive iron deposition in the liver, lipid peroxidation was induced locally as reflected by the significant increase in MDA

concentrations. Moreover, there was a consistent accumulation of MDA in hepatic microsomes as well as in the plasma and bile of iron-overload rats compared with diet-restricted controls (Fig. 2). We also evaluated the glutathione status of the liver. Iron treatment produced an 84% rise of total glutathione when compared with values of control animals. Both forms of reduced and oxidized glutathione were enhanced, the former (100%) more than the second (55%) (Table 2).

**Plasma Lipid Profile.** Several alterations in plasma lipids were observed in the experimental group (Table 3). In fact, iron supplementation increased the lipid classes significantly: TG by 46% ( $P < .005$ ) and total cholesterol by 59% ( $P < .003$ ). As expected, because of the high total plasma cholesterol concentration, both cholesterol fractions were markedly increased: FC by 37% ( $P < .005$ ) and CE by ~72% ( $P < .007$ ). In addition, hypercholesterolemia was closely associated with a significant ( $P < .03$ ) increment in VLDL-cholesterol (300%) and HDL-cholesterol (83%) (Table 3).

**Lipoprotein Lipid and Apoprotein Composition.** The relative content of plasma lipoproteins, isolated by sequential ultracentrifugation, is shown in Table 4. Both VLDL (1.006 g/mL) and HDL (1.21 g/mL) particles from iron-overload rats displayed a higher proportion of TG and CE and a lower percentage of protein than control rats. These alterations led to greater ratios of TG/PR, CE/PR and TG+CE/FC+PL+PR, indicative of larger VLDL and HDL particles in iron-overload rats. Despite the decrease in FC, the composition of LDL particles was largely unaffected by iron supplementation.

In addition to the changes observed in the lipid composition of iron-overload rats, the SDS-PAGE presented an apolipoprotein distribution that differed from that obtained from control rats (Fig. 3). More specifically, the gels displayed an enrichment in apo B-100, apo B-48, and apo E in VLDL, whereas a reduction in apo B-100, apo B-48, and apo A-II with a different apo E isoform pattern characterized the LDL fraction (Fig. 3A). In addition, HDL particles disclosed a slight increase in apo E content along with a reduction in apo A-IV (Fig. 3B).

**Liver Lipid Content.** To examine the association of iron overload and hepatic endogenous lipids, liver homogenates and microsomes were prepared. The amount of total lipids was significantly decreased (48%) in the liver homogenates of iron-overload rats compared with controls (Table 5). Analysis of the different lipid classes reveals that the low lipid values were totally accounted for by the pronounced drop (45%) in TG. Similar data were recorded in isolated microsomes. There does not appear to be a substantial effect on free cholesterol and cholesteryl ester.

TABLE 2. Hepatobiliary Glutathione Levels

Rats	Liver			Bile			
	Total GSH	Reduced GSH	Oxidized GSH	Total GSH	Reduced GSH	Oxidized GSH	
		(µmol/g of liver)				(µmol/g of bile)	
Control	0.076 $\pm$ 0.015	0.054 $\pm$ 0.012	0.022 $\pm$ 0.004	0.31 $\pm$ 0.04	0.14 $\pm$ 0.02	0.17 $\pm$ 0.03	
Iron overload	0.161 $\pm$ 0.026*	0.123 $\pm$ 0.019*	0.037 $\pm$ 0.006*	0.57 $\pm$ 0.04†	0.44 $\pm$ 0.04†	0.14 $\pm$ 0.02	

NOTE. Values are means  $\pm$  SEM for  $n = 5$ /group.

\* $P < .05$ .

† $P < .001$ .

TABLE 3. Effect of Iron Treatment on Plasma Lipids and Lipoproteins

	Lipids				Lipoprotein-cholesterol		
	TC	FC	CE	TC	VLDL	LDL	HDL
	(mmol/L)				(mmol/L)		
Control	1.42 ± 0.09	0.46 ± 0.03	0.96 ± 0.07	0.29 ± 0.03	0.21 ± 0.001	0.209 ± 0.013	0.792 ± 0.051
Iron overload	2.27 ± 0.19*	0.63 ± 0.02†	1.65 ± 0.18‡	0.54 ± 0.06§	0.087 ± 0.007	0.205 ± 0.017	1.446 ± 0.122¶

NOTE. The biochemical determinations were performed in control and iron-loaded rats for 12 weeks. Data are means ± SEM for n = 10/group.

Abbreviations: TC, total cholesterol; FC, free cholesterol; CE, cholesteryl ester.

\*P ≤ .0039.

†P ≤ .0006.

‡P ≤ .0068.

§P ≤ .0051.

||P ≤ 2.17 × 10<sup>5</sup>.

¶P ≤ .0011.

**Biliary Lipids.** To further characterize biliary lipids and bile acids, the common bile duct was cannulated and bile collected at hourly intervals for 6 hours. Whereas no differences were noticeable regarding the output of phospholipids, a pattern of decrease was apparent for bile acid secretion rate and cholesterol delivery in iron-overload rats (Fig. 4). The reduction of these processes reached statistical significance from 5 hours onwards.

**Sterol Enzymes.** To further pursue the question of whether the iron regimen affects the regulatory enzymes that coordinate hepatic sterol metabolism, the activity of three key enzymes was measured. Data documented in Fig. 5 indicate that iron treatment diminished HMG-CoA reductase by 66% (P < .005) and cholesterol 7 $\alpha$ -hydroxylase by 58% (P < .0004). In contrast, ACAT activity was enhanced by 62%.

**Membrane Fluidity.** Because lipid peroxidation can affect the physical properties of membranes, thereby altering enzymatic activities, it was important to determine membrane fluidity in our studies. Table 6 depicts the changes noted in membrane fluidity in the control and experimental groups. Following iron overload, a significant (P < .029) decrease (38%) in membrane fluidity characterized hepatic microsomal fractions.

**Correlation Between Hepatic MDA, Plasma, and Liver Parameters.** As mentioned previously, iron overload produced unequivocally lipid peroxidation documented by the high levels of MDA in plasma and liver (Fig. 2). Under iron treatment,

there were remarkably significant alterations in plasma and liver lipid metabolism as shown by our data. The reported correlations between MDA and various parameters (Table 7) suggest that the mechanisms underlying lipid and enzymatic changes are related to lipid peroxidation.

#### DISCUSSION

From the data presented above, it is possible to note profound disorders in circulating and hepatobiliary lipid components provoked by iron overload. First, marked hyperlipidemia was evidenced by the concomitant increment in plasma triglycerides and cholesterol. Second, the development of hypercholesterolemia was associated with striking changes in cholesteryl ester and HDL-C levels. Third, several chemical abnormalities characterized the composition and size of lipoproteins, VLDL and HDL particularly. Fourth, a significant decline in the pool size of biliary lipids was an accompanying feature. Fifth, the activity of the three regulatory enzymes of cholesterol homeostasis was considerably affected. Finally, most of these disturbances were correlated with lipid peroxidation as reflected by elevated MDA concentrations. To our knowledge, this is the first investigation, that has systematically explored the repercussions of iron-induced peroxidation on hepatic cholesterol metabolism and biliary sterol output as well as lipoprotein lipid and apoprotein composition.

Several laboratories have shown a link between chronic

TABLE 4. Chemical Composition of Lipoproteins

Lipoproteins	Composition					Weight Ratios		
	TC	CE	FC	PL	PR	TC/PR	CE/PR	(TC + CE)/(FC + PL + PR)
<b>VLDL (1.006 g/mL)</b>								
Control	47.71 ± 2.80	3.64 ± 0.17	3.18 ± 0.12	12.03 ± 0.71	33.44 ± 3.33	1.57 ± 0.24	0.12 ± 0.02	1.10 ± 0.12
I.O.	57.73 ± 2.08	5.24 ± 0.51	3.32 ± 0.26	12.59 ± 0.88	21.11 ± 1.07	2.80 ± 0.23	0.25 ± 0.03	1.74 ± 0.14
P<	.0134	.0123	NS	NS	.0026	NS	.0011	.0046
<b>LDL (1.063 g/mL)</b>								
Control	9.79 ± 2.08	26.37 ± 0.70	7.90 ± 0.45	25.72 ± 1.35	30.22 ± 2.03	0.36 ± 0.10	0.90 ± 0.06	0.58 ± 0.06
I.O.	13.66 ± 1.06	24.71 ± 1.28	6.77 ± 0.28	23.87 ± 0.87	30.99 ± 1.78	0.45 ± 0.05	0.83 ± 0.09	0.63 ± 0.04
P<	NS	NS	.0518	NS	NS	NS	NS	NS
<b>HDL (1.21 g/mL)</b>								
Control	0.22 ± 0.03	26.81 ± 2.26	3.47 ± 0.31	30.42 ± 1.02	39.07 ± 3.03	0.006 ± 0.001	0.76 ± 0.15	0.38 ± 0.05
I.O.	0.50 ± 0.07	34.69 ± 0.89	2.73 ± 0.32	29.20 ± 0.45	32.87 ± 0.38	0.015 ± 0.002	1.06 ± 0.04	0.55 ± 0.02
P<	.0018	.0071	NS	NS	.0651	.0016	NS	.0071

NOTE. Mean X ± SEM of total plasma lipoproteins for n = 6 in control and iron-overload (IO) rats.

Abbreviations: TC, triglyceride; FC, free cholesterol; CE, cholesteryl ester; PL, phospholipid; PR, protein; NS, not significant.

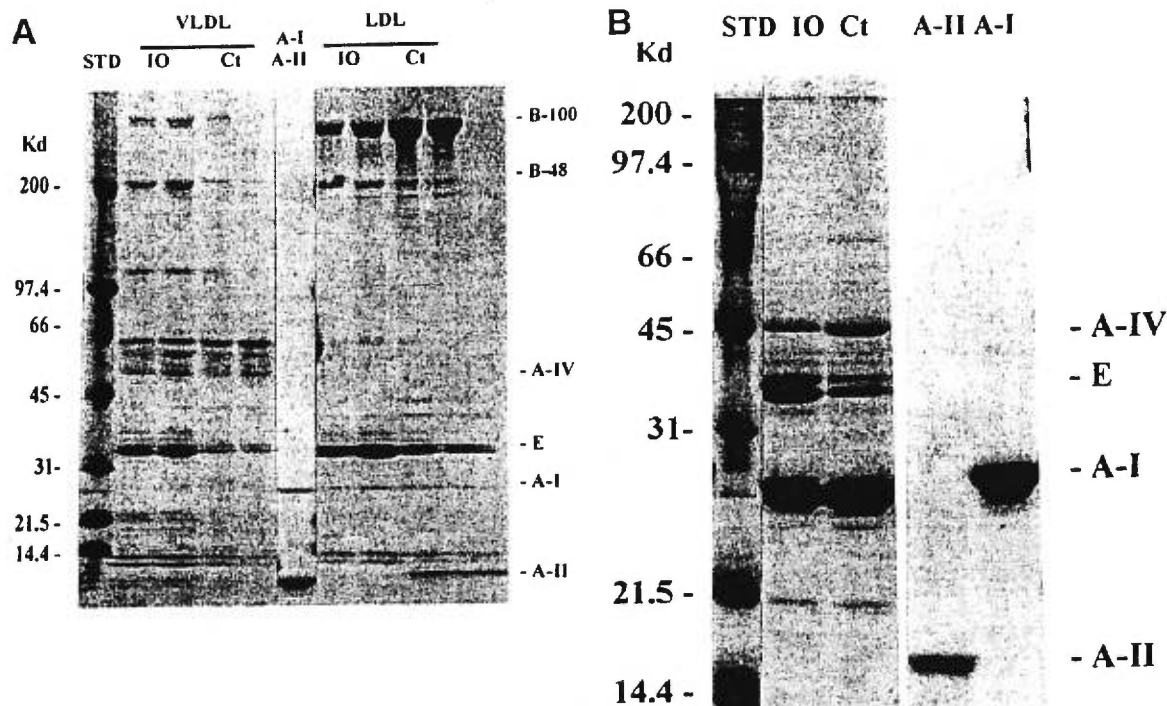


FIG. 3. SDS-PAGE showing the apolipoprotein composition of lipoproteins. VLDL and LDL (A) as well as HDL (B) were isolated from iron-treated (IO) and control (Ct) animals by ultracentrifugation, delipidated, and applied on gels. Apolipoproteins (A-I, A-II) and molecular weight standards (STD) were used as reference proteins: phosphorylase B (97,400), BSA (66,200), ovalbumin (46,000), carbonic anhydrase (30,000), Trypsin inhibitor (21,500), lysozyme (14,300).

iron overload and lipid peroxidation.<sup>6,7,10-15</sup> This association has been shown to take place under two conditions: the uptake of iron by the liver and a minimal threshold value of 2,000  $\mu\text{g}$  non-hem iron/gr liver.<sup>6</sup> In the current study, rats fed a 3% carbonyl iron-enriched diet displayed a hepatic iron level above 4,000  $\mu\text{g}/\text{g}$  liver. This iron deposition induces a 400% raise in liver MDA when compared with control animals. In view of the reported iron-induced peroxidative tissue injury, it was mandatory to ascertain the liver condition

before examining lipid metabolism. Using transaminases, albumin and bilirubin as sensitive indicators of the severity of liver injury, we noticed no substantial damage to or loss of hepatic function. Accordingly, excess iron present in chronically iron-loaded rats did not cause an apparent decrease in reduced glutathione, essential for the defense against oxygen and iron toxicity.<sup>35</sup> On the contrary, our parameters indicated that the endogenous antioxidant protective capacity was not only compromised, but also increased because of the appro-

TABLE 5. Lipid Content in Liver and Microsomes From Iron Overloaded Rats and Controls

Rats	Liver				Microsomal Fraction			
	TC	TC	FC	CE	TC	TC	FC	CE
	( $\mu\text{g}/\text{mg}$ Liver Proteins)				( $\mu\text{g}/\text{mg}$ Proteins)			
Control	49.48 $\pm$ 9.10	23.03 $\pm$ 1.62	18.90 $\pm$ 1.46	4.18 $\pm$ 0.24	40.90 $\pm$ 6.36	34.42 $\pm$ 4.59	24.99 $\pm$ 2.57	9.43 $\pm$ 3.72
Iron overload	26.40 $\pm$ 2.07*	21.81 $\pm$ 1.44	17.50 $\pm$ 1.06	4.31 $\pm$ 0.59	31.65 $\pm$ 3.55†	30.32 $\pm$ 2.63	20.48 $\pm$ 2.75	9.84 $\pm$ 1.54

NOTE. Lipids were extracted from liver homogenates and microsomal fractions and quantified by enzymatic techniques. Values are means  $\pm$  SEM for n = 10/group.

Abbreviations: TC, triglycerides; TC, total cholesterol; FC, free cholesterol; CE, cholesteryl ester.

\*P < .038.

†P < .047.

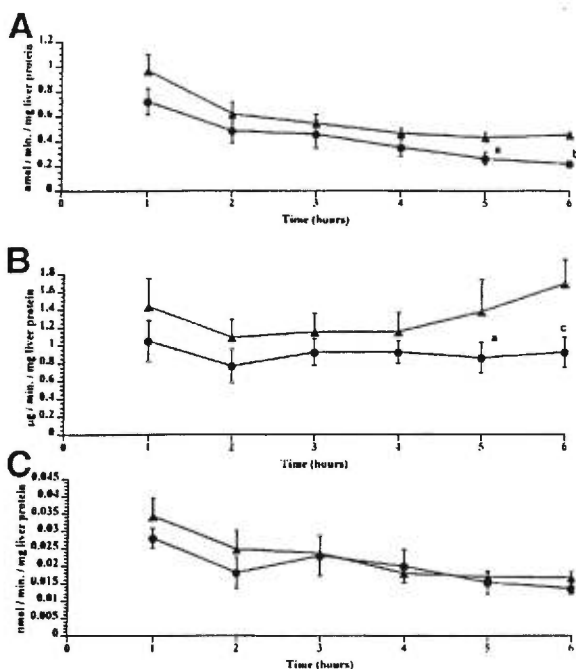


FIG. 4. Effect of iron overload on biliary lipids and bile acids following bile duct cannulation. (A) bile acid secretion rate, (B) cholesterol, and (C) phospholipids. Values are means  $\pm$  SEM for 6 rats/group of control ( $\blacktriangle$ - $\blacktriangle$ ) and iron-overloaded ( $\bullet$ - $\bullet$ ). <sup>a</sup> $P < .05$ , <sup>b</sup> $P < .003$ , <sup>c</sup> $P < .002$

appropriate liver response to oxidative stress, and was exceeded at the levels of iron loading achieved in this study. This enhanced glutathione production had already been reported by other groups who attributed it to a defense or adaptation mechanism.<sup>36,37</sup> Nevertheless, the latter could not prevent the occurrence of the abnormalities seen in lipid metabolism.

Unlike Antébi et al.,<sup>38</sup> our experiments document increased levels of lipids in the plasma and confirm the data of Dabbagh et al.<sup>39</sup> Normally, the magnitude of these components depends on liver production and peripheral metabolism. It is, therefore, possible that one of these processes is altered by iron overload. Although we cannot conclusively identify the pathway affected by iron overload, it is tempting to suggest that overall lipid profile modification is the result of liver

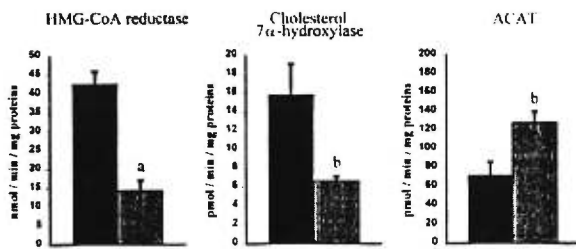


FIG. 5. Activity of liver HMG-CoA reductase, cholesterol 7 $\alpha$ -hydroxylase and ACAT. Rat liver microsomes were isolated and assayed for the three key regulatory enzymes. The data represent means  $\pm$  SEM of 5 rats/group. <sup>a</sup> $P < .004$ , <sup>b</sup> $P < .002$

TABLE 6. Membrane Fluidity in Microsomal Membranes

Rats	Fluidity (1/polarization)
Control	8.20 $\pm$ 0.03
Iron overload	5.00 $\pm$ 0.01*

NOTE. Membrane fluidity in microsomal membranes was determined on microsomes isolated from livers of control and iron overload rats. Membrane fluidity was assayed as described in Materials and Methods. Values are means  $\pm$  SEM for n = 5/group.

\* $P < .029$ .

hypersecretion and/or defective uptake preventing hepatic lipid accumulation as emphasized by our data.

Concurrent to iron-catalyzed peroxidation, functional anomalies were detected in the membranes of liver microsomes. The inspection of our data revealed that the activity of HMG-CoA reductase and cholesterol 7 $\alpha$ -hydroxylase was suppressed and that of ACAT was enhanced. Potential mechanisms involved in these changes may include the direct effects of free radicals on the physical properties of the membrane in which the three enzymes are embedded. The peroxidative reactions may result in an alteration in the unsaturation/saturation ratio of membrane phospholipid fatty acids, in the chain length distribution of fatty acids and, possibly, in the induction of covalent cross-links between adjacent lipid radicals in the membrane,<sup>40</sup> which, in turn, may influence membrane enzyme activity. In this context, our recent studies have underlined the importance of polyunsaturated fatty acids in the maintenance of these key enzymes.<sup>21</sup> Furthermore, data from other laboratories showed that *in vivo* chronic iron overload induces a modification of fatty acid profile in cellular structures consistent with the occurrence of lipid peroxidation.<sup>41</sup> Functional anomalies were concomitantly detected in the mitochondrial membranes, e.g., oxidative metabolism,<sup>14,42,43</sup> calcium transport,<sup>44</sup> and porphyrin pathway.<sup>45</sup> Similarly, adverse effects of iron-induced peroxidation were reported in microsomal function, including reduced concentrations of cytochromes P-450 and b5 as well as a decline in aminopyrine demethylase activity.<sup>15</sup> On the other hand, protection was observed with the supplementation of antioxidants or iron chelators.<sup>10</sup> As probably a consequence of membrane lipid changes, subsequent to peroxidation, our results showed decreased fluidity in microsomal membranes. There is growing evidence as to the impact of fluidity alterations on the activity of physiologically important membrane enzymes.<sup>46</sup>

Hepatic cholesterol secretion into plasma occurs principally through the output of free and cholesteryl esters contained in VLDL.<sup>47,48</sup> Nascent VLDL CE are derived from the intrahepatic esterification of free cholesterol catalyzed by the ACAT reaction. The rate of cholesteryl ester formation by

TABLE 7. Correlation Between Hepatic MDA and Various Parameters

	Plasma			Liver	
	R <sup>2</sup>	P		R <sup>2</sup>	P
TC	0.4691	0.0284	Iron	0.7690	.0009
FC	0.5822	0.0102	ACAT	0.3615	.066
CE	0.4276	0.0403	Cholesterol 7 $\alpha$ -hydroxylase	0.5305	.0169
TC	0.5343	0.0163	HMG-CoA reductase	0.7764	.0008

Abbreviations: TC, total cholesterol; FC, free cholesterol; CE, cholesteryl ester; TG, triglycerides.

ACAT has been shown as a determinant of the amount of VLDL cholesterol secreted.<sup>49</sup> The obvious stimulation of ACAT activity likely resulted in the secretion of VLDL enriched with cholesteryl esters, thus confirming the crucial contribution of intrahepatic cholesterol esterification by ACAT to VLDL CE content.<sup>49</sup>

Careful scrutiny of the biliary pool size following chronic dietary iron overload discloses the depletion in cholesterol and bile acid output. The decreased activity of cholesterol 7 $\alpha$ -hydroxylase may be behind the reduction in rat biliary secretion. Because bile acids represent the major product of cholesterol catabolism, diminishing their yield can also lead to the accumulation of plasma cholesterol. Support for this relationship between the activity of cholesterol 7 $\alpha$ -hydroxylase, bile acid secretion and plasma cholesterol concentrations comes from other metabolic states. Increasing hepatic cholesterol 7 $\alpha$ -hydroxylase and bile acid synthesis in hypercholesterolemic rabbits reduces plasma cholesterol concentrations.<sup>50</sup> In contrast, decreased cholesterol 7 $\alpha$ -hydroxylase activity and bile acid synthesis were associated with increased plasma cholesterol concentrations in human<sup>51,52</sup> and animals.<sup>53-56</sup> Therefore, iron-induced peroxidation may have repercussions on cholesterolemia by altering cholesterol 7 $\alpha$ -hydroxylase, which regulates the biogenesis of bile acids, the major product of cholesterol catabolism.

Iron-loading by carbonyl iron in the rat has advantages over various iron-overload models. The dietary supplementation with carbonyl iron in the rat was found to be efficient, because only 3 months of oral-loading resulted in hepatic iron concentrations comparable to iron levels in genetic hemochromatosis.<sup>35,57</sup> Furthermore, several studies documented that iron deposition was limited almost entirely to hepatocytes<sup>13,14,36,41,57</sup> as occurs in idiopathic hemochromatosis.<sup>5</sup> Using this experimental model, Bacon et al were able to achieve gradually increasing concentrations of liver parenchymal storage iron,<sup>15</sup> similarly to genetic hemochromatosis. Besides, other iron overload methods using parenteral injections of iron chelates led to iron deposition in reticuloendothelial cells.<sup>6,58</sup> Finally, even with prolonged iron loading, overt hepatic fibrosis was minimal in the livers of rats fed carbonyl iron,<sup>59</sup> a situation permitting to examine liver lipid metabolism without confounding hepatocellular damage, fibrosis, or additional interferences that clouded data interpretation.

Animal diets containing high levels of sucrose, such as AIN-76, could lead to liver steatosis. Indeed, hepatic abnormalities are usually observed when animals are allowed semi-purified diets *ad libitum* for very long periods.<sup>60</sup> However, it is also established that rats receiving the iron-supplemented diet reduce their total dietary intake.<sup>6,7</sup> For this reason, control rats in our experiments were fed in a restricted manner such that their rate of growth paralleled the iron-overloaded group. Apparently, this dietary restriction prevented both groups of animals from presenting morphological abnormalities in their liver as a consequence of the semi-purified diet administration. Besides the pair-feeding, we were very cautious and carried out additional light and electron microscopy examinations of liver tissues. Our data did not reveal any morphological or histological abnormalities (Fig. 1). Finally, the data included in Table 5 did not disclose increased values of control lipids (indicative of liver steatosis) confirming the observations reported by numerous studies. In this study, feeding rats diets supplemented with

carbonyl iron resulted in abnormal lipid profile and lipoprotein composition. These findings are consistent with the view that iron overload can lead to atherosclerosis and worsen high-cholesterol-related coronary artery disease.<sup>61</sup> Furthermore, significant disturbed sterol enzyme activities were seen in our animal model concomitant with defective biliary output of cholesterol and bile acids. This may contribute to the well-documented manifestations of chronic liver disease observed in patients with iron overload. The key question of whether lipid peroxidation is the major cause of these defects needs further exploration.

In summary, marked perturbations in plasma lipid transport and hepatobiliary sterol metabolism occur in dietary carbonyl iron overload. Although substantial evidence links excess circulating and hepatic iron to lipid peroxidation, the role of the latter in the reported manifestations should be delineated. Additional experimental studies employing antioxidants, such as vitamin E, may provide further insights into this issue.

#### REFERENCES

- Powell LW, Bassett ML, Halliday JW. Hemochromatosis: 1980 update. *Gastroenterology* 1980;78:374-381.
- Bassett ML, Halliday JW, Powell LW. Genetic hemochromatosis. *Semin Liver Dis* 1984;4:217-227.
- Gordeuk VR, McLaren GD, Samowitz W. Etiologies, consequences and treatment of iron overload. *Critical Reviews in Clinical Laboratory Sciences* 1994;31:89-133.
- Friedman SL. The cellular basis of hepatic fibrosis. *N Engl J Med* 1993;328:1828-1835.
- McLaren GD, Muir WA, Kiellermeier RW. Iron overload disorders: natural history, pathogenesis, diagnosis and therapy. *CRC Crit Rev Clin Lab Sci* 1983;19:205-266.
- Bacon BR, Tavill AS, Brittenham GM, Park CH, Recknagel RO. Hepatic lipid peroxidation *in vivo* in rats with chronic iron overload. *J Clin Invest* 1983;71:429-439.
- Bacon BR, Britton RS. The pathology of hepatic iron overload: A free radical-mediated process? *HEPATOLOGY* 1990;11:127-137.
- Freeman BA, Crapo JD. Biology of disease: free radical and tissue injury. *Lab Invest* 1982;47:412-426.
- Tien M, Svingen BA, Aust SD. Superoxide dependent lipid peroxidation. *Fed Proc* 1981;40:179-182.
- Bacon BR, O'Neill R, Park CH. Iron-induced peroxidative injury to isolated rat hepatic mitochondria. *J Free Rad Biol Med* 1986;2:339-347.
- Hogberg J, Bergstrand A, Jacobsson SV. Lipid peroxidation of rat-liver microsomes. Its effect on the microsomal membrane and some membrane-bound microsomal enzymes. *Eur J Biochem* 1973;37:51-59.
- Myers BM, Prendergast FC, Holman R, Kuntz SM, LaRusso NF. Alterations in the structure, physicochemical properties, and pH of hepatocyte lysosomes in experimental iron overload. *J Clin Invest* 1991;88:1207-1215.
- Park CH, Bacon BR, Brittenham GM, Tavill AS. Pathology of diet carbonyl iron overload in rats. *Lab Invest* 1987;57:555-563.
- Bacon BR, Park CH, Brittenham GM, O'Neill R, Tavill AS. Hepatic mitochondrial oxidative metabolism in rats with chronic dietary iron overload. *HEPATOLOGY* 1985;5:789-797.
- Bacon BR, Healey JF, Brittenham GM, Park CH, Nunnari J, Tavill AS, Bonkovski HL. Hepatic microsomal function in rats with chronic dietary iron overload. *Gastroenterology* 1986;90:1844-1853.
- Turley SD, Dietschy JM. The metabolism and excretion of cholesterol by the liver. In: Arias IM, Jacoby H, Popper d, Schachter D, Schafritz DA, eds. *The liver: biology and pathology*. New York: Raven, 1988;617-641.
- Packard CJ, Shepherd J. The hepatobiliary axis and lipoprotein metabolism: effects of bile acid sequestrants and ileal bypass surgery. *J Lipid Res* 1982;23:1081-1099.
- Wanon J, Guertin F, Gavino V, Delvin E, Bouthillier D, Lairon D, Levy E. The effects of cholesterol uptake from high-density lipoprotein subfractions on biliary sterol secretion in essential fatty acid deficiency. *HEPATOLOGY* 1998;27:779-786.
- Havel RJ, Hamilton RL. Hepatocytic lipoprotein receptors and intracellular lipoprotein catabolism. *HEPATOLOGY* 1988;8:1689-1704.




20. Cooper AD. Role of the liver in the degradation of lipoproteins. *Gastroenterology* 1985;88:192-205.
21. Levy E, Garofalo C, Rouleau T, Gavino V, Bendayan M. Impact of essential fatty acid deficiency on hepatic sterol metabolism in rats. *HEPATOLOGY* 1996;23:848-857.
22. Beg ZH, Stonik JA, Brewer HB Jr. Modulation of the enzymic activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase by multiple kinase systems involving reversible phosphorylation: a review. *Metabolism* 1987;36:900-917.
23. Levy E, Thibault L, Garofalo C, Messier M, Lepage G, Ronco N, Roy CC. Combined (n-3 and n-6) essential fatty acid deficiency is a potent modulator of plasma lipids, lipoprotein composition, and lipolytic enzymes. *J Lipid Res* 1990;31:2009-2017.
24. Levy E, Lepage G, Bendayan M, Ronco N, Thibault L, Galéano N, Smith L, et al. Relationship of decreased hepatic lipase activity and lipoprotein abnormalities to essential fatty acid deficiency in cystic fibrosis patients. *J Lipid Res* 1989;30:1197-1209.
25. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-275.
26. Bartlett GR. Phosphorus assay in column chromatography. *J Biol Chem* 1959;234:466-468.
27. Levy E, Garofalo C, Daoust L, Dionne S, Roy CC. Intraluminal and intracellular phases of fat absorption are impaired in essential fatty acid deficiency. *Am J Physiol* 1992;262:G319-G326.
28. Chirico S. High performance liquid chromatography-based thiobarbituric acid tests. *Methods in Enzymology* 1994;223:314-318.
29. Lepage G, Munoz G, Champagne J, Roy CC. Preparative steps necessary for the accurate measurement of malondialdehyde by high performance liquid chromatography. *Anal Biochem* 1991;197:277-283.
30. Tietze F. Enzymatic method for quantitative determination of monogram amount of total and oxidized glutathione. *Analyt Biochem* 1968;27:502-522.
31. Torrance JD, Bothwell TH. Tissue iron stores. In: Cook J, ed. *Iron*. New York: Churchill-Livingstone, 1980:90-115.
32. Kapitulnik J, Tshershedsky M, Barenholz Y. Fluidity of the rat liver microsomal membrane: increase at birth. *Science (Wash, DC)* 1979;206:843-844.
33. Brown MS, Goldstein JL, Dietschy JM. Active and inactive forms of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver of the rat. *J Biol Chem* 1979;254:5144-5149.
34. Hylemon PB, Studer ES, Pandak WM, Heuman DM, Vlahcevic ZR, Chiang JYL. Simultaneous measurement of cholesterol 7 $\alpha$ -hydroxylase activity by reverse-phase high-performance liquid chromatography using both endogenous and exogenous [4-<sup>14</sup>C] cholesterol as substrate. *Anal Biochem* 1989;182:212-216.
35. Kosower EM. Chemical properties of glutathione. In: Arias IM, Jacoby WB, eds. *Glutathione: Metabolism and Function*. New York: Raven Press, 1976:1-15.
36. Brown KE, Kinter MT, Oberley TD, Freeman ML, Frierson HF, Ridmour LA, Tao Y, et al. Enhanced  $\gamma$ -glutamyl transpeptidase expression and selective loss of CuZn superoxide dismutase in hepatic iron overload. *Free Radic Biol Med* 1998;24:545-555.
37. Adams JD, Bernhard JR, Lauerburg H, Mitchell JR. Plasma glutathione and glutathione disulfide in the rat: Regulation and response to oxidative stress. *J Pharmacol Exp Ther* 1983;227:749-754.
38. Antébi H, Pagès N, Zimmermann L, Bourcier C, Flechet B, Alcindor LG. Resistance to oxidation of native lipoproteins and erythrocyte membrane lipids in rats with iron overload. *Ann Nutr Metab* 1995;39:63-68.
39. Dabbagh AJ, Mannion T, Lynch SM, Frei B. The effect of iron overload on rat plasma and liver oxidant status in vivo. *Biochem J* 1994;300:799-803.
40. Curtis MT, Gilfor D, Farber J. Lipid peroxidation increases the molecular order of microsomal membranes. *Arch Biochem Biophys* 1984;235:644-649.
41. Pietrangelo A, Grandi R, Tripodi A, Tomasi A, Ceccarelli D, Ventura E, Masini A. Lipid composition and fluidity of liver mitochondria, microsomes and plasma membrane of rats with chronic dietary iron overload. *Biochim Pharmacol* 1990;39:123-128.
42. Masini A, Ceccarelli-Stanzani D, Trenti T, Ventura E. Transmembrane potential of liver mitochondrial from hexachlorobenzene and iron treated rats. *Biochim Biophys Acta* 1984;802:253-258.
43. Masini A, Trenti T, Ventura E, Ceccarelli-Stanzani D, Muscatello U. Functional efficacy of mitochondrial membrane of rats with hepatic chronic iron overload. *Biochem Biophys Res Commun* 1984;24:462-469.
44. Masini A, Trenti T, Ceccarelli-Stanzani D, Muscatello U. Mitochondrial involvement in causing cell injury in experimental hepatic iron overload. *Ann NY Acad Sci* 1986;88:517-519.
45. Masini A, Trenti T, Ceccarelli D, Muscatello U. The effect of iron overload on the mitochondrial porphyrin level in the hexachlorobenzene induced experimental porphyria. *Biochem Biophys Res Commun* 1988;151:320-326.
46. Schachter D. Fluidity and function of hepatocyte plasma membranes. *HEPATOLOGY* 1984;4:140-151.
47. Hamilton RL, Williams MC, Fielding CJ, Havel RJ. Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver. *J Clin Invest* 1976;58:667-680.
48. Cooper AD. Hepatic lipoprotein and cholesterol metabolism. In: Zakim D, Boyer TD, eds. *Hepatology: A textbook of liver disease*. Tokyo, WB Saunders Company, 1990:96-124.
49. Drevon CA, Engelhorn SC, Steinberg D. Secretion of very low density lipoproteins enriched in cholesteryl esters by cultured rat hepatocytes during stimulation of intracellular cholesterol esterification. *J Lipid Res* 1980;21:1065-1071.
50. Xu G, Salen G, Shefer S, Ness GC, Nguyen LB, Tint S, Parker TS, et al. Increasing hepatic cholesterol 7 $\alpha$ -hydroxylase reduces plasma cholesterol concentrations in normocholesterolemic and hypercholesterolemic rabbits. *HEPATOLOGY* 1996;24:882-887.
51. Salen G, Colalilo A, Verga D, Bagan E, Tint CS, Shefer S. The effect of high and low doses of ursodeoxycholic acid on gallstone dissolution in humans. *Gastroenterology* 1980;78:1412-1418.
52. Hellström K, Einarsson K. Bile acid metabolism in hyperlipoproteinemia. *Clin Gastroenterol* 1977;6:103-128.
53. Xu G, Salen G, Shefer S, Ness GC, Nguyen LB, Parker TS, Chen TS, et al. Unexpected inhibition of cholesterol 7 $\alpha$ -hydroxylase by cholesterol in New Zealand white and Watanabe heritable hyperlipidemic rabbits. *J Clin Invest* 1995;95:1497-1504.
54. Rudel L, Decheiman C, Wilson M, Scobey M, Anderson R. Dietary cholesterol and downregulation of cholesterol 7 $\alpha$ -hydroxylase and cholesterol absorption in African green monkeys. *J Clin Invest* 1994;93:2463-2472.
55. Horton JD, Cuthbert JA, Spady DK. Regulation of hepatic 7 $\alpha$ -hydroxylase expression and response to dietary cholesterol in the rat and hamster. *J Biol Chem* 1995;270:5381-5387.
56. Pandak WM, Doerner M, Heuman DM, Hylemon PB, Chiang JYL, Vlahcevic ZR. Expression of cholesterol 7 $\alpha$ -hydroxylase in response to cholesterol and bile acid feeding in the hamster and rat. *Gastroenterology* 1995;108:A1141.
57. LeSage GD, Kost LJ, Barham SS, LaRusso NF. Biliary excretion of iron from hepatocyte lysosomes in the rat. *J Clin Invest* 1986;77:90-97.
58. Pechet GS. Parenteral iron overload. Organ and cell distribution in rats. *Lab Invest* 1969;20:119-126.
59. Brown KE, Puolos JE, Li L, Soweid AM, Ramn GA, O'Neill R, Britton RS, Bacon BR. The effect of vitamin E supplementation on hepatic fibrogenesis in chronic dietary iron overload. *Am J Physiol* 1997;272:G116-G123.
60. Bacon BR, Park CH, Fowell EM, McLaren CE. Hepatic steatosis in rats fed diets with varying concentrations of sucrose. *Fundam Appl Toxicol* 1984;4:819-826.
61. Salonen JT, Nyyssonen K, Korpola H, Taomilehto J, Seppanen R, Salonen R. High stored iron levels are associated with excess risk of myocardial infarction in Eastern Finnish men. *Circulation* 1992;86:803-811.

**LIPOPROTEIN METABOLISM ALTERATIONS  
IN IRON-LOADED HepG2 CELLS**

**Sylvain Brunet, Isabelle Suc, Nadia Dubé, Louise Thibault, Emile Levy**

Centre de Recherche, Hôpital Sainte-Justine  
and Département de Nutrition, Université de Montréal  
Montréal, Québec, Canada

**Address for correspondence:** Dr. Emile Levy  
GI-Nutrition Unit  
Research Center  
Hôpital Sainte-Justine  
3175 Côte Ste-Catherine  
Montreal, Quebec H3T 1C5  
Tel.: (514) 345-4626  
Fax: (514) 345-4999



**ABSTRACT**

Iron-catalyzed free radical-mediated lipid peroxidation elicited marked in vivo perturbations in plasma lipid transport (Hepatology 29:1809-1817, 1999). Since the liver plays a central role in cholesterol homeostasis, experiments were carried out in HepG2 cells in order to evaluate its implication in cholesterol synthesis, lipoprotein secretion and LDL-receptor activity. The incubation of HepG2 cells with iron (10 µg/ml) produces a substantial increase in iron (71%,  $p < 0.05$ ), malondialdehyde (86%,  $p < 0.01$ ) and the oxidation of the fluorescent probe DCFH-DA (60%, 0.005) content. Concomitantly, iron loading enhanced [<sup>14</sup>C]-acetate incorporation into free and esterified cholesterol ( $p < 0.01$ ). In addition, iron treatment promoted the secretion of VLDL (76%,  $p < 0.005$ ) and LDL (41%,  $p < 0.05$ ) and inhibited LDL binding/internalization (16%,  $p < 0.05$ ), while it did not alter LDL receptor number. Conversely, the presence of iron decreased plasma membrane fluidity ( $p < 0.005$ ), a potential mechanism by which lipid peroxidation may affect LDL receptor function. These data might be relevant in understanding the association between iron-induced peroxidation and atherosclerosis.

## **INTRODUCTION**

It is now well established that iron plays an extensive role in lipid peroxidation. Iron-catalyzed generation of lipid peroxidation displays a wide variety of damaging actions (1,2). An increasing body of literature emphasizes its implication in the pathogenesis of numerous disorders, including coronary artery disease (3), ischemia reperfusion injury (4,5) and hepatocellular disorders secondary to hemochromatosis (6). By virtue of its pro-oxidant properties, iron promotes early atherogenesis primarily by acting as a catalyst for the atherogenic modification of LDL (7) and by modulating the reactivity of blood cell components, in particular platelets (8). Nevertheless, knowledge of the role of iron-induced lipid peroxidation in liver cholesterol homeostasis and circulating lipoprotein metabolism is still limited. Recently, we have shown that rats, chronically administered a diet enriched with carbonyl iron, developed hyperlipidemia accompanied with marked perturbations in plasma lipid transport (9). These *in vivo* studies, however, did not allow us to dissect the direct contribution of hepatocytes, the major cellular site of iron storage (10), to the various lipid abnormalities reported. Towards this end, human hepatoma cell (HepG2) cultures were submitted to iron treatment for 7 days in order to mimic chronic iron loading. Then, the effects of iron were assessed on cholesterol synthesis and esterification, LDL binding/internalization and lipoprotein.

## **MATERIAL AND METHODS**

**Cell culture:** HepG 2 cells were grown as a monolayer in culture with 10% fetal calf serum (FCS) MEM (gibco BRL) and PEN STREP as described previously (11). Experimental cells were incubated with 10 ug / ml of iron in the form of FeSO<sub>4</sub>.7H<sub>2</sub>O (sigma) for 7 days after. All the experiments were carried out at the eighth day. Media were collected, centrifuged (2500 rpm, 4°C, 10 min.) to discard cell debris and cells were harvested following trypsinisation and centrifugation.

**Plasma lipid and lipoprotein analysis:** HepG2 cells were incubated with [<sup>14</sup>C]-acetate in order to evaluate lipoprotein secretion. Very low-(VLDL, 1.006 g/ml), low-(LDL, 1.063 g/ml) and high-(HDL, 1.21 g/ml) density lipoproteins were isolated from fresh medium by conventional discontinuous density gradient ultracentrifugation as described previously (12). Each fraction was dialysed intensively against Phosphate Buffered Saline (PBS) with 0.001 M EDTA, pH 7.0 at 4°C, and radioactivity was measured by scintillation counting. Aliquots of lipoproteins were lipid-extracted with chloroform-methanol (2:1, v/v) and their lipid classes were separated by TLC, using a solvent mixture of hexane, diethyl ether, and acetic acid (80:20:3, vol/vol/vol) (13).

**LDL receptor assay by Dil-LDL uptake:** Blood was obtained from healthy volunteers and LDL were isolated as aforementioned. Labelling of LDL with fluorescent probe DiI (Molecular Probe, Eugene, OR) was carried out according to Verhoeve et al (14). Labeled LDL was then added at a final concentrations of 50 ug/ml and to the incubation medium and cells were maintained at either 4° C or 37° C for 2 hours. After incubation with DIL-LDL, cells were washed and resuspended in PBS for flow cytometric analysis (15). Indirect immunofluorescence staining was used to determine surface LDL receptor enumeration (16).

**Other determinations:** Iron and malondialdehyde were determined by chromogen reagent and high-performance liquid chromatography, respectively (9). *De novo* cholesterol synthesis was evaluated by following [ $^{14}\text{C}$ ]-acetate incorporation into cellular lipids (17). Fluidity of cell membranes was estimated by the incorporation of a fluorescent probe (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluene-sulfonate) (TMA-DPH) and measurement of polarization (18). Viability of hepatocytes was assessed by trypan blue exclusion. Peroxide levels within HepG2 cells were also determined using the oxidation of the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) according to Ikeda et al. (19).

## **RESULTS**

**Cellular iron content and lipid peroxidation:** The concentration of iron added to the culture medium was chosen because it did not cause any harmful effects to HepG2 cell morphology or viability (results not shown). After 7 d incubation, the intracellular content was found to be over 71% higher as compared to untreated HepG2 cell controls (Fig. 1). In the same cultures, an increase in the cellular MDA (86%) was noted. Similarly, HepG2 cells containing iron displayed a significant ( $p < 0.0005$ ) enhancement (60%) in cellular DCFH. These data evidenced the lipid peroxidation induced in HepG2 cells by the inclusion of iron in the culture medium.

**Lipid secretion:** HepG2 cells were incubated with [ $^{14}\text{C}$ ]-acetate in order to examine *de novo* lipid synthesis and secretion. Iron loading resulted in a marked incorporation of [ $^{14}\text{C}$ ]-acetate into newly synthesized lipids (Fig. 2). In fact, iron supplementation increased triglyceride by 48% ( $p < 0.01$ ) and total cholesterol by 47% ( $p < 0.01$ ). Even if the levels of both cholesterol fractions was higher in iron-loaded hepatocytes than in controls, cholesteryl ester was more increased (66%) than free cholesterol (43%).

**Lipoprotein production and composition:** The levels of lipoproteins and their content were also assessed using [ $^{14}\text{C}$ ]-acetate. From the data presented in Table 1, it seems evident that iron-loaded HepG2 cells secrete more VLDL (76%,  $p < 0.005$ ) and LDL (41%,  $p < 0.05$ ) particles than control cells. On the other hand, HDL fraction was unaffected by iron supplementation. From the data presented in Table 1, it was also possible to note profound disorders in lipoprotein-lipid composition of iron-loaded HepG2. All the lipoprotein fractions isolated by ultracentrifugation (VLDL, LDL and HDL) displayed a higher proportion of cholesteryl ester and a lower percentage of free cholesterol. In addition, the relative TG content in VLDL was higher with iron supplementation.

**LDL receptor activity and number:** LDL receptor plays a key role in cholesterol homeostasis in the liver. In order to determine whether it is affected by iron-induced peroxidation, LDL receptor activity and number were assessed in HepG2 cells. It is possible to note in Fig. 3 that iron supplementation reduced LDL receptor activity without altering the number of LDL receptors.

**Membrane fluidity:** Since iron may affect LDL receptor activity through lipid peroxidation, which alters membrane physical properties, it was important to determine membrane fluidity in our studies. Table 2 depicts the significant ( $p < 0.005$ ) decrease in membrane fluidity of iron-loaded hepatocytes.



## **DISCUSSION**

With the view to understand the suggested involvement of peroxidation in cholesterol homeostasis, the effects of iron were tested in Hep G2 cells, the most commonly used cell line for examining the regulation of cholesterol metabolism. This cellular model was chosen since previous reports showed that 1) the addition of iron to Hep G2 cells resulted in a high rate of iron transport coupled with considerable deposition in microsomal and cytosolic compartments (20); 2) HepG2 cells are known to synthesize apolipoproteins and lipoproteins (21); and 3) these hepatocytes express several lipoproteins receptor (21). Similarly, iron-catalyzed peroxidation has been employed in our investigation given its proved advantages over various models of peroxidation. Our findings indicated that increased lipoprotein secretion and decreased LDL receptor activity occurred in the presence of iron-catalysed peroxidation. These changes were associated in the one hand with the evidence of increased lipid MDA and DCFH-DA as well as high cellular iron content, and in the other hand with membrane fluidity modification.

LDL receptor, as an integral part of cellular cholesterol homeostasis, is a freely mobile transmembrane glycoprotein that could potentially be influenced by alterations in the physico-chemical properties of the surrounding plasma membrane. Previous studies demonstrated an increase in LDL binding, uptake and degradation in Hep G2 cells, when plasma membrane was enriched with unsaturated fatty acids (11). An elevation in plasma membrane fluidity was invoked as an explanation for these observations (11). Our data provide additional argument in favor of the regulation of LDL receptor function by changes in membrane lipid composition. The investigation of the capacity of iron-catalysed peroxidation to modify LDL metabolism revealed defective LDL binding/internalization in association with diminished membrane fluidity. The mechanisms involved could be related to the peroxidation of polyunsaturated fatty acids in proximity of LDL receptors. Accordingly, LDL receptor number was not modified in response to iron-catalysed peroxidation, which

confirmed local alterations in membrane microdomain resulting in abnormal LDL receptor conformation.

The highly reactive oxygen species generated by iron, combined with lipoperoxidative influence on membrane lipids, has been shown to cause functional abnormalities in hepatic subcellular organelles (22-24). The effects of iron-induced lipid peroxidation include microsomal decrease in cytochrome P-450 drug metabolizing activities (25), glucose-6-phosphatase (22) and ATPase (8). We have previously showed that even microsomal enzymes, assuring cholesterol balance, were strongly affected by iron-catalyzed free radical-mediated lipid peroxidation (9). From our experiments, it seems that an association exists between iron-induced peroxidation and enhanced lipoprotein secretion. This could account for by the perturbations of lipoprotein synthesis system located on the luminal surface of the endoplasmic reticulum.

In summary, we have documented evidence that lipoprotein production and LDL uptake are impaired by iron-induced peroxidation. Therefore, liver hypersecretion and/or defective uptake of lipoproteins may explain the overall lipid modification observed under iron overload peroxidation condition. Our findings are in agreement with other reports showing an elevated risk of acute myocardial infarction in men with chronic hepatic iron overload (1). Additional work is necessary to evaluate the contribution of the aforementioned processes to the cardiovascular manifestations noted in patients with iron overload.

#### **ACKNOWLEDGMENTS**

This work was supported by grants from the Medical Research Council of Canada and the Canadian Foundation for Crohn's and Colitis. We thank Danielle St-Cyr for the skillful preparation of the manuscript.

## **REFERENCES**

1. Britton, R.S., Bacon, B.R., and Recknagel, R.O. (1987) *Chem Phys Lipids* **45**, 207-239.
2. Bacon, B.R., O'Neill, R., and Park, C.H. (1986) *J Free Rad Biol Med* **2**, 339-347.
3. de Valk, B., and Marx, J.J. (1999) *Arch Intern Med* **159**, 1541-1548.
4. Aust, S.D., and White, B.C. (1985) *Adv Free Radicals Biol Med* **1**, 1-7.
5. Katoh, S., Toyama, J., Kodama, I., Akita, T., and Abe, T. (1992) *J Mol Cell Cardiol* **24**, 1267-1275.
6. Bacon, B.R., and Britton, R.S. (1990) *Hepatology* **11**, 131-137.
7. van Jaarsveld, H., Pool, G.F., and Barnard, H.C. (1998) *Res Commun Mol Pathol Pharmacol* **99**, 69-80.
8. Pratico, D., Pasin, M., Barry, O.P., Ghiselli, A., Sabatino, G., Iuliano, L., FitzGerald, G.A., and Violi, F. (1999) *Circulation* **99**, 3118-3124.
9. Brunet, S., Thibault, L., Delvin, E., Yotov, W., Bendayan, M., and Levy, E. (1999) *Hepatology* **29**, 1809-1817.
10. Tribble, D.L., Aw, T.Y., and Jones, D.P. (1987) *Hepatology* **7**, 377-387.
11. Kuo, P., Weinfeld, M., and Loscalzo, J. (1990) *Biochemistry* **29**, 6626-6632.
12. Levy, E., Thibault, L., Garofalo, C., Messier, M., Lepage, G., Ronco, N., and Roy, C.C. (1990) *J Lipid Res* **31**, 2009-2177.
13. Spalinger, J.H., Seidman, E.G., Lepage, G., Ménard, D., Gavino, V., and Levy, E. (1998) *Am J Physiol* **275**, G652-G659.

14. Verhoeve, F.R., Descamps, O., Husson, B., Hondekijn, J.C., Ronveaux-Dupal, M.F., Lontie, J.F., and Heller, F.R. (1996) *J Lipid Res* **37**, 1377-1844.
15. Ranganathan, S., Hattori, H., and Kashyap, M.L. (1995) *J Lab Clin Med* **125**, 479-486.
16. Raungaard, B., Heath, F., Brorholt-Petersen, J.U., Jensen, H.K., and Faergeman, O. (1998) *Clin Chem* **44**, 966-972.
17. Loirdighi, N., Ménard, D., Delvin, E., and Levy, E. (1997) *J Cell Biochem* **66**, 65-76.
18. Kapitulnik, J., Tshershedsky, M., and Barenholz, Y. (1979) *Science* **206**, 843-844.
19. Ikeda, M., Kodama, H., Fukuda, J., Shimizu, Y., Murata, M., Kumagai, J., and Tanaka, T. (1999) *Biol Reprod* **61**, 393-399.
20. Hann, H.-W.L., Stahlhut, M.W, and Hann, C.L. (1990) *Hepatology* **11**, 566-569.
21. Brisette L, Charest MC, Falstraalt. *Biochem J* 1996;318:841-847.
22. Wills, E.D. (1971) *Biochem J* **123**, 983-991.
23. Kanazawa, K., Ashida, H., Inoue, N., and Natake, M. (1989) *J Nutr Sci Vitaminol* **34**, 25-27.
24. LeSage, G.D., Kost, L.J., Barham, S.S., (1986) LaRusso, N.F. *J Clin Invest* **77**, 90-97.
25. Cheeseman, K.H., Milia, A., Chiarpotto, E., Biasi, F., Albano, E., Diangoni, M.U., Stater, T.J. (1984) *IRCS Med Sci* **12**, 61-62.

**LEGENDS**

**Figure 1:** Cellular iron uptake and lipid peroxidation in HepG2 cells. Human hepatoma cells were cultured with iron (10 µg/ml). Following the incubation period, cells were washed and prepared for the determination of iron content and lipid peroxidation assessed by the measurement of malondialdehyde (MDA) and the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH). Data of DCFH are expressed as mean channel representing the intensity of fluorescence. Results are mean ± SEM of 4 experiments.

\* p<0.05

\*\* p<0.01

\*\*\* p<0.005

**Figure 2:** Effect of iron on *de novo* lipid synthesis by HepG2 cells. Human hepatoma cell cultures were submitted to iron-induced lipid peroxidation in the presence of [<sup>14</sup>C]-acetate. Lipids were extracted from culture media, and measured as reported in Methods. Data are mean ± SEM of 4 experiments.

\* p<0.05

\*\* p<0.01

**Figure 3:** LDL binding and internalization for determination of receptor activity (A) and LDL receptor number (B) in HepG2 cells. HepG2 were cultured with iron as in Figure 1. Binding and uptake of LDL were assayed by incubating cells for 2h with Dil-LDL at 37°C. Non specific binding was determined by adding 50-fold excess unlabeled ligand. Receptor activity was expressed in arbitrary units.

\* p<0.05

**Table 1: Distribution of lipid constituents in secreted lipoprotein particles from HepG2 cells exposed to iron-induced lipid peroxidation**

Lipoprotein	TG	CE	FC	PL	Total dpm/mg protein
	% distribution				
<b>VLDL</b>					
Control	24.33±1.43	3.42±.14	45.6±1.51	26.65±.54	41,777±3,324
Iron overload	32.59±0.96 <sup>b</sup>	4.59±0.8 <sup>c</sup>	37.99±.24 <sup>a</sup>	24.83±.78	73,570±7,456 <sup>c</sup>
<b>LDL</b>					
Control	52.08±0.5	6.91±.34	18.04±.27	22.97±.21	291,437±15,763
Iron overload	53.43±.54	8.33±.38 <sup>a</sup>	16.55±.39 <sup>a</sup>	21.69±.72	412,288±43,392 <sup>a</sup>
<b>HDL</b>					
Control	21.25±1.55	8.77±.52	11.48±.95	58.5±2.21	99,704±5,226
Iron overload	21.37±1.01	10.99±.9 <sup>a</sup>	15.1±1.06 <sup>a</sup>	52.55±2.75	92,062±19,078

HepG2 cells were submitted to iron-induced lipid peroxidation in the presence of [<sup>14</sup>C]-oleate. At the end of the incubation period, media were collected and ultracentrifuged in order to isolate VLDL (1.006 g/ml), LDL (1.063 g/ml) and HDL (1.21 g/ml). Lipoprotein constituents were separated by TLC and counted. Data are mean ± SEM of 4 experiments. They are expressed as percentage of total lipids. <sup>a</sup>p<0.05; <sup>b</sup>p<0.01; <sup>c</sup>p<0.005.

TG, triglyceride; CE, cholesteryl ester; FC, free cholesterol; PL, phospholipid.

**Table 2: Membrane fluidity of HepG2 cells exposed to iron-induced lipid peroxidation.**

<b>HepG2</b>	<b>Fluidity (1/polarization)</b>
Controls	5.560±0.118
Iron-treated	5.209±0.041*

Fluidity in plasma membrane was assessed as described in Methods.

Values are means ± SEM for 4 experiments.

\* p<0.05.

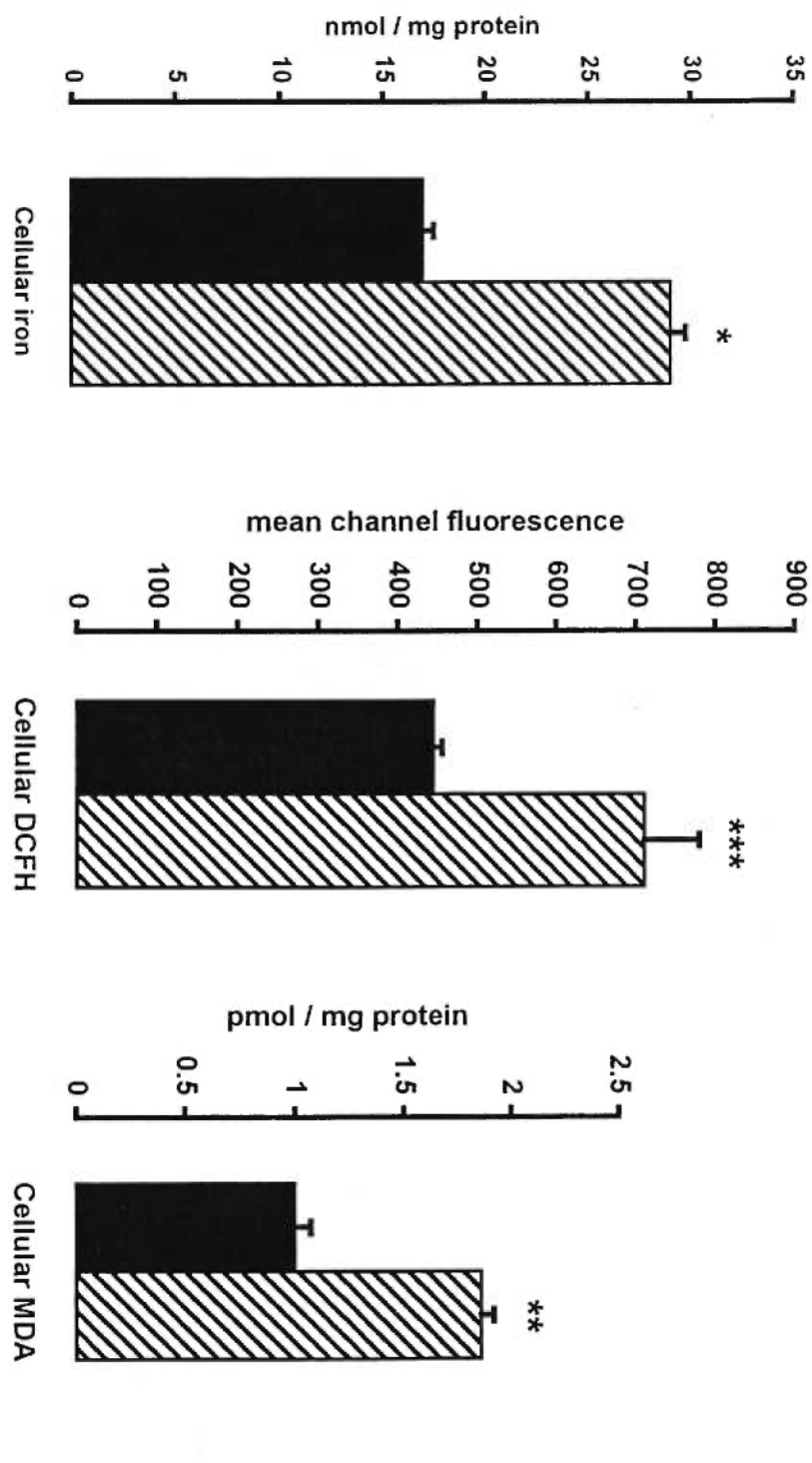


Figure 1



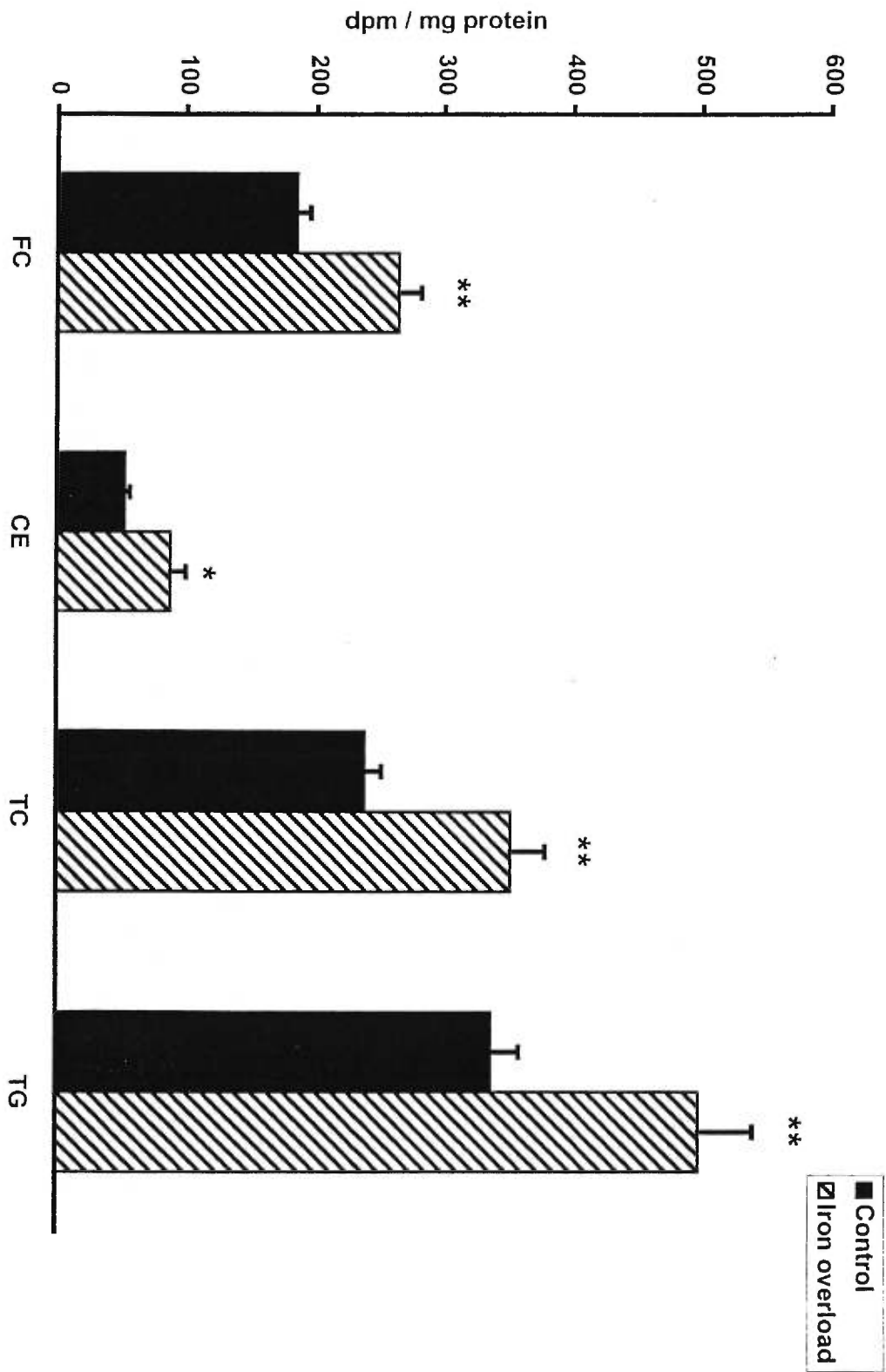


Figure 2

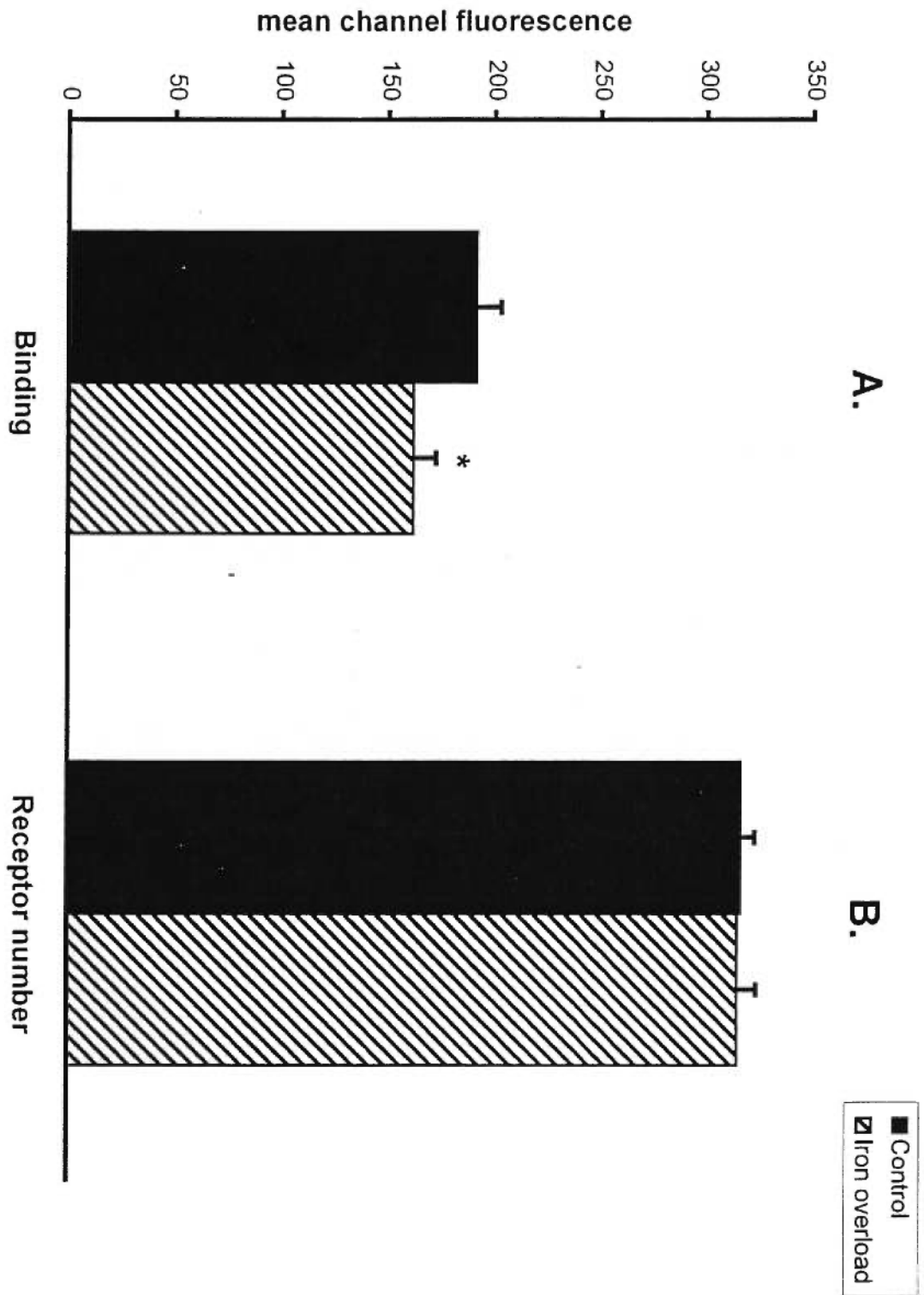


Figure 3

#### IV. DISCUSSION

Comme nous l'avons souligné dans l'introduction, il ne fait plus aucun doute que la peroxydation intervient activement dans la physiologie normale et dans plusieurs maladies humaines. D'excellentes études se sont affairées à vérifier son existence et à préciser le rôle de la peroxydation dans des conditions pathophysiologiques[1, 10, 71, 87, 88].

Plus précisément, certains désordres dont l'athérosclérose, la rétinopathie du nouveau-né et la maladie de Crohn pourraient recevoir le qualificatif de «maladie radicalaire » à cause des événements clairement peroxydatifs qui les président[2, 10, 71].

Toutefois, il n'est pas toujours facile de distinguer la contribution directe ou indirecte de la peroxydation dans la plupart des pathologies. Ainsi, les fortes concentrations de fer tissulaire notées dans l'hémochromatose, rassemblent à priori les conditions idéales pour mettre en relief le rôle du stress oxydatif dans la multitude des anomalies inhérentes à cette maladie. Malgré tout, et même dans ce cas, il peut être difficile de spécifier la part véritable de la peroxydation dans le tableau de la maladie. C'est dans cette réflexion que s'inscrit l'objectif majeur de notre programme de recherche. Cette dernière

consiste à définir le devenir du carrefour métabolique du cholestérol sous l'influence du stress oxydatif.

Il est intéressant de remarquer que plusieurs pathologies que l'on soupçonne à composantes radicalaires, présentent une association plus ou moins forte avec le fer. En effet, ce dernier se retrouve souvent sous forme libre dans la plaque d'athérosclérose, dans les lésions neurologiques des malades souffrant de Parkinson ou d'Alzheimer[15, 17-21, 89]. On le retrouve encore dans le foie, le cœur et la rate de patients, présentant une hémochromatose. Sa disponibilité dans tous ces exemples catalyse des événements peroxydatifs.

Il n'est, donc, pas étonnant de constater que de nombreuses études l'utilisent comme modèle de peroxydation autant *in vitro* que *in vivo*. Dans certaines conditions, le fer est utilisé directement pour initier la peroxydation[90-93], alors que dans d'autres on l'emploie, comme élément suppléant[94, 95], afin d'accentuer les dérèglements peroxydatifs provoqués par d'autres inducteurs. Par exemple, on combine souvent le fer à l'éthanol pour suivre les dommages peroxydatifs de l'alcool au niveau du foie.

L'addition du fer permet d'accentuer les niveaux du stress oxydatif et d'analyser subséquemment les événements hépatiques[95]. De plus, la

surcharge hépatique en fer représente un excellent outil pour examiner l'adaptation du foie à l'augmentation progressive du stress oxydatif[96]. Le dépassement du seuil de tolérance permet alors d'étudier les réactions enzymatiques qui prennent place dans des grands systèmes biochimiques : tel que celui contrôlant les voies métaboliques du cholestérol hépatique.

Dans le présent projet, nous avons étudié l'influence de la peroxydation initiée, par le fer (en absence ou présence de l'ascorbate), sur le métabolisme du cholestérol hépatique. Nous avons concentré notre attention au niveau des microsomes, des cellules en culture et de l'organisme entier. L'ampleur de la peroxydation obtenue témoigne du potentiel puissant du fer et de son rôle. En effet, la mesure des concentrations des marqueurs de peroxydation, notamment le MDA, a révélé de façon constante l'existence du stress dans des conditions de surcharge en fer dans tous les systèmes expérimentaux.

Même si le MDA est, à ce jour, le marqueur de peroxydation le plus utilisé, sa pertinence dans l'évaluation d'un état de stress oxydatif est souvent remise en question[97]. C'est pourquoi, il convient d'adjoindre d'autres formes de mesures de peroxydation pour souligner et confirmer l'existence d'un stress oxydatif.

A cette fin, nous avons mesuré, dans nos différentes études, la peroxydation par les dienes conjugués, l'oxydation du DCFH-DA et le profil des acides gras, souvent la cible de la peroxydation. Finalement, l'ajout d'antioxydant (tocophérol) aux préparations microsomiales et cellulaires, suivi de la diminution des marqueurs de peroxydation, témoigne du rôle et de l'influence du stress oxydatif dans les dérangements notés dans les lipides, les lipoprotéines et les enzymes étudiés.

Il est de toute première importance, une fois l'existence d'une peroxydation établie, de caractériser l'amplitude du stress oxydatif imposé au foie. En effet, si cet organe est trop fortement peroxydé, il risque de perdre toute forme de régulation et de s'enfoncer dans un état de fibrose et de nécrose. Pour nous assurer que nos modèles in vivo, exposés à la surcharge en fer, demeurent dans des limites acceptables, nous avons mesuré les niveaux de transaminases à titre d'indicateur d'un dysfonctionnement hépatique sévère. L'absence d'augmentation importante des transaminases était rassurante. De même, l'examen de la bilirubine montrait des valeurs normales. En outre, l'analyse des coupes de foie au microscope ne présentait pas de signe classique de fibrose et / ou de nécrose hépatique. Finalement, la mesure directe ou indirecte du glutathion n'a jamais montré de diminution anormale.

L'ensemble de ces résultats indiquent que l'ampleur de la peroxydation à laquelle le foie a été soumis, ne cause pas de perturbations profondes et permet ainsi l'exploration des mécanismes de régulation du cholestérol sous le stress oxydatif initié par le fer. Lorsque l'on considère l'homéostasie du cholestérol, il faut d'emblée évaluer la contribution hépatique. En effet, on constate souvent que les altérations du cholestérol en périphérie sont dictées par des événements hépatiques[37, 73]. En quelques sorte, il convient de dire que le devenir métabolique du cholestérol en périphérie est indissociable de sa condition au niveau du foie. Nous avons donc jugé important d'examiner le profil du cholestérol dans la circulation.

De façon unanime, dans la plupart de nos modèles, l'analyse détaillée des lipoprotéines transporteuses de cholestérol, a révélé des perturbations profondes tant dans leur composition lipidique que protéique. Ce type d'altérations n'est pas sans conséquence sur les transformations dynamiques qui prennent place dans la circulation. Si les lipoprotéines s'activent à échanger leur contenu en lipides et en protéines au gré des besoins physiologiques, la moindre altération de densité lipoprotéinique ou d'apoprotéines peut entraver l'interaction normale de ces dernières avec les enzymes du plasma et les récepteurs cellulaires susceptibles de présider à leur transformation[98]. Par exemple, nous avons démontré qu'une modification des HDL,

épurateurs de cholestérol, entraîne une série de dérangements dans le parcours métabolique et le retour du cholestérol au foie[51-53].

Il est clair que les HDL modifiées exercent leur influence sur la voie de synthèse des acides biliaires, et qu'elles provoquent une diminution nette des quantités de cholestérol et d'acides biliaires excrétées[51, 53]. Ceci illustre bien le risque potentiel des modifications des HDL, y compris celles des apoprotéines, sur le devenir métabolique du cholestérol en périphérie ou dans le système hépatobiliaire. Le choix du mode d'induction du stress oxydatif à l'aide du fer produit tout de même des variations quant au déséquilibre du profil plasmatique des lipides obtenus.

En effet, en situation aiguë (fer/salicylate), nous avons constaté une diminution des esters de cholestérol. Dans leur étude utilisant le fer comme inducteur de peroxydation aiguë, Antebi et al.[99] ont aussi noté de telles modifications des lipoprotéines circulantes d'origine hépatique. Toutefois, avec l'administration chronique du fer carbonyle, nous n'avons pas observé de pareils changements. Au contraire, la tendance notée va dans le sens d'une augmentation des concentrations plasmatiques de cholestérol et de triglycérides. Nous avons même pu constater l'augmentation importante de la forme estérifiée au dépend de la forme libre.



De façon générale, les lipoprotéines présentent une composition enrichie en esters de cholestérol et une déplétion en cholestérol libre. Ce genre d'altération a aussi été observé, dans un contexte de surcharge chronique en fer, par plusieurs autres équipes[97, 100, 101]. Il semble donc que ces dérèglements soient une conséquence étroitement liée au stress oxydatif chronique causé par le fer au niveau du foie. Les variations, observées entre les deux modèles, sont probablement inhérentes au mode d'administration et à la période de surcharge en fer. Cependant, la peroxydation entraîne dans tous les cas des changements profonds dans le profil lipidique et la composition des lipoprotéines.

Qu'en est-il du contenu hépatique en cholestérol ? Lorsque l'on compare le contenu en lipide des foies exposés en aiguë et celui des foies exposés chroniquement, on remarque dans les deux cas une diminution significative du contenu en triglycérides. L'analyse des différentes formes de cholestérol, à savoir libre ou estérifié, a montré des diminutions similaires à celles observées dans le plasma seulement. Il est possible que les diminutions en cholestérol ester et en triglycérides, deux formes estérifiées d'acides gras, reflètent l'abaissement des acides gras polyinsaturés. En effet, le stress oxydatif, réalisé grâce à 48 heures de surcharge en fer, a peut-être été suffisant pour causer une attaque radicalaire sur les acides gras essentiels, se

répercutant ainsi sur les molécules de cholestérol esters et de triglycérides.

Toutefois, dans le contexte de peroxydation chronique, seuls les triglycérides ont montré une diminution de leur concentration hépatique. La sensibilité de la voie de synthèse hépatique des triglycérides, sous le contrôle du glycerol-3-phosphate acyltransferase (GPAT), face au stress oxydatif *in-vitro* est bien documentée[93]. Nos résultats confirment *in vivo* la sensibilité de cette enzyme et/ou de ses voies de régulations face au stress oxydant et concluent à l'incapacité de ce système à contrer efficacement l'effet de la peroxydation au fer.

Par ailleurs, toujours en situation chronique de peroxydation et malgré l'absence de dérèglement du profil hépatique en cholestérol, l'analyse des activités enzymatiques qui supportent sa synthèse, sa transformation et sa sécrétion indiquent clairement qu'une perturbation hépatique des voies du cholestérol a lieu.

En effet, *in vivo* nous avons observé une augmentation de l'activité d'estérification du cholestérol, une diminution nette des activités de synthèse du cholestérol et enfin de sa transformation en acide biliaire.

L'entrave à la voie des acides biliaires exerce donc une pression hépatique à disposer du cholestérol pour éviter la fibrose hépatique.

D'une part, le tissu hépatique devrait diminuer la synthèse endogène de cholestérol en réduisant l'activité de l'HMG-CoA reductase, ce qu'indiquent nos résultats. D'autre part, puisque la seule voie hépatique d'évitement de la fibrose dans cette situation consiste à synthétiser et assembler des lipoprotéines en vue de leur export, le foie devra augmenter l'activité d'estérification du cholestérol via l'ACAT. Ceci a pour résultante d'augmenter les niveaux circulants (ou extra-hépatique) en cholestérol (VLDL). L'étude de Drevon CA et coll.[78] souligne précisément la nécessité absolue de l'ACAT dans la sécrétion des VLDL d'origine hépatique. De plus, il convient de rappeler l'association fréquente entre hypercholestérolémie et cholestase hépatique[102-104], aussi notée dans nos expériences.

La suite logique de notre recherche impliquait l'examen de la sensibilité *in vitro* des trois enzymes contrôlant le métabolisme des stérols au niveau du foie face au stress peroxydatif. L'évaluation de l'ACAT, de l'HMG CoA reductase et de la cholestérol 7  $\alpha$ -hydroxylase permet de dégager la contribution hépatique aux dérèglements lipidiques observés *in vivo* et potentiellement de cerner les mécanismes sous-jacents.

Des trois enzymes, l'ACAT présentait la plus grande sensibilité au stress oxydatif; la supplémentation en tocophérol rétablissait complètement son activité. De nombreuses études ont souligné l'importance de préserver l'intégrité de la composition des phospholipides et des acides gras du réticulum endoplasmique pour maintenir de façon optimale l'activité d'estérification de l'ACAT, une enzyme microsomiale[105-108]. En effet, l'addition de la vitamine E a protégé ces classes de lipides contre la peroxydation, particulièrement les acides gras essentiels, tout en rétablissant l'activité de l'ACAT. Il semble donc que l'environnement membranaire est crucial pour le fonctionnement optimal de cette enzyme.

Ceci contraste fortement avec l'HMG CoA reductase et la cholestérol 7  $\alpha$ -hydroxylase. Dans le cas de l'HMG CoA reductase, le tocophérol n'a pu rétablir l'activité qu'après correction de la fluidité des membranes microsomiales. Nos résultats suggèrent, tout comme ceux de Liscum et coll., que le site d'ancrage de cet enzyme au réticulum endoplasmique est sensible aux variations de fluidité membranaire et est relié directement à l'activité catalytique de l'enzyme. De plus, nos observations précisent qu'à part l'insaturation des acides gras, les autres composantes de la membrane (les types de phospholipides, le ratio de phospholipide/cholestérol, etc) influençant la fluidité

membranaire, sont aussi extrêmement importants pour stabiliser l'activité de l'HMG CoA reductase.

La cholestérol 7  $\alpha$ -hydroxylase s'est distingué, davantage, des deux enzymes précédents; non seulement le tocophérol n'a pas permis de restaurer l'activité de l'enzyme comparativement aux contrôles, mais encore la correction de la fluidité membranaire aggravait sa perturbation. La possibilité que des ponts dissulfides, essentiels à la fonction de cette enzyme, soient influencés par des interactions directes avec le fer n'est pas à écarter.

Finalement, l'ajout de fer, au milieu de culture des cellules HepG2, constitue un modèle cellulaire valable pour reproduire et approfondir les dérèglements de la surcharge chronique en fer, observés *in vivo*. Cette approche nous a permis d'isoler la contribution hépatique des désordres périphériques. En effet, notre modèle reproduit l'augmentation de cholestérol en périphérie sous la forme d'une plus grande sécrétion de cholestérol, dans le milieu de culture. De plus, similairement au modèle chronique de peroxydation *in vivo*, les fractions VLDL et LDL augmentent toutes deux dans les milieux de culture des cellules soumises à une surcharge chronique en fer. L'analyse du contenu des différentes lipoprotéines a, de plus, révélé une composition enrichie en

esters de cholestérol et en triglycérides. La similarité des effets, observés ici, avec les perturbations, notées *in vivo*, permet d'affirmer que les altérations observées lors de la surcharge en fer chronique ont bel et bien une origine hépatique. Il est important de souligner la diminution d'activité de liaison et d'internalisation du récepteur LDL dans nos cellules en culture chargées en fer. Cette situation aggraverait l'homéostasie du cholestérol hépatique, en privant l'hépatocyte d'une voie de «signalisation» quant aux surplus de cholestérol dans le milieu. Il conviendrait sûrement, dans le futur, de valider *in vivo* cette observation novatrice.

## V. CONCLUSIONS ET PERSPECTIVES

Il ressort donc, de l'ensemble de nos résultats, qu'une augmentation du stress oxydatif à l'aide du fer perturbe profondément l'homéostasie normale du cholestérol hépatique. L'atteinte se situe au niveau des voies de synthèse et de sécrétion des acides biliaires, et semble être l'une des principales conséquences de l'agression des radicaux libres sur le système hépatique. L'incapacité du foie à normaliser les anomalies de la cholestérol 7  $\alpha$ -hydroxylase et des sentiers métaboliques relatifs, force, alors, l'induction de l'ACAT et l'inhibition de l'HMG CoA reductase. Le tout accentue la sécrétion de lipoprotéines transporteurs de cholestérol. En plus de contribuer à l'hypercholestérolémie, un abaissement de l'efficacité d'épuration des lipoprotéines circulantes via le récepteur apo B/E au niveau du foie, augmente le risque d'athérosclérose.

Plusieurs aspects du système de régulation du cholestérol hépatique, dans le contexte d'un stress oxydatif, n'ont pas encore été résolus. Il faudra, notamment, caractériser plus à fond les mécanismes qui ont cours dans l'activation / diminution des enzymes d'intérêt.

Ainsi, l'influence de la peroxydation sur les états de phosphorylation / déphosphorylation, de même que les réactions transcriptionnelles des gènes impliqués devront être scrutées de très près. Il est à parier qu'une analyse des réponses moléculaires à l'agression des radicaux libres nous éclairera davantage sur les mécanismes sous-jacents à l'apparente hypercholestérolémie, provoquée par la peroxydation chronique au fer. De même, lorsque les rôles spécifiques des différentes formes de ACAT seront mieux connus, nous pourrons sûrement dégager la contribution de chacune des variantes aux désordres observés.

Il conviendrait, aussi, de développer une stratégie antioxydante permettant de contrer la peroxydation en milieu lipidique et en milieu aqueux. Avec de tels outils, nous pourrons mieux caractériser les sites d'atteintes de la peroxydation et cela permettra également de préciser la résistance peroxydative au tocophérol. Enfin, il demeure tout de même possible que certains effets observés dans nos modèles soient attribuables au fer *per se* et non reliés à son caractère pro-oxydant. Il importe donc de développer davantage l'analyse biochimique et moléculaire de nos modèles. Grâce à cette analyse, nous pourrons mieux caractériser l'origine peroxydative des effets observés sous l'induction du stress oxydatif à l'aide du fer et d'autres pro-oxydants.



## VI. BIBLIOGRAPHIE

- [1] C. Rice-Evans and R. Burdon, *Free radical-lipid interactions and their pathological consequences*. Progress in Lipid Research 32, 71-110 (1993).
- [2] O. I. Aruoma, *Nutrition and health aspects of free radicals and antioxidants* Food & Chemical Toxicology 32, 671-683 (1994).
- [3] J. Pre, [*Lipid peroxidation*]. Pathologie Biologie 39, 716-736 (1991).
- [4] C. E. Cross, B. Halliwell, E. T. Borish, W. A. Pryor, B. N. Ames, R. L. Saul, J. M. McCord and D. Harman, *Oxygen radicals and human disease*. Annals of Internal Medicine 107, 526-545 (1987).
- [5] J. A. Berliner, M. C. Territo, A. Sevanian, S. Ramin, J. A. Kim, B. Bamshad, M. Esterson and A. M. Fogelman, - *Minimally modified low density lipoprotein stimulates monocyte endothelial interactions*. - Journal of Clinical Investigation. Apr;85(4):1260-6 (1990)

- [6] J. T. Curnutte, - *Molecular basis of the autosomal recessive forms of chronic granulomatous disease*. - Immunodeficiency Reviews ;3(2):149-72 149-722 (1992)
- [7] Kehrer James P. and Smith Charles V., *Free Radicals in Biology: Sources, Reactivities, and Roles in the Etiology of Human Diseases* . In *Natural Antioxidants in Human Health and Disease*. (Ed. B. Frei) pp. 25-62, Academic Press, San Diego, California (1994).
- [8] D. Armstrong and R. Browne, *The analysis of free radicals, lipid peroxides, antioxidant enzymes and compounds related to oxidative stress as applied to the clinical chemistry laboratory*. Advances in Experimental Medicine & Biology 366, 43-58 (1994).
- [9] G. Minotti and S. D. Aust, *The role of iron in oxygen radical mediated lipid peroxidation*. Chemico-Biological Interactions 71, 1-19 (1989).
- [10] M. Martinez-Cayuela, *Oxygen free radicals and human disease*. Biochimie 77, 147-161 (1995).
- [11] J. M. McCord, *The superoxide free radical: its biochemistry and pathophysiology*. Surgery 94, 412-414 (1983).

- [12] O. I. Aruoma, *Characterization of drugs as antioxidant prophylactics*. *Free Radical Biology & Medicine* 20, 675-705 (1996).
- [13] S. J. Stohs and D. Bagchi, - *Oxidative mechanisms in the toxicity of metal ions*. - *Free Radic Biol Med* 18(2): 321-366. (1995)
- [14] B. Halliwell, *Oxidants and the central nervous system: some fundamental questions. Is oxidant damage relevant to Parkinson's disease, Alzheimer's disease, traumatic injury or stroke?* *Acta Neurologica Scandinavica Supplementum*. 126, 23-33 (1989).
- [15] B. Halliwell, *Reactive oxygen species and the central nervous system*. *Journal of Neurochemistry* 59, 1609-1623 (1992).
- [16] B. Halliwell, *Oxygen radicals as key mediators in neurological disease: fact or fiction?* *Annals of Neurology* 32 Suppl, S10-S15 (1992).
- [17] G. Logroscino, K. Marder, J. Graziano, G. Freyer, V. Slavkovich, Lolacono, L. Cote and R. Mayeux, *Altered systemic iron metabolism in Parkinson's disease*. *Neurology* 49, 714-717 (1997).

- [18] J. E. Nielsen, L. N. Jensen and K. Krabbe, *Hereditary haemochromatosis: a case of iron accumulation in the basal ganglia associated with a parkinsonian syndrome*. *Journal of Neurology, Neurosurgery & Psychiatry* 59, 318-321 (1995).
- [19] J. P. Spencer, A. Jenner, O. I. Aruoma, P. J. Evans, H. Kaur, D. T. Dexter, Jenner, A. J. Lees, D. C. Marsden and B. Halliwell, *Intense oxidative DNA damage promoted by L-dopa and its metabolites. Implications for neurodegenerative disease*. *FEBS Letters* 353, 246-250 (1994).
- [20] J. P. Spencer, P. Jenner and B. Halliwell, *Superoxide-dependent depletion of reduced glutathione by L-DOPA and dopamine. Relevance to Parkinson's disease*. *Neuroreport* 6, 1480-1484 (1995).
- [21] C. N. Still, *Postmenopausal Parkinsonism: brain iron overload?* *Advances in Experimental Medicine & Biology* 90, 291-296 (1977).
- [22] B. Demple and L. Harrison, - *Repair of oxidative damage to DNA: enzymology and biology*. - *Annu Rev Biochem* ;63:915-48 915-488, (1994)

- [23] T. Jabs, *Reactive oxygen intermediates as mediators of programmed cell death in plants and animals*. *Biochemical Pharmacology* 57, 231-245 (1999).
- [24] L. Iacovitti, N. D. Stull and A. Mishizen, *Neurotransmitters, KCl and antioxidants rescue striatal neurons from apoptotic cell death in culture*. *Brain Research* 816, 276-285 (1999).
- [25] P. Johnson, Y. Wei, M. J. Huentelman, C. M. Peters and A. A. Boldyrev, *Hydralazine, but not captopril, decreases free radical production and apoptosis in neurons and thymocytes*. *Free Radical Research* 28, 393-402 (1998).
- [26] K. Oishi and K. Machida, *Inhibition of neutrophil apoptosis by antioxidants in culture medium*. *Scandinavian Journal of Immunology* 45, 21-27 (1997).
- [27] T. Patel and G. J. Gores, *Inhibition of bile-salt-induced hepatocyte apoptosis by the antioxidant lazaroid U83836E*. *Toxicology & Applied Pharmacology* 142, 116-122 (1997).
- [28] M. Tagami, K. Yamagata, K. Ikeda, Y. Nara, H. Fujino, A. Kubota, F. Numano and Y. Yamori, *Vitamin E prevents apoptosis in cortical neurons during hypoxia and oxygen reperfusion*. *Laboratory Investigation* 78, 1415-1429 (1998).

- [29] B. Frei, L. England and B. N. Ames, *Ascorbate is an outstanding antioxidant in human blood plasma*. Proceedings of the National Academy of Sciences of the United States of America 86, 6377-6381 (1989).
- [30] G. R. Buettner and B. A. Jurkiewicz, - *Catalytic metals, ascorbate and free radicals: combinations to avoid*. - Radiat Res May;145(5):532-41 532-411, (1996)
- [31] T. M. Berger, M. C. Polidori, A. Dabbagh, P. J. Evans, B. Halliwell, J. D. Morrow, L. J. Roberts and B. Frei, *Antioxidant activity of vitamin C in iron-overloaded human plasma*. Journal of Biological Chemistry 272, 15656-15660 (1997).
- [32] A. Hatta and B. Frei, *Oxidative modification and antioxidant protection of human low density lipoprotein at high and low oxygen partial pressures*. Journal of Lipid Research 36, 2383-2393 (1995).
- [33] P. Palozza and N. I. Krinsky, *The inhibition of radical-initiated peroxidation of microsomal lipids by both alpha-tocopherol and beta-carotene*. Free Radical Biology & Medicine 11, 407-414 (1991).

- [34] P. Palozza, S. Moualla and N. I. Krinsky, *Effects of beta-carotene and alpha-tocopherol on radical-initiated peroxidation of microsomes*. Free Radical Biology & Medicine 13, 127-136 (1992).
- [35] P. Palozza and N. I. Krinsky, *beta-Carotene and alpha-tocopherol are synergistic antioxidants*. Archives of Biochemistry & Biophysics 297, 184-187 (1992).
- [36] H. J. Kayden and M. G. Traber, - *Absorption, lipoprotein transport, and regulation of plasma concentrations of vitamin E in humans*. - J Lipid Res 34(3):343-358, (1993).
- [37] R. Ross, - *Atherosclerosis: current understanding of mechanisms and future strategies in therapy*. - Transplantation Proceedings;25(2):2041-33,(1993)
- [38] L. B. Nielsen, - *Transfer of low density lipoprotein into the arterial wall and risk of atherosclerosis*. - Atherosclerosis;123(1-2):1-15, (1996)
- [39] J. A. Kim, M. C. Territo, E. Wayner, T. M. Carlos, F. Parhami, C. W. Smith, M. E. Haberland, A. M. Fogelman and J. A. Berliner, - *Partial characterization of leukocyte binding molecules on*

*endothelial cells induced by minimally oxidized LDL. -*

*Arteriosclerosis & Thrombosis ;14(3):427-433 (1994)*

[40] M. S. Brown and J. L. Goldstein, *Scavenger cell receptor shared*.  
*Nature 316, 680-681 (1985).*

[41] R. Ross, - *The pathogenesis of atherosclerosis: a perspective for  
the 1990s.* - *Nature 362:801-809, (1993)*

[42] E. W. Raines and R. Ross, - *Smooth muscle cells and the  
pathogenesis of the lesions of atherosclerosis. [Review] [32 refs].*  
- *British Heart Journal; 69,S30-S77 (1993).*

[43] V. W. Bowry, K. K. Stanley and R. Stocker, *High density  
lipoprotein is the major carrier of lipid hydroperoxides in human  
blood plasma from fasting donors.* *Proceedings of the National  
Academy of Sciences of the United States of America 89, 10316-  
10320 (1992).*

[44] J. K. Christison, K. A. Rye and R. Stocker, *Exchange of oxidized  
cholesteryl linoleate between LDL and HDL mediated by  
cholesteryl ester transfer protein.* *Journal of Lipid Research 36,  
2017-2026 (1995).*

[45] W. Sattler and R. Stocker, *Greater selective uptake by Hep G2  
cells of high-density lipoprotein cholesteryl ester hydroperoxides*



- than of unoxidized cholesteryl esters. Biochemical Journal* 294, 771-778 (1993).
- [46] K. Osada, E. Sasaki and M. Sugano, *Lymphatic absorption of oxidized cholesterol in rats. Lipids* 29, 555-559 (1994).
- [47] I. Staprans, X. M. Pan, M. Miller and J. H. Rapp, *Effect of dietary lipid peroxides on metabolism of serum chylomicrons in rats. American Journal of Physiology* 264, t-8 (1993).
- [48] I. Staprans, J. H. Rapp, X. M. Pan, K. Y. Kim and K. R. Feingold, *Oxidized lipids in the diet are a source of oxidized lipid in chylomicrons of human serum. Arteriosclerosis & Thrombosis* 14, 1900-1905 (1994).
- [49] I. Staprans, J. H. Rapp, X. M. Pan and K. R. Feingold, *Oxidized lipids in the diet are incorporated by the liver into very low density lipoprotein in rats. Journal of Lipid Research* 37, 420-430 (1996).
- [50] Y. Nagano, H. Arai and T. Kita, *High density lipoprotein loses its effect to stimulate efflux of cholesterol from foam cells after oxidative modification. Proceedings of the National Academy of Sciences of the United States of America* 88, 6457-6461 (1991).
- [51] F. Guertin, S. Brunet, V. Gavino, B. Tuchweber and E. Levy, *Malondialdehyde-modified high-density lipoprotein cholesterol:*

- plasma removal, tissue distribution and biliary sterol secretion in rats. Biochimica et Biophysica Acta 1214, 137-142 (1994).*
- [52] F. Guertin, S. Brunet, V. Gavino, B. Tuchweber and E. Levy, *Malondialdehyde-modified HDL leads to accumulation of cholesterol in rat liver endothelial cells. Biochemical & Biophysical Research Communications 212, 1-8 (1995).*
- [53] F. Guertin, S. Brunet, D. Lairon and E. Levy, *Oxidative tyrosylation of high density lipoprotein impairs biliary sterol secretion in rats. Atherosclerosis 131, 35-41 (1997).*
- [54] G. Poli, *Liver damage due to free radicals. British Medical Bulletin 49, 604-620 (1993).*
- [55] T. Amimoto, T. Matura, S. Y. Koyama, T. Nakanishi, K. Yamada and G. Kajiyama, *Acetaminophen-induced hepatic injury in mice: the role of lipid peroxidation and effects of pretreatment with coenzyme Q10 and alpha-tocopherol. Free Radical Biology & Medicine 19, 169-176 (1995).*
- [56] A. P. Kourounakis, E. A. Rekka and P. N. Kourounakis, *Antioxidant activity of guaiazulene and protection against paracetamol hepatotoxicity in rats. Journal of Pharmacy & Pharmacology 49, 938-942 (1997).*

- [57] G. Vendemiale, I. Grattagliano, E. Altomare, N. Turturro and F. Guerrieri, *Effect of acetaminophen administration on hepatic glutathione compartmentation and mitochondrial energy metabolism in the rat*. *Biochemical Pharmacology* 52, 1147-1154 (1996).
- [58] A. Garrido, C. Arancibia, R. Campos and A. Valenzuela, *Acetaminophen does not induce oxidative stress in isolated rat hepatocytes: its probable antioxidant effect is potentiated by the flavonoid silybin*. *Pharmacology & Toxicology* 69, 9-12 (1991).
- [59] M. Younes, I. Eberhardt and R. Lemoine, *Spontaneous and xenobiotic-induced in vivo-lipid peroxidation in iron-overloaded rats*. *Archives of Toxicology Supplement*. 13, 282-286 (1989).
- [60] M. G. Irving, C. J. Booth, C. M. Devlin, J. W. Halliday and L. W. Powell, *The effect of iron and ethanol on rat hepatocyte collagen synthesis*. *Comparative Biochemistry & Physiology - C: Comparative Pharmacology & Toxicology* 100, 583-590 (1991).
- [61] H. Ishii, I. Kurose and S. Kato, *Pathogenesis of alcoholic liver disease with particular emphasis on oxidative stress*. *Journal of Gastroenterology & Hepatology* 12, S272-S282 (1997).

- [62] K. T. Knecht, R. G. Thurman and R. P. Mason, *Role of superoxide and trace transition metals in the production of alpha-hydroxyethyl radical from ethanol by microsomes from alcohol dehydrogenase-deficient deer mice*. *Archives of Biochemistry & Biophysics* 303, 339-348 (1993).
- [63] R. Nordmann, C. Ribiere and H. Rouach, *Involvement of iron and iron-catalyzed free radical production in ethanol metabolism and toxicity*. *Enzyme* 37, 57-69 (1987).
- [64] O. Sergent, I. Morel, P. Cogrel, M. Chevanne, N. Padeloup, P. Brissot, G. Lescoat, P. Cillard and J. Cillard, *Increase in cellular pool of low-molecular-weight iron during ethanol metabolism in rat hepatocyte cultures. Relationship with lipid peroxidation*. *Biological Trace Element Research* 47, 185-192 (1995).
- [65] M. Younes, I. Eberhardt and R. Lemoine, *Effect of iron overload on spontaneous and xenobiotic-induced lipid peroxidation in vivo*. *Journal of Applied Toxicology* 9, 103-108 (1989).
- [66] G. Minotti, *tert-butyl hydroperoxide-dependent microsomal release of iron and lipid peroxidation. I. Evidence for the reductive release of nonheme, nonferritin iron*. *Archives of Biochemistry & Biophysics* 273, 137-143 (1989).

- [67] G. Minotti, *tert-butyl hydroperoxide-dependent microsomal release of iron and lipid peroxidation. II. Evidence for the involvement of nonheme, nonferritin iron in lipid peroxidation.* Archives of Biochemistry & Biophysics 273, 144-147 (1989).
- [68] M. Younes and A. Wess, *The role of iron in t-butyl hydroperoxide-induced lipid peroxidation and hepatotoxicity in rats.* Journal of Applied Toxicology 10, 313-317 (1990).
- [69] T. H. Bothwell, - *Overview and mechanisms of iron regulation.* - Nutr Rev ;53(9):237-45 237-455.(1995)
- [70] B. Halliwell and J. M. Gutteridge, *Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts.* Archives of Biochemistry & Biophysics 246, 501-514 (1986).
- [71] B. Halliwell, J. M. Gutteridge and C. E. Cross, *Free radicals, antioxidants, and human disease: where are we now?.* Journal of Laboratory & Clinical Medicine 119, 598-620 (1992).
- [72] J. L. Farber, *Mechanisms of cell injury by activated oxygen species.* Environmental Health Perspectives 102, Suppl-24 (1994).

- [73] G. Luc, J.-M. Leclerc, J.-M. Bard, E. Hachulla, J.-C. Fruchart and B. Devulder, *Cholestérol et Athérosclérose*, p. -246, Masson, Paris, France 1991.
- [74] R. Z. Vlahcevis, D. M. Heuman and P. B. Hylemon. Regulation of Bile Acid Synthesis. *Hepatology* 13, 590-600. (1991).
- [75] R. Asslan, A. Pradines, C. Pratz, C. Allal, G. Favre and F. Le Gaillard, - *Epidermal growth factor stimulates 3-hydroxy-3-methylglutaryl-coenzyme A reductase expression via the ErbB-2 pathway in human breast adenocarcinoma cells*. - *Biochemical & Biophysical Research Communications* ;260(3):699-706 (1999)
- [76] L. W. Lecureux and B. W. Wattenberg, - *The regulated degradation of a 3-hydroxy-3-methylglutaryl-coenzyme A reductase reporter construct occurs in the endoplasmic reticulum*. - *Journal of Cell Science*;107:2635-42. (1994).
- [77] R. Y. Hampton, - *Genetic analysis of hydroxymethylglutaryl-coenzyme A reductase regulated degradation. [Review] [19 refs]*. - *Current Opinion In Lipidology*;9(2):93-97.(1998)
- [78] C. A. Drevon, S. C. Engelhorn and D. Steinberg, - *Secretion of very low density lipoproteins enriched in cholesteryl esters by cultured rat hepatocytes during simulation of intracellular*

*cholesterol esterification*. - Journal of Lipid Research ;21(8):1065-71. (1980)

- [79] A. A. Spector, S. N. Mathur and T. L. Kaduce, - *Role of acylcoenzyme A: cholesterol o-acyltransferase in cholesterol metabolism*. - Progress in Lipid Research;18(1):531-533. (1979)
- [80] T. Y. Chang, C. C. Chang and D. Cheng, - *Acyl-coenzyme A:cholesterol acyltransferase*. - Annual Review of Biochemistry ;66:613-638. (1997)
- [81] J. M. Corton and D. G. Hardie, - *Evidence against a role for phosphorylation/dephosphorylation in the regulation of acyl-CoA:cholesterol acyl transferase*. - European Journal of Biochemistry ;204(1), 203-288. (1992).
- [82] R. A. Anderson, C. Joyce, M. Davis, J. W. Reagan, M. Clark, G. S. Shelness and L. L. Rudel, - *Identification of a form of acyl-CoA:cholesterol acyltransferase specific to liver and intestine in nonhuman primates*. - Journal of Biological Chemistry; 273:26747-26754. (1998)
- [83] C. Joyce, K. Skinner, R. A. Anderson and L. L. Rudel, - *Acyl-coenzyme A:cholesteryl acyltransferase 2*. - Current Opinion In Lipidology;10(2):89-95.(1999).

- [84] M. Schwarz, E. G. Lund and D. W. Russell, - *Two 7 alpha-hydroxylase enzymes in bile acid biosynthesis. [Review] [56 refs].* - Current Opinion In Lipidology;9(2):113-118.(1998).
- [85] L. B. Nguyen, S. Shefer, G. Salen, J. Y. Chiang and M. Patel, - *Cholesterol 7alpha-hydroxylase activities from human and rat liver are modulated in vitro posttranslationally by phosphorylation/dephosphorylation.* - Hepatology 1996 ;24(6):1468-74. (1996).
- [86] P. M. Tang and J. Y. Chiang, - *Modulation of reconstituted cholesterol 7 alpha-hydroxylase by phosphatase and protein kinase.* - Biochemical & Biophysical Research Communications ;134(2):797-802. (1986)
- [87] B. Halliwell, *Free radicals, antioxidants, and human disease: curiosity, cause, or consequence?* Lancet 344, 721-724 (1994).
- [88] B. Halliwell and C. E. Cross, *Oxygen-derived species: their relation to human disease and environmental stress.* Environmental Health Perspectives 102 Suppl 10, 5-12 (1994).
- [89] M. B. Youdim, D. Ben-Shachar and P. Riederer, *The possible role of iron in the etiopathology of Parkinson's disease.* Movement Disorders 8, 1-12 (1993).



- [90] W. R. Bidlack and A. L. Tappel, - *Damage to microsomal membrane by lipid peroxidation.* - *Lipids* ;8(4):177-82 177-822 (1973).
- [91] P. K. Dudeja and T. A. Brasitus, - *Inactivation of rat small intestinal brush-border membrane alkaline phosphatase by oxygen free radicals.* - *Gastroenterology*;105(2):357-366 (1993).
- [92] D. Jour'd'Heuil, P. Vaananen and J. B. Meddings, - *Lipid peroxidation of the brush-border membrane: membrane physical properties and glucose transport.* - *Am J Physiol*;264(6 Pt 1): G1009-G1155 (1993).
- [93] P. D. Thomas and M. J. Poznansky, - *Lipid peroxidation inactivates rat liver microsomal glycerol-3- phosphate acyl transferase. Effect of iron and copper salts and carbon tetrachloride.* - *J Biol Chem*;265(5):2684-2691 (1990).
- [94] H. Muliawan and H. Kappus, - *Ferrous ion-stimulated alkane expiration in rats treated with carbon tetrachloride.* - *Toxicology* ;28(1-2):29-36 (1983)
- [95] P. Stal and R. Hultcrantz, - *Iron increases ethanol toxicity in rat liver.* - *J Hepatol* ;17(1):108-15 (1993).

- [96] B. R. Bacon and R. S. Britton, - *Hepatic injury in chronic iron overload. Role of lipid peroxidation.* - *Chemico-Biological Interactions* ;70(3-4):183-226 (1989).
- [97] A. J. Dabbagh, T. Mannion, S. M. Lynch and B. Frei, *The effect of iron overload on rat plasma and liver oxidant status in vivo.* *Biochemical Journal* ; 300, 799-803 (1994).
- [98] S. Yokoyama, - *Apolipoprotein-mediated cellular cholesterol efflux* *Biochim Biophys Acta*;1392(1):1-15 (1998).
- [99] H. Antebi, N. Pages, L. Zimmermann, C. Bourcier, B. Flechet and L. G. Alcindor, - *Resistance to oxidation of native lipoproteins and erythrocyte membrane lipids in rats with iron overload.* - *Annals of Nutrition & Metabolism*;39(1):63-68 (1995).
- [100] M. Fields and C. G. Lewis, - *Hepatic iron overload may contribute to hypertriglyceridemia and hypercholesterolemia in copper-deficient rats.* - *Metabolism*;46(4):377-381 (1997).
- [101] P. Whittaker, W. G. Wamer, R. F. Chanderbhan and V. C. Dunkel, - *Effects of alpha-tocopherol and beta-carotene on hepatic lipid peroxidation and blood lipids in rats with dietary iron overload.* - *Nutrition & Cancer*;25(2):119-128 (1996).

- [102] J. S. Crippin, K. D. Lindor, R. Jorgensen, B. A. Kottke, J. M. Harrison, P. A. Murtaugh and E. R. Dickson, -  
*Hypercholesterolemia and atherosclerosis in primary biliary cirrhosis: what is the risk?* - *Hepatology*;15(5):858-862 (1992).
- [103] P. Soros, J. Bottcher, H. Maschek, O. Selberg and M. J. Muller, -  
*Lipoprotein-X in patients with cirrhosis: its relationship to cholestasis and hypercholesterolemia.* - *Hepatology* ;28(5):1199-1205 (1998).
- [104] W. E. Stehbens and E. Wierzbicki, - *The relationship of hypercholesterolemia to atherosclerosis with particular emphasis on familial hypercholesterolemia, diabetes mellitus, obstructive jaundice, myxedema, and the nephrotic syndrome.* - *Prog Cardiovasc Dis* ;30(4):289-306 (1988).
- [105] S. N. Mathur, I. Simon, B. R. Lokesh and A. A. Spector, -  
*Phospholipid fatty acid modification of rat liver microsomes affects acylcoenzyme A:cholesterol acyltransferase activity.* - *Biochim Biophys Acta* ;751(3):401-411 (1983).
- [106] M. R. Johnson, S. N. Mathur, C. Coffman and A. A. Spector, -  
*Dietary fat saturation and hepatic acylcoenzyme A:cholesterol acyltransferase activity. Effect of n-3 polyunsaturated and long-chain saturated fat.* - *Arteriosclerosis*;3(3):242-248 (1983).

- [107] M. Chautan, E. Termine, N. Amirayan, J. Leonardi, A. M. Pauli, H. Portugal and H. Lafont, - *Compared effect of n-3 and n-6 dietary fatty acids on rat intestinal acyl-CoA:cholesterol acyltransferase activity*. - Scand J Gastroenterol ;24(5):632-640 (1989).
- [108] S. Pal and P. J. Davis, - *Effects of different types of polyunsaturated fatty acids on cholesterol esterification in human fibroblasts*. - Biochem Int;25(2):281-288 (1991).







