Université de Montréal

PROTECTION OF PRIMARY CULTURES OF MOUSE HEPATOCYTES AGAINST FAS-INDUCED APOPTOSIS

Role of EGF receptor intrinsic activity and intracellular redox state

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PROTECTION OF PRIMARY CULTURES OF MOUSE HEPATOCYTES AGAINST FAS-INDUCED APOPTOSIS: Role of EGF receptor intrinsic activity and intracellular redox state

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Résumé

Le foie est très sensible à l'apoptose. De nombreuses études ont rapporté une induction marquée de l'apoptose des hépatocytes au cours des infections virales (hépatites B, C et fulminante) et dans les hépatopathies alcooliques. Étant donné que les hépatocytes expriment le récepteur Fas de façon constitutive, ceci soulève son implication Fas dans le développement des pathologies hépatiques. Ainsi, le but de cette thèse est d'étudier les mécanismes de protection activés par le facteur de croissance épidermique (EGF) contre l'apoptose induite par le Fas chez les hépatocytes de souris en culture primaire. En particulier, ces études ont concentré sur l'implication de l'état rédox et de l'activité tyrosine kinase (TK) du récepteur EGF. Il est à noter que l'injection du Fas in vivo résulte en apoptose massive du foie (~100%) alors que seulement 5-10% de hépatocytes en culture meurent par apoptose suite à leur traitement avec le Fas. Nos résultats démontrent ici que la résistance relative des hépatocytes en culture à l'apoptose est due à la perte importante du contenu cellulaire en glutathion (GSH) durant l'isolation et la culture des cellules (totalisant ~75% de perte après seulement 2h en culture). L'augmentation des niveaux de GSH intracellulaire et surtout du ratio de glutathion réduit (GSH)/total (GSx), en enrichissant le milieu avec la cysteine et méthionine (deux acides aminés précurseurs de GSH) a amené une sensibilité accrue à l'apoptose induite par le Fas. En plus, l'EGF, qui est connu pour son effet anti-apoptotique, a significativement diminué les niveaux de GSH intracellulaires, le ratio GSH/GSx ainsi que l'activité de GSSG réductase. Ces changements se sont produits

en parallèle à l'effet protecteur de l'EGF contre l'apoptose et ils étaient contrecarrés par PD168393, un inhibiteur de l'activité TK du récepteur EGF. La réplétion de la cellule en GSH a diminué l'effet protecteur de l'EGF contre l'apoptose induite par le Fas. Cette modulation de l'apoptose par l'état rédox semble être due à la régulation de l'activité des caspases. Il est à noter cependant, que malgré le fait que PD168393 a aboli l'autophosphorylation du R-EGF, l'activation des voies de MAPK et PI3K ainsi que l'augmentation de l'expression de l'ARNm et de la protéine de BCL-x_L, il n'a pas complètement inhibé l'effet anti-apoptotique de l'EGF. Ceci suggère que des mécanismes indépendants de l'activité TK du récepteur EGF participe aussi dans la protéger contre l'apoptose. En conclusion, un état rédox oxydative semble protéger contre l'apoptose induite par le Fas dans les cultures primaires d'hépatocytes de souris. Ce processus est même utilisé par les facteurs de croissance, tel que l'EGF, et il est dépendant sur l'activité TK du récepteur.

Mots-clés : apoptose, hepatocytes, Fas, EGF, BCL-x_L, caspases, glutathion, état rédox, protection, activité tyrosine kinase

Abstract

The liver is highly sensitive towards apoptosis. Numerous injurious events result in the loss of hepatocytes by apoptosis, including hepatitis C, B and alcoholic liver disease. Since hepatocytes constitutively express Fas-R, the Fas system has been involved in the development of liver pathologies. Therefore, the aim of these studies was to investigate the mechanisms of protection afforded by epidermal growth factor (EGF) against Fas-induced apoptosis in primary mouse hepatocyte cultures. In particular, these studies focused on the involvement of the intracellular redox state and of the tyrosine kinase (TK) activity of the EGF receptor. Of note, *in vivo* injection of anti-Fas results in massive apoptosis of the liver (~100 %) while exposure of primary hepatocytes cultures to Fas causes 5-10 % apoptotic bodies only. Here, our findings indicate that the relative resistance of mouse hepatocytes in culture toward Fas-induced apoptosis is due to the loss of the important stocks of glutathione (GSH) during the isolation procedure and culture (totalling ~75% loss after 2h culture). The increase of intracellular GSH levels and especially of the reduced (GSH)/ total (GSx) glutathione ratio, by supplementing the media with cysteine and methionine (precursor amino acids of GSH), enhanced cell sensitivity toward Fas-induced apoptosis. Furthermore, EGF, a known anti-apoptotic agent, significantly lowered GSH levels and GSH/GSx ratio in a time- and dose-dependent manner. In fact, EGF treatment decreased GSSG reductase activity, which may account for the decline in GSH/GSx ratio. These changes occurred in parallel to the anti-apoptotic effect of EGF on mouse hepatocytes in culture and were inhibited by PD168393, a specific inhibitor of the TK activity of the EGF

receptor. Cell replenishment with GSH diminished the protective effect of EGF against Fas-induced apoptosis. This redox modulation of apoptosis seems to occur through regulation of the activity of caspases. Of note, and in spite of the fact that PD168393 has effectively abolished the autophosphorylation of the EGF-R, the activation of MAPK and PI3K pathways as well as the induction of BCL-x_L mRNA and protein expressions, it did not completely inhibit the anti-apoptotic effect of EGF. This indicated that some TK-independent pathways are involved in the anti-apoptotic signal of EGF against Fas-induced apoptosis. In conclusion, an intracellular oxidative state seems to protect against Fas-induced apoptosis in primary mouse hepatocyte cultures. This mechanism is used by growth factors such as EGF to protect against apoptosis and it is dependent on the TK activity of the receptor.

Keywords : apoptosis, Fas, EGF, BCL-x_L, caspases, glutathione, redox state, protection, tyrosine kinase activity.

TABLE OF CONTENT

TABLE OF CONTENT ..... **vii**

LIST OF TABLES ..... **xii**

LIST OF FIGURES ..... **xiii**

LIST OF ABBRIVIATIONS ..... **xvii**

GENERAL INTRODUCTION **1**

A.CELL DEATH BY APOPTOSIS **2**

1. CELL DEATH: A POINT OF NO RETURN 2

2. TYPES OF CELL DEATH: NECROSIS VS APOPTOSIS 3

2.1. Necrosis *3*

2.2. Apoptosis *4*

3. ROLE OF APOPTOSIS IN HEALTH AND DISEASE 8

3.1. Apoptosis in physiology *8*

3.2. Apoptosis in pathology *10*

4. APOPTOSIS AND THE LIVER 10

4.1. The liver *10*

4.2. Physiological and pathological relevance of liver cell apoptosis *11*

B.APOPTOSIS : CELLULAR MECHANISMS 13

1. INDUCTION OF APOPTOSIS 13

2. EXECUTION OF APOPTOSIS 18

2.1. THE FAMILY OF ICE-PROTEASES (CASPASES)..... 18

2.2. THE PRO-APOPTOTIC PROTEINS OF THE BCL-2 FAMILY 21

2.3. Diablo/Smac proteins..... 23

3. DEGRADATION AND ELIMINATION OF APOPTOTIC CELLS 23

C.FAS RECEPTOR / FAS LIGAND SYSTEM..... 25

1. FAS SYSTEM AND ITS ROLE IN DISEASE..... 25

2. FAS RECEPTOR SIGNAL TRANSDUCTION 26

2.1. FADD pathway: 30

2.2. Fas signalling in hepatocytes (type II cells): 30

2.3. FADD-independent signalling:..... 31

D.ANTI-APOPTOTIC MECHANISMS..... 33

1. GROWTH FACTORS AND PROTECTION AGAINST APOPTOSIS..... 33

2. ANTI-APOPTOTIC PROTEINS OF THE BCL-2 FAMILY 34

3. PHOSPHATIDYLINOSITOL 3-OH KINASE/ Akt PATHWAY 36

4. FLICE-INHIBITORY PROTEIN (FLIP) 37

5. INHIBITOR OF APOPTOSIS PROTEINS (IAP) 38

E. EPIDERMAL GROWTH FACTOR (EGF) 40

1. STRUCTURE OF THE EGF RECEPTOR 40

2. ACTIVATION OF EGF-R : 41

3. EGF-R SIGNAL TRANSDUCTION PATHWAYS 42

3.1. Mitogen-Activated Protein Kinases Pathway (MAPK): 42

3.2. Phosphatidyl Inositol 3-OH Kinase Pathway (PI 3-K): 46

3.3. Phospholipase Cγ Pathway (PLCγ): 46

3.4. Reactive oxygen species (ROS): 47

F. INTRACELLULAR REDOX STATE 48

1. REACTIVE OXYGEN SPECIES (ROS) 49

2. CYTOTOXIC EFFECTS OF ROS 52

3. INTRACELLULAR ANTI-OXIDANT MECHANISMS AND REDOX STATE 56

3.1. Scavengers 56

3.2. Enzymes 57

3.3. GSH system 58

4. REDOX SIGNALLING 62

4.1. Alterations in intracellular redox state 62

4.2. Oxidative modifications of proteins *63*

4.3. Signalling pathways targeted by ROS *64*

4.4. Ligand-induced ROS production *66*

5. REDOX REGULATION OF APOPTOSIS 67

5.1. Oxidative stress sensitizes cells toward apoptosis *67*

5.2. ROS protect cells against apoptosis *68*

CHAPTER 2 OBJECTIVES AND HYPOTHESIS 70

A.RATIONAL 71

B.HYPOTHESES 72

C.OBJECTIVES 73

CHAPTER 3 EXPERIMENTAL RESULTS 74

3.1 ARTICLE NO. 1 RESISTANCE TO FAS INDUCED APOPTOSIS IN HEPATOCYTES: ROLE OF GSH DEPLETION BY CELL ISOLATION AND CULTURE 75

3.2 ARTICLE NO. 2 ROLE OF EGF RECEPTOR TYROSINE ACTIVITY IN ANTI-APOPTOTIC EFFECT OF EGF ON MOUSE HEPATOCYTES 122

3.3 ARTICLE NO. 3 EGF MEDIATES PROTECTION AGAINST FAS-INDUCED APOPTOSIS BY DEPLETING AND OXIDIZING INTRACELLULAR GSH STOCKS 173

CHAPTER 4 DISCUSSION & CONCLUSION **220**

CONCLUSION **230**

BIBLIOGRAPHY **231**

LIST OF TABLES

CHAPTER 1 INTRODUCTION

Table 1 *Comparative table between the morphological and biochemical properties of apoptosis and necrosis* 5

CHAPTER 3 EXPERIMENTAL RESULTS

ARTICLE NO. 2

Table 1 *Effect of PD168393 and Tyrphostin AG1478 on $H_3{}^{32}PO_4$ incorporation into mouse hepatocytes treated with EGF* 172

ARTICLE NO. 3

Table 1 *Effect of GSHmee on the intracellular levels of GSx, GSH and GSH/GSx ratio in mouse hepatocytes treated with EGF* 207

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

Figure 1 *Morphology of dying cells by necrosis and apoptosis* 6

Figure 2 *Mechanisms of induction and inhibition of apoptosis* 16

Figure 3 *Fas receptor signalling pathways* 28

Figure 4 *EGF receptor signalling pathways* 44

Figure 5 *Sources of reactive oxygen species (ROS) and their mechanisms of elimination* 50

Figure 6 *ROS cytotoxic effects* 54

CHAPTER 3 EXPERIMENTAL RESULTS

ARTICLE NO. 1

Figure 1 *Effect of cell isolation and culture on total glutathione (GSx) levels* 108

Figure 2 *Effect of medium N and C+M on Fas-induced cell death* 110

Figure 3 *Effect of medium N and C+M on the expression levels of procapsase-8, Bid and BCL-x_L proteins* 112

Figure 4 *Effect of medium N and C+M on Fas-induced caspase-8 activation* 114

Figure 5 *Effect of extended time in culture on GSx, and GSH levels as well as GSH/GSx ratio in cells cultured in medium C+M* 116

Figure 6 *Effect of time in culture on Fas ability to induce caspase-8 activation and apoptosis in medium C+M* 118

Figure 7 *Modulation of the protease activity of human recombinant activated caspase-8 by GSH/GSx ratio* 120

ARTICLE NO. 2

Figure 1 *Effect Kinetics of EGF-R phosphorylation in mouse hepatocytes treated with EGF in the absence and presence of Tyrphostin AG1478* 160

Figure 2 *Anti-apoptotic effect of EGF on primary mouse hepatocyte cultures subjected to Fas receptor stimulation* 162

Figure 3 *Effect PD168393 on EGF-R phosphorylation in mouse hepatocytes treated with EGF* 164

Figure 4 *Effect of PD168393 on post-receptor events in mouse hepatocytes treated with EGF* 166

Figure 5 *Morphology of EGF-treated mouse hepatocytes in the presence and absence of PD168393* 168

Figure 6 *Effect of PD168393 and Tyrphostin AG1478 on the anti-apoptotic response of EGF in mouse hepatocytes* 170

ARTICLE NO. 3

Figure 1 *Effect of anti-Fas and/or EGF on intracellular glutathione levels and GSH/GSx ratio* 208

Figure 2 *Effect of anti-Fas and/or EGF on the activity of glutathione reductase (GR)* 210

Figure 3 *Time-course and dose-response curves of intracellular GSx levels and GSH/GSx ratio following exposure to EGF* 212

Figure 4 *Effect of PD168393 on EGF-induced decrease of GSx, GSH levels as well as GSH/GSx ratio and GR activity* 214

Figure 5 *Effect of glutathione monotheyl ester (GSHmee) on protective effect of EGF against Fas-induced caspase-3 activation and cell death by apoptosis* 216

Figure 6 *Effect of GSHmee on EGF receptor (EGF-R) autophosphorylation and procaspase-3 protein expression in EGF treated cultures* 218

LIST OF ABBRIVIATIONS

AIF-1	:	Apoptosis inducing factor-1
ALT	:	Alanine aminotransferase
Apaf-1	:	Apoptosis protease-activating factor-1
ASK-1	:	Apoptosis signal regulating kinase-1
AST	:	Aspartate aminotransferase
ATP	:	Adenosine tris-phosphate
BCL-2	:	B-cell lymphoma
BIR	:	Baculovirus repeats
CAD	:	Caspase dependent DNase
CARD	:	Caspase recruitment domain
Caspases	:	Cysteinyl aspartate-specific protease

DAG : 1,2-di-acyl gllycerol

DAXX : Death associated protein

DD : Death domain

DED : Death effector domain

Diablo : Direct IAP binding protein with low pI

DISC : Death inducing signalling complex

EGF : Epidermal growth factor

EGF-R : EGF receptor

ERK : Extracellular signal regulated kinase

FADD : Fas associated protein with death domain

Fas-L : Fas ligand

Fas-R : Fas receptor

FLIP : Flice inhibitory protein

GCS	:	γ-glutamylcysteine synthetase
GDP	:	Guanyl dis-phosphate
GF	:	Growth factors
GS	:	Glutathion synthetase
GSH	:	Glutathione, reduced form
GSSG	:	Glutathione, oxidized form
GSx	:	Total Glutathione
GTP	:	Guanyl tris-phosphate
HGF	:	Hepatocyte growth factor
IAP	:	Inhibitors of apoptosis proteins
ICAD	:	Inhibitor of caspase dependent DNase
ICE	:	Interleukin 1β converting enzyme
IL-3	:	Interleukin 3

IP_3	:	Inositol 1,4,5- tris-phosphate
JNK	:	Jun-amino terminal kinase
LDH	:	Lactate dehydrogenase
MAPK	:	Mitogen-activated protein kinase
MEK-1	:	MAPK kinase-1
MPT	:	Mitochondrial permeability transition
NAD(P)H	:	Nicotimide adenine denucleotide (phosphate), reduced form
NGF	:	Nerve growth factor
O_2^{-}	:	Superoxide dismutase
OH·	:	Hydroxyl radical
PARP	:	Poly(ADP-ribose)polymerase
PI	:	Phosphatidylinositol
PI 3-K	:	Phosphatidylinositol 3'-OH kinase

PIP_2	:	Phosphatidylinositol 4,5-dis-phosphate
PKC	:	Protein kinase C
PLCγ	:	Phopholipase Cγ
PTH	:	Phosphotyrosine homology domain
ROS	:	Reactive oxygen species
RTK	:	Receptors tyrosine kinase
SH-	:	Sulfhydryl group
SH-2	:	Src-homology-2 domain
Smac	:	Second mitochondrial activator of caspases
SOS	:	Son of Sevenless
TGF-α	:	Tumor growth factor-α
TK	:	Tyrosine kinase

TNFα : Tumor necrosis factor α

TRADD : TNF receptor associated protein with death domain

TRX : Thiorodoxin

To my parents,

To my brothers and sister,

To all my family and friends,

For their support and for the good and the bad

moments that we shared during this adventure.

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CHAPTER 1

GENERAL INTRODUCTION

A. CELL DEATH BY APOPTOSIS

1. CELL DEATH: A POINT OF NO RETURN

Cell structure and function are maintained in dynamic stability by cell's genetic program. Preserving this intracellular dynamic equilibrium in spite of the normal physiological demands, the constrains of neighbouring cells and the availability of metabolic substrates is called homeostasis. Disturbance of this equilibrium by more excessive physiological stresses or pathological stimuli may bring about a number of physiological and morphological cellular adaptations, in which a new but altered steady state is achieved to preserve the viability of the cell and to modulate its function as a response to such stimuli. If the limits of adaptive response to a stimulus are exceeded, or in certain instances when adaptation is not possible, a sequence of events follows, loosely termed cell injury. Cell injury is reversible up to a certain point, but if the stimulus persists, or if the stimulus is severe enough from the beginning, the cell reaches the point of no return and suffers irreversible cell injury and cell death.(1)

Cell death, the ultimate result of cell injury, is one of the most crucial events in pathology, affecting every cell type and being the major consequence of ischemia, infection, toxins and immune reaction. In addition, it is critical during embryogenesis, lymphoid tissue development and hormonally induced involution. Finally, it is the aim of cancer radiotherapy and chemotherapy.(1)

2. TYPES OF CELL DEATH: NECROSIS VS APOPTOSIS

The causes of cell injury and cell death range from external stimuli (such as physical, chemical, and infectious agents,) to internal causes (such as hypoxia, immunological reactions and genetic alterations). There are two major morphological types of cell death, necrosis and apoptosis. Cell death by necrosis is normally observed after a pathological or injurious event. Apoptosis is a programmed type of cell death which necessitates the active participation of the cell and one which, in contrast to necrosis, is associated with normal physiology in addition to pathological conditions (2-4).

2.1. Necrosis

Morphologically, necrosis is characterized by organelle and general cellular swelling, loss of membrane integrity (organelles and plasmatic) and rupture of lyzosomes leading to membrane breakdown and cell lysis (see figure 1) (2;3;5). These morphological phenomena are the result of important biochemical disturbances of intracellular homeostasis including depletion of adenosine tris-phosphate (ATP), loss of selective membrane permeability and impairment of ionic pumps function. These cause the loss of ionic homeostasis (calcium, potassium and sodium) and the leak of cytoplasmic enzymes (e.g. alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH)). Necrosis induces a local inflammatory reaction aimed at eliminating the necrotic cells. However, this induction ends up aggravating the tissue damages triggered by the initial insult (3;5).

2.2. Apoptosis

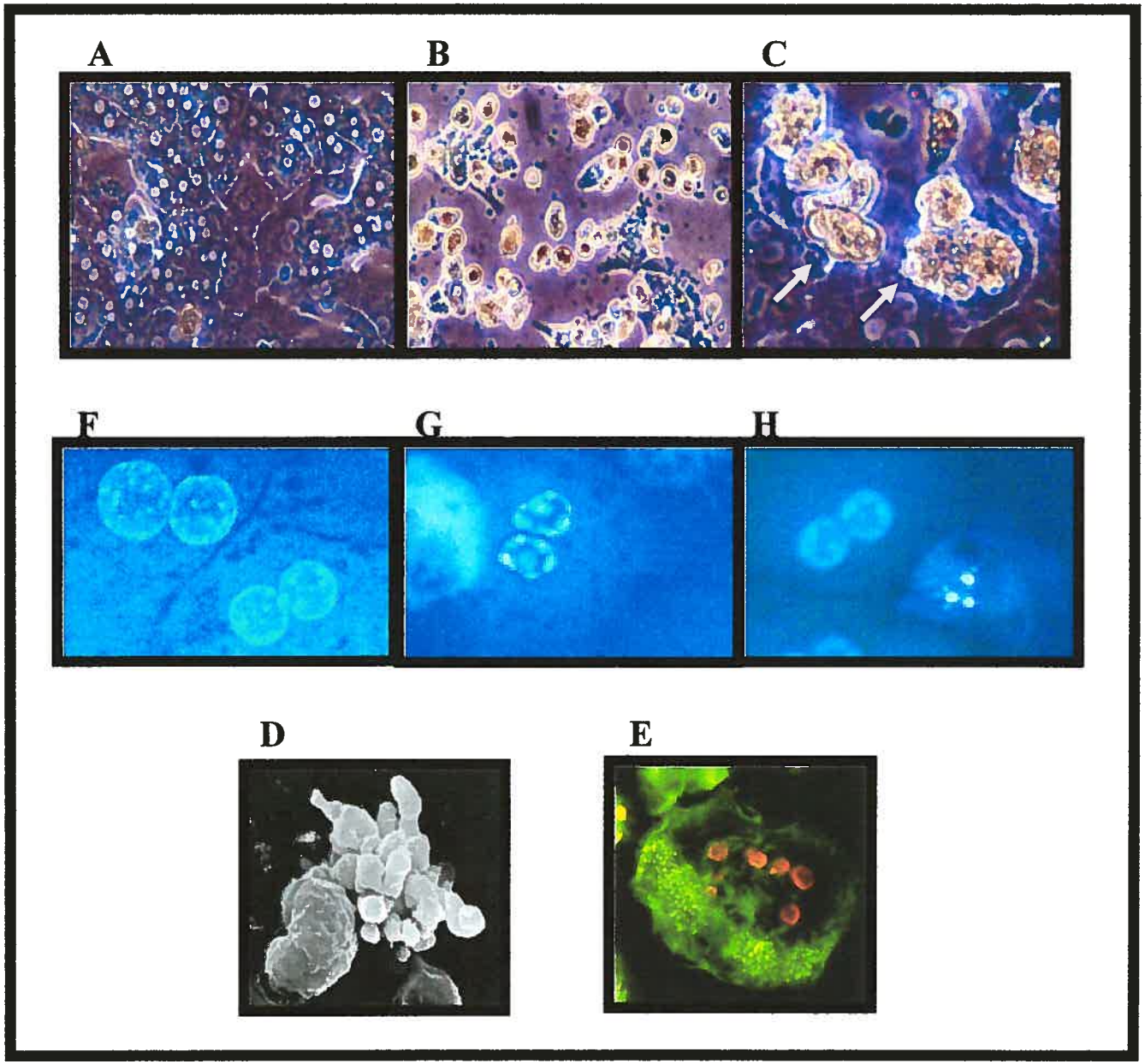
The second form of cell death is apoptosis. The term apoptosis was first used by Kerr, Wyllie and Currie in 1972 (6), to distinguish a form of cell death morphologically distinct from necrosis. Apoptosis is characterized by cytoplasmic shrinking, membrane blebbing, dilated endoplasmic reticulum, and nuclear chromatin condensation and fragmentation (see Figure 1)(7). Mitochondria remain unchanged morphologically, but its energetic function is severely compromised since mitochondrial potential dissipates. Activation of caspases (Cysteinyl aspartate-specific protease; (8)), endonucleases (9;10) and transglutaminases (11) are responsible for these morphological changes (3;12;13). In final stages of apoptosis, the cell separates into intact, membrane-bound apoptotic bodies (14) (see Figure 1). This type of cell death is often hard to observe *in vivo* under physiological conditions. Indeed, apoptosis does not normally induce an inflammatory reaction and the dying cells are rapidly phagocytosed by non-activated tissue macrophages. (3;6;15)

Table 1 ***Comparative table between the morphological and biochemical properties of apoptosis and necrosis***

	Necrosis	**Apoptosis**
Inducers	Pathological	Physiological or pathological
Regulation	No	Yes
Morphological criteria		
Cell volume	Oedema	Retraction
Nucleus	Chromatin precipitation	Chromatin condensation and fragmentation
Plasmatic membrane	Loss of impermeability/ Ruptured	Impermeability preserved/ Intact but with blebbs
Inflammatory reaction	Present	Physiologically - absent Pathologically -present
Biochemical criteria		
Nuclear DNA	Random digestion	Internucleosomal fragmentation
Mitochondrial anomalies	Present	Present
Intracellular Ca^{+2} anomalies	Present	Present
Proteases activation	Non specific proteases	Specific proteases *(caspases)*

Figure 1 ***Morphology of dying cells by necrosis and apoptosis***

Morphology of normal **(A)**, necrotic **(B)** and apoptotic **(C)** mouse hepatocytes, where arrows indicate apoptotic bodies. Hepatocyte nuclei can be visualized under a microscope equipped with ultraviolet epifluorescence following staining with Hoechst 33258, a specific DNA fluorochome. Normal nuclei appear homogenous and intact **(D)**, as opposed to apoptotic nuclei which are condensed **(E)**, fragmented and very bright **(F)**. In panel **(G)**, a T-cell undergoing apoptosis show the characteristic blebbing of the cytoplasmic membrane (photo from *Zimmermann, K.C. et al. Pharmacology & Therapeutics (2001) 92: 57-70*). Non-activated tissue macrophages **(panel H)**, stained with fluorescein, ingest apoptotic bodies (stained with rodamine) to prevent inflammation (photo from *Savill, J. et al. Nature (2000) 407: 784-788*).


A
B
C
F
G
H
D
E

3. ROLE OF APOPTOSIS IN HEALTH AND DISEASE

3.1. Apoptosis in physiology

The concept of spontaneous cell death as a physiological event was first reported by Walther Flemming in 1885 who noticed that many of the epithelial cells lining the regressing ovarian follicules showed fragmented nuclei typical of what is now known as apoptosis that he termed "chromatolysis". (2) Later on, embryologists also understood the importance of "chromatolysis" as a morphogenetic mechanism during embryogenesis. Indeed, the formation of digits, the development of the immune (through the elimination of self-reactive lymphocytes) and of the nervous systems were all shown to necessitate this special kind of cell death. However, these finding were thought to be limited to embryonic tissue. (2)

Why the explosion in studies in articles related to apoptosis? In 1972, Kerr et al. (6) have documented the diversity of the occurrence of apoptosis in different tissues and in a wide range of physiological and pathological conditions. Later, Wyllie (10) has demonstrated that nuclear apoptotic events included the activation of an endonuclease that resulted in the degradation of chromatin into nucleosome-sized DNA ladders that have become a biochemical hallmark of apoptosis. However, these findings remained without significant recognition for almost a decade. The discovery that the adult hermaphrodite *Caenorhabditis elegans* forms 1090 somatic cells, of which precisely 131 cells die by

apoptosis, hurtled apoptosis into hot topic status in the 1990s. (16) The study of mutants of *C. elegans* in the apoptotic processes identified 3 genes which are involved in the regulation of apoptosis in all somatic cells (ced-3, ced-4 and ced-9). In addition, these genes have been shown to have mammalian homologues (caspases (17), apoptosis protease-activating factor-1 [Apaf-1] (18) and B-cell lymphoma [BCL-2] (19) families of genes respectively)(20). With this regain of interest in apoptosis, researchers have focused on the physiological aspects of apoptosis as a widespread phenomena. Indeed, apoptosis has been shown to be involved in several physiological processes, such as:

- Acquisition of immunological self tolerance in T cells (21),
- Elimination of virally-infected cells by cytotoxic T cells (21),
- Down-regulation of the immune reaction after inflammatory reaction (Activated-induced cell death) (21;22),
- Organ atrophy following hormonal retrieval (23),
- Establishment of the immune privileged sites (such as testes, ovaries, uterus and retina) (22;24)
- Maintenance of tissue homeostasis (i.e. appropriate number of cells) and is therefore considered as the counterpart of mitosis (25).

3.2. Apoptosis in pathology

Given the widespread and critical role of apoptosis in biology, it is not surprising that deregulation of apoptosis occurs frequently during pathological conditions. Indeed, the aberrant activation of apoptosis may contribute to a number of diseases such as neurodegenerative disorders (e.g. Alzheimer and Parkinson) (26) and viral infection. Several viruses were shown capable of inducing (27-29) or inhibiting (30-32) apoptosis directly in infected cells or to induce their elimination by cytotoxic T cells through activation of the Fas receptor (Fas-R) and/or Granzyme B systems (33-35). In contrast, impaired apoptosis may be a significant factor in the etiology of diseases such as cancer (36;37) and autoimmune disorders (systemic lupus erythematosis (34) and lymphoproliferative disorders (38; 39;40)).

4. APOPTOSIS AND THE LIVER

4.1. The liver

The liver, which constitutes approximately 2% of body weight in adult humans, resides at the crossroads between the digestive tract and the rest of the body. Hence, it plays a major role in maintaining the body's metabolic homeostasis. This includes ***1)*** the uptake of dietary amino acids, carbohydrates, lipids and vitamins, their subsequent storage, metabolism and release into the blood and the bile; ***2)*** the biotransformation of hydrophobic substances (circulating metabolites, endogenous waste products and xenobiotics (1)) into

water-soluble derivatives that can be excreted into the bile or the urine; ***3)*** the synthesis of serum proteins such as albumin, vitamin transporters and blood clotting factors. In addition, the liver is integrated into the reticuloendothelial system of defence of the body against foreign macromolecules and microorganisms. (41)

Hepatic disorders therefore have severe consequences on overall health. The liver is vulnerable to a wide variety of metabolic, toxic, microbial, circulatory and neoplastic insults that cause liver injury both by necrosis and apoptosis (1;41;42).

4.2. Physiological and pathological relevance of liver cell apoptosis

In normal liver, apoptosis is a rare event. Indeed, only about 1-5 apoptotic cells are found out of 10 000 hepatocytes in the rodent liver. (43) This very low rate could be explained by the low rate of turnover of hepatocytes under physiological conditions. However, this rate is increased during liver involution to remove excessive hepatocytes. This can occu following withdrawal of hypertrophic or hyperplasic stimuli (e.g. increased functional demand, over feeding, pregnancy or during severe protein loss) (42) or during liver atrophy following left portal vein ligation (14;44). Furthermore, aberrant activation of apoptosis has been noted in metabolic liver diseases such as alcoholic liver disease (where high levels of Fas ligand were detected, see below). (45) In addition, several studies have reported a marked increase in the levels of apoptosis in fulminant hepatitis (46) and viral infections such as hepatitis B(42;47) and hepatitis C(48), as well as in immune-mediated liver diseases, such as host-versus-graft reaction and allograft rejection (42). On the other hand,

failure of apoptosis to delete genetically altered cells appears to contribute to the development of hepatocellular cancer and cholangiocarcinoma. (37)

Based on this evidence, new therapeutic approaches could be developed to treat theses diseases. Indeed, pharmacological inhibition of apoptosis may ameliorate liver injury and/or promote liver regeneration. On the other hand, purposeful induction of apoptosis in malignant cells may be useful in treating hepatobiliary cancers. Thus, understanding the mechanisms of apoptotic induction and inhibition is highly relevant for the clinical diagnosis and treatment of liver diseases. (49)

B. APOPTOSIS : CELLULAR MECHANISMS

The apoptotic process is divided into three phases: induction, execution, and finally degradation and elimination. During the induction phase, cells receive signals to initiate the apoptotic process, decode and interpret them. When the cell takes the decision to commit suicide, it activates the executioner caspases: this is the point of no return. These caspases then cleave multiple intracellular targets (such as endonucleases, transglutaminases, other caspases) to produce the biochemical (DNA ladder, protein precipitation and degradation of cytoskeleton components) and morphological (chromatin fragmentation, membrane blebbing, cytoplasm disintegration) alterations characteristic of apoptosis. Finally, apoptotic bodies are phagocytosed by macrophages and neighbouring cells. (7;15) (see Figure 1)

1. INDUCTION OF APOPTOSIS

Apoptosis can be initiated by different signals from outside as well as inside the cell :

1. Cell-surface death receptors of the Tumour Necrosis Factor α (TNFα) family, such as Fas and TNF receptors, are among the best characterized extracellular inducers of apoptosis. Binding of ligands to their receptors initiates a fairly well-defined caspase signalling cascade that activates, in an amplification loop, other executioner caspases directly (type I cells) or through cleavage of pro-apoptotic proteins of the

BCL-2 family. The latter then transmit the signal through activation of the mitochondrial apoptotic pathway (type II cells, see below). (50-53)

2. Granule-mediated apoptosis: granules released by cytotoxic T cells contain perforin (a pore-forming protein which facilitates the entry of granules components into the target cell) and Granzyme B (a serine protease which cleaves after an aspartate residue) (54). It has been suggested that Granzyme B can directly cleave and therefore activate caspase-3, which then activates caspase-7. (55)

3. Genotoxic agents, such as topoisomerase inhibitors (e.g. etoposide), anti-metabolites (e.g. 5-fluorouracil), DNA damaging agents (like *cis*platin) and γ–irradiation, are among the best-studied initiators of apoptosis. Damage to DNA is sensed by p53, which upregulates Bax and therefore activates the mitochondrial apoptotic cascade. (53;56-58)

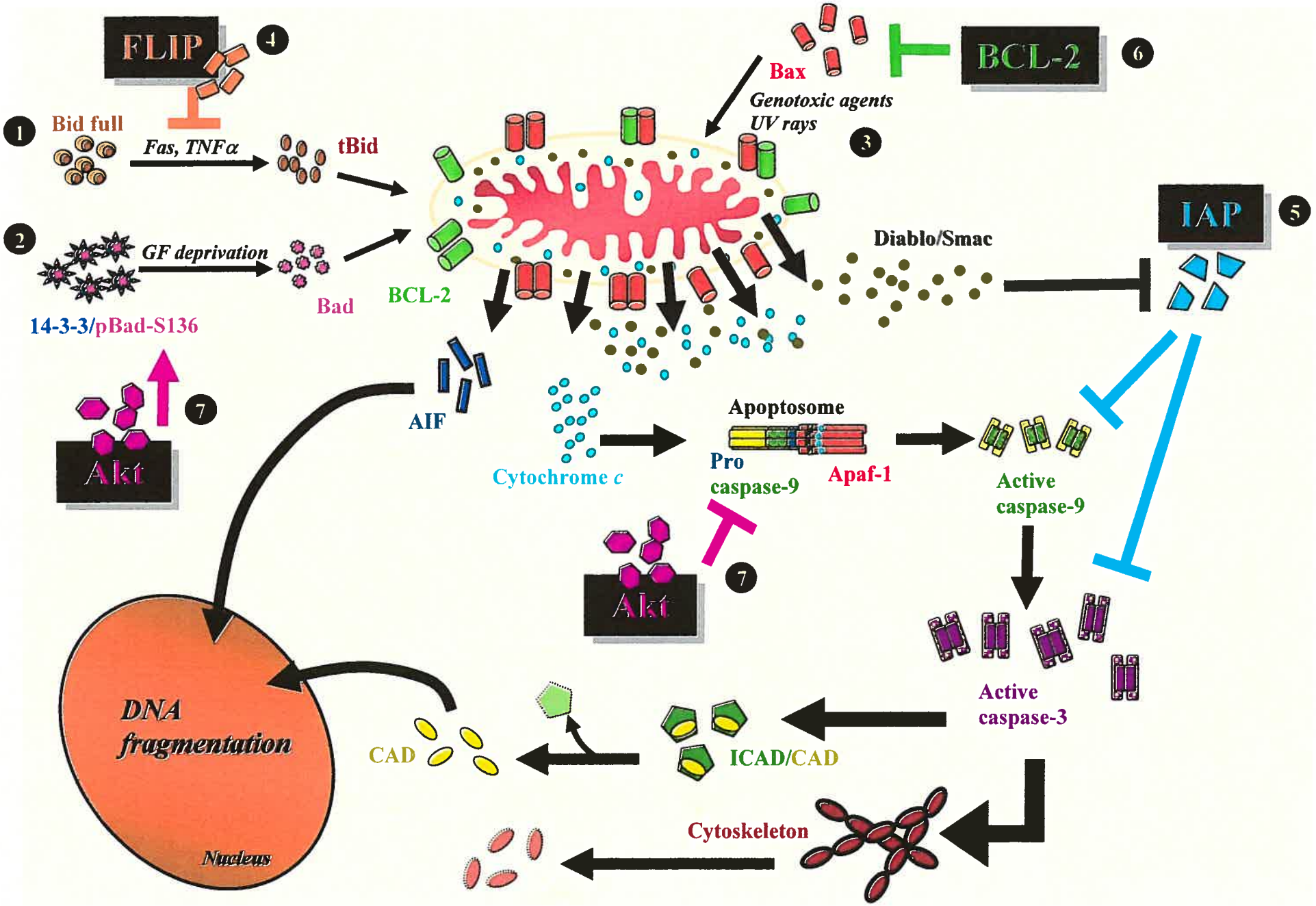
4. Loss of survival signals, such as growth factors (e.g. epidermal growth factor [EGF], hepatocyte growth factor [HGF], nerve growth factor [NGF], etc) and contact with the extracellular matrix, which are normally perceived by cells, activates the apoptotic program by default. (59-61) This has been shown to occur through the association of Bad with Bax, two pro-apoptotic proteins of the BCL-2 family (see below), in the cytoplasm thus favouring the oligomerization and the translocation of the complex to the mitochondria. The sequence culminates with the initiation of the downstream caspases. (62-66)

5. Mitochondrial-elicited death pathway: numerous pro-apoptotic pathways converge on the mitochondria to induce mitochondrial permeability transition (MPT): the outer membrane becomes protein-permeable while the inner membrane continues to retain matrix proteins but dissipates the mitochondrial transmembrane permeability ($\Delta\Psi$). MPT triggers the activation of caspases and nucleases through the release of pro-apoptotic proteins normally confined to the mitochondrial intermembrane space, such as cytochrome *c* and Apoptosis Inducing Factor-1 (AIF-1). (13). The release of cytochrome *c* stimulates the assembly of the apoptosome (procaspase-9, cytochrome *c* and Apaf-1) in the cytoplasm and therefore the activation of the downstream caspase-3. On the other hand, AIF-1 translocates to the nucleus and activates a nuclear DNase (reviewed in (67;68)).

Figure 2 ***Mechanisms of induction and inhibition of apoptosis***

Pro-apoptotic mechanisms: **(1)** Cell surface death receptors (e.g. Fas, TNFα) activate initiator caspases (e.g. caspas-8) to cleave Bid and generate tBid. **(2)** Growth factors (GF) deprivation induces the dephosphorylation of Bad and its dissociation of 14-3-3 proteins. **(3)** Genotoxic agents increase the expression of Bax in a p53-dependent manner. All these apoptotic stimuli induce the translocation of Bax from the cytosol to the mitochondria. This culminates in the release of cytochrome c and Diablo/Smac. Diablo/Smac relieves the inhibitory effect of IAP. Cytochrome c forms the apoptosome with Apaf-1, procaspase-9 and ATP. This activates caspase-9, which then activates caspase-3. The latter caspase is responsible for the degradation of cytoskeleton components (e.g. fodrin, gelsolin) as well as ICAD (inhibitor of CAD), thus releasing CAD (Caspase Activated DNase). CAD is the DNase responsible for the fragmentation of DNA into 180-200 bp, which are the hallmark of apoptosis.

Anti-apoptotic mechanisms (in Black boxes): There are several checkpoints for where apoptotic signal can be inhibited. **(4)** FLIP is a natural inhibitor of the formation of the DISC complex. **(5)** IAPs (Inhibitors of apoptosis proteins) are natural inhibitors of active caspases. **(6)** Anti-apoptotic members of the BCL-2 (e.g. BCL-2) act directly on Bax activity in the mitochondria. **(7)** Akt, a serine/threonine kinase, phosphorylates Bad on ser136, thus favouring its sequestration with 14-3-3 proteins, and procaspase-9, thus preventing its activation.


FLIP
4
Bid full
1
Fas, TNFα
tBid
2
GF deprivation
Bad
14-3-3/pBad-S136
Akt
7
Bax
Genotoxic agents
UV rays
3
BCL-2
6
BCL-2
IAP
5
Diablo/Smac
AIF
Cytochrome c
Apoptosome
Pro
caspase-9
Apaf-1
Active
caspase-9
Akt
7
Active
caspase-3
DNA
fragmentation
Nucleus
CAD
ICAD/CAD
Cytoskeleton

2. EXECUTION OF APOPTOSIS

During this phase, the fate of the cell is set. Indeed, the cellular response to the above mentioned apoptotic signals is reversible and depends on the intracellular context. Irreversible activation of the apoptotic process can be facilitated (pro-apoptotic) or abrogated (anti-apoptotic) by specific proteins whose expression and activity are under tight control. In this section, the pro-apoptotic proteins will be discussed. The anti-apoptotic proteins will be detailed in section D.

There are three key pro-apoptotic families of genes:

1. ICE (interleukin-1 converting enzyme) family, which are now best known as caspases;
2. Pro-apoptotic members of the BCL-2 family (e.g. Bid, Bax);
3. Inhibitors of Inhibitor Apoptosis Proteins (e.g. Diablo/Smac)

2.1. THE FAMILY OF ICE-PROTEASES (CASPASES)

Proteases: Proteases participate in protein destruction and in the regulation of protein activities in different contexts. They are involved in protein maturation (e.g. caspase-1 in the context of pro-IL-1β processing), regulate and amplify specific reactions (e.g. the clotting and complement cascades) and allow cells to migrate (e.g. proteases of the

extracellular matrix). In addition, they are responsible for the degradation of proteins outside the cell (digestive proteases) and in a similar albeit more controlled and discriminating manner inside the cell (proteosome). Finally, in the context of cell death, they act as triggers of the apoptotic process, as regulatory elements within it, and ultimately as a subset of the effector elements of the machinery itself. (54)

Caspases: Ced-3 encodes a protein which resembles the mammalian interleukin 1β-converting enzyme (ICE), commonly called caspase-1.(69) They are proteases with a reduced cysteine residue in their active site (QAC̲XG) which confers them the specificity of cleavage after an aspartate residue.(70) This specificity of cleavage is shared with another protease, Granzyme B (see section B.1).

To date, 14 caspases have been identified in mammals. Caspases are constitutively expressed and are normally present as inactive precursors in cells. Caspases have a very conserved structure composed of a N-terminal prodomain of varying length, and a domain that, after processing, will give rise to two subunits: a large subunit (17-21 KDa) which contains the active site, and a small subunit (10-14 KDa). (70-72)

Upon receiving an apoptotic signal, the pro-forms (zymogens) of caspases undergo sequential proteolytic processing at internal aspartate residues (releasing the small subunit then the prodomain) to generate the active enzyme. Caspases then assemble into two large subunits and two small subunits thus forming the active caspases.(12;73;74) In addition, the cysteine in the active site must be reduced in order for the caspases to function. Indeed,

several *in vitro* as well as *in vivo* studies have reported that oxidants inhibit the proteolytic activity of caspases while anti-oxidants restore or maintain their activity. (75-77)

Based on the length of their prodomain, caspases can be divided into two groups: class I, which contains a relatively long prodomain (e.g. caspases 1, 2, 4, 5, 8, 9, 10, 12, and 13) and class II containing a short prodomain (e.g. caspases 3, 6, 7).(54;70) The long prodomains in many class I caspases contain protein-protein interaction domains that play a crucial role in the recruitment of caspases to specific death complexes. This triggers oligomerization of procaspase molecules, thus facilitating their activation by autocleavage. For example, the prodomain of caspases-8 and -10 contain a Death Effector Domain (DED), which mediates their binding to adaptor molecules FADD (Fas Associated protein with Death Domain) (78;79) or TRADD (TNF Receptor Associated protein with Death Domain) (80). Other caspases (such as 1, 2, 4, and 9) contain a Caspase Recruitment Domain (CARD, (81), which plays an important role in the interaction between different caspases as well as with various adaptor proteins. Class II caspases, which lack a long prodomain, also lack the ability to auto-activate and require cleavage by class I caspases to get activated.(54;70;74) For this reason, class I caspases are referred to as initiators caspases while class II as executioner ones. The fact that caspases can auto-activate and/or activate others constitutes a caspase cascade that not only amplifies the process but also transmits the signal from one compartment to another within the cell. Caspases are activated or inactivated through a series of intracellular steps, or pathways, in response to death or survival signals, which are subject to multiple regulations (see Section D).

2.2. THE PRO-APOPTOTIC PROTEINS OF THE BCL-2 FAMILY

As mentioned above, mitochondria play an important role in apoptosis. In particular, they release AIF-1 and cytochrome *c* (64;68;82), which are responsible for the mediation of the apoptotic signal to downstream effectors. BCL-2 family members (mammalian homologue of ced-9) have been identified as key regulators of cytochrome *c* release.(13;68;83;84)

The BCL-2 family is characterized by conserved motifs known as the BCL-2 homology domain (BH). Members of this family have at least one of the four BH identified. According to their function and structure, these proteins have been subdivided into three subfamilies (68;85):

1. Anti-apoptotic proteins, such as BCL-2, BCL-x_L, BCL-w and MCL-1. BCL-2 and BCL-x_L contain all four BH (BH1-BH4), but other members contain at least BH1 and BH2. (discussed in detail in Section D.2)

2. BH3-only containing pro-apoptotic proteins which include Bid, Bad, Blk.

3. Classical pro-apoptotic proteins such as Bax and Bak, which share sequence homology in all BH domains except BH4.

These proteins can form homo- and heterodimers that involves the BH domains (84;86;87). The BH3 domain of pro-apoptotic proteins appears to be required for the interaction between anti- and pro-apoptotic proteins (88).

BH3-only containing pro-apoptotic proteins: Members of the BH3-only subset of the BCL-2 family connect proximal signals to the core apoptotic machinery. These proteins, of which Bid is a prototype, are capable of heterodimerizing with members of other subsets of the BCL-2 family.(73) They are found in the cytosol under non-apoptotic conditions. Upon receiving the apoptotic signal, they become activated. For example, Bid activation by cleavage (following Fas-R stimulation) favours the interaction of truncated Bid with Bax. This interaction induces a conformational change of Bax leading to its oligomerization and insertion into the outer mitochondrial membrane (65;89;90) and finally culminating in the activation of the downstream caspase cascade.

Classical pro-apoptotic proteins: The expression of Bax and Bak has been associated with high sensitivity toward apoptosis. In fact, genotoxic agents induce apoptosis by increasing the expression levels of Bax. Bax has been shown to form pores in synthetic lipid bilayers that resemble the channel-forming properties of the bacterial toxins *colicins* and *diphteria*.(91) Of note, the channel forming activity of Bax (and Bak) has not been yet demonstrated *in vivo*. Bax translocation to the mitochondria has been shown to cause cytochrome *c* release and activation of caspases, leading to cell death by apoptosis. (64-66) However, the exact mechanism responsible for these proteins' function are not yet elucidated.(64-66;89;90)

2.3. Diablo/Smac proteins

Diablo (direct IAP binding protein with low pI) /Smac (second mitochondrial activator of caspases) is a mammalian protein analogous to the pro-apoptotic Drosophila molecules, Grim, Reaper, and HID. (92;93) It is released from the mitochondria following apoptotic stimulation in a Bid-dependent manner to relieve inhibition of caspases by the inhibitor of apoptosis proteins (IAPs; see section D.5; (94)). Once released from the mitochondria, Diablo/Smac binds to IAPs and antagonizes their anti-apoptotic effect. It therefore assists the initiator caspase-9 and effector caspases (caspase-3, caspase-6, and caspase-7) in becoming active, ultimately leading to cell death (95;96). Extra-mitochondrial translocation of Diablo/Smac along with cytochrome c and other pro-apoptotic proteins represent important regulatory checkpoints for mitochondria-mediated apoptosis.

3. DEGRADATION AND ELIMINATION OF APOPTOTIC CELLS

When the full complement of caspases that are necessary for the proper execution of the cell death program have become activated, the final disintegration phase begins. In this phase, the biochemical bases for the morphological changes take place.

Nucleus: Active caspase-3 is responsible for the cleavage of the Inhibitor of Caspase Dependant DNase (ICAD), an inhibitory protein which retains Caspase Dependent DNase (CAD) in the cytoplasm (97). This degradation releases CAD, which translocates to the nucleus. This nuclease cuts the DNA between the nucleosomes thus producing 180-200 bp DNA fragments. The activity of CAD is responsible for the DNA laddering that is a

hallmark of apoptosis.(98) Lamin A (99), Lamin B (100) and NuMA (101) are also degraded by caspases which translocates to the nucleus (e.g. caspase-6). This leads to the disassembly and the destruction of the nuclear cytoskeleton thus further contributing to nuclear fragmentation. In order to ensure that the damage done by the apoptotic process is not repaired, PARP (poly (ADP-ribose) polymerase), which is involved in the repair of damaged DNA, is also degraded by caspase-3. (102) The mitochondrial protein AIF released in parallel to cytochrome *c* following apoptotic stimulation induces high molecular weight DNA fragments in a caspase-independent manner. (103)

Plasma membrane: Structural breakdown of the cell includes cleavage of fodrin (104), membrane-associated cytoskeletal protein, as well as direct cleavage of gelsolin (105), an actin regulatory protein, resulting the disruption of actin filaments. Taken together these two events can be held responsible for dissociation of the submembrane cytoskeleton and the destabilization of the plasma membrane (membrane blebbing). In addition, the asymmetrical distribution of phosphatidylserine (PS) is lost resulting in the presentation of PS on the external surface of apoptotic cells. This has been shown to facilitate the phagocytosis of apoptotic cells by macrophages and neighbouring cells thus reducing the possibility of an inflammatory response (106;107).

C. FAS RECEPTOR / FAS LIGAND SYSTEM

1. FAS SYSTEM AND ITS ROLE IN DISEASE

In 1989, two groups independently isolated mouse-derived antibodies that were cytotoxic for various human cell lines (108). The cell surface proteins recognized by these antibodies were named Fas and APO-1. The Fas system is composed of Fas receptor (Fas-R) and Fas ligand (Fas-L) (109). Fas-R is highly expressed in activated mature lymphocytes (110) and human lymphocytes transfected with Epstein-Barr virus or human immunodeficiency virus (108;111). In addition, many nonlymphoid tissues express high levels of Fas-R, including liver, heart, lung, kidney and ovary (112). Fas-L is primarily expressed by activated T cells and plays a role in peripheral T- and B-cell homeostasis. (14;34;47;113)

The apoptotic potential of Fas-R / Fas-L system has been mostly studied in the immune system. Indeed, it was observed the *lpr* (lymphoproliferative) and *gld* (generalized lymphoproliferative disorder) phenotypes (39) were associated with progressive lymphadenopathy and immune complex syndrome that resemble the human autoimmune disease systemic lupus erythematosus (38;40). A major role of Fas system in lymphoid cells lies in the control the immune response. It acts as a self-limiting feedback in activated lymphocytes through a process called activation-induced cell death. Indeed, after antigen-driven expansion of lymphocytes, there is upregulation of Fas-L on activated T cells that is required for subsequent death and removal of activated lymphocytes. (20;114) In parallel,

cytotoxic T cells eliminate virally infected and genetically altered cells by activating the Fas apoptotic death signal. (33;34)

One of the early indications that the Fas system plays an important role in hepatic homeostasis and therefore in the development of hepatic disorders is the observation that injection of anti-Fas antibody to mice caused massive hepatic apoptosis and animal demise shortly thereafter (115). Hepatocytes constitutively express Fas-R and may upregulate its expression in a variety of liver diseases (14;42;47). Numerous studies have implicated Fas-R and Fas-L in both hepatitis B (42;47) and C (48), where infected cells appear to be extremely sensitive to Fas-mediated apoptosis (52). In alcoholic liver disease (116) as well as Wilson's disease (117), Fas-L mRNA was found to be expressed in hepatocytes. This expression is hypothesized to mediate hepatocyte loss observed in these disorders. Therefore, the mechanisms of apoptotic induction of the Fas system as well as protection against it in the liver should be of great therapeutic interest.

2. FAS RECEPTOR SIGNAL TRANSDUCTION

The structure of Fas-R indicated that it is a type I transmembrane protein (46 KDa), which belongs to the TNF and NGF receptor family. It possesses 3 cysteine-rich extracellular domains involved in the recognition and binding of the Fas-L. The cytoplasmic domain contains a conserved region (about 70 amino acids) that is necessary and sufficient to transduce the apoptotic signal (118). This domain is called the Death Domain (DD). It is

involved in the recruitment of and the protein-protein interaction with FADD (20;108;113), an adaptor protein that also contains a DD region.

It is well documented that cell death induced by Fas-R stimulation is dependent on caspase activation.(20;51;119) However, there some reports describing a caspase-independent killing.(51;120-122) This led to the hypothesis of two independent signal transduction pathways activated at the level of Fas-R: one is mediated by the adaptor protein DAXX (death associated protein; (123)) which can enhance Fas-induced apoptosis through the activation of jun-amino terminal kinase (JNK) cascade; the other involves the adaptor protein FADD and the subsequent recruitment of caspases to the Death Inducing Signalling Complex (DISC) and therefore the activation of the caspase cascade (51;78;79;124).

Figure 5 ***Fas receptor signalling pathways***

Binding of Fas ligand (Fas-L) to Fas receptor (Fas-R) induces the trimerization of the latter. This activates Daxx- and FADD- dependent signalling pathways. **(1)** In the former, Daxx associates with the death domain (DD) of the Fas-R. This activates Apoptosis Signal regulating Kinase-1 (ASK-1), which results in the activation of the jun-amino terminal kinase (JNK) pathway leading to cell death by apoptosis. **(2)** The FADD pathway is initiated by its recruitment to the Fas-R. Procaspase-8 molecules are then recruited to form the Death Inducing Signalling Complex (DISC) and therefore get activated. **(2a)** In type II cells (e.g. hepatocytes), caspase-8 cleaves Bid to generate truncated Bid (tBid). tBid associates with Bax protein in the cytoplasm and induce its translocation to the mitochondria resulting in the release of cytochrome c and Diablo/Smac proteins into the cytoplasm. Diablo/Smac relieve the inhibitory effect of IAP (Inhibitors of Apoptosis Proteins) thus facilitating the activation of caspases. Cytochrome c along with Apaf-1 (apoptosis protease activating factor-1), procaspase-9 and ATP form the apoptosome, which culminate in the activation of caspase-9. Caspase-9 then cleaves and activates caspase-3. **(2b)** In type I cells (e.g. lymphocytes), caspase-8 directly cleaves and activates caspase-3. Active caspase-3 degrades several cellular components leading to cell death by apoptosis.

2.1. FADD pathway:

Fas-induced activation of the caspase signalling cascade also involves two major pathways termed type I and type II. Basically, they differ in the involvement of the mitochondria in Fas signal transduction. In type I cells, such as lymphocytes, there is activation of a sufficient amount of initiator caspases at the receptor level to directly transmit the signal to effector caspases. (20;125) Hepatocytes are type II cells, where the signal generated at the receptor is not strong enough to transduce the signal and needs to be amplified. Therefore, the weak apoptotic signal is transmitted to the mitochondria where it induces MPT and activates downstream caspases (see Section B.1 and below).(51;119)

2.2. Fas signalling in hepatocytes (type II cells):

Binding of the Fas-L or of anti-Fas antibodies to Fas-R in hepatocytes results in receptor trimerization. The clustering of the DD in the intracellular portion of the receptors recruits the adapter molecule called FADD via the DD of the latter (50;79). Procaspase-8 molecules (FLICE) are recruited to the Fas-R/FADD complex through an interaction mediated by the DED present on FADD along with the prodomain of caspase-8, thus forming the DISC complex. The close proximity of procaspase molecules favours their oligomerization, and stimulates their autocleavage resulting in their activation.(79;119) Active caspase-8 then cleaves Bid, a BH3 only pro-apoptotic protein of the BCL-2 family. This generates a p15-Bid fragment (tBid) which translocates to the outer membrane of the mitochondria.(73) tBid can interact, through its BH3 domain, with the anti-apoptotic BCL-x_L which

destabilizes the BCL-x_L/BAX heterodimers.(126) This favours the homo-oligomerization of BAX. In addition, this association prevents the formation of the anti-apoptotic complex between BCL-x_L and Apaf-1/procaspase-9, leading to the release of the latter. (73;127) Consequently, cytochrome *c* (82)as well as Diablo/Smac (92;93) are released from the mitochondria, although the mechanisms of this liberation are not yet fully elucidated. (51;82;119;128)

Once released from the mitochondria, cytochrome *c* interacts with Apaf-1 and procaspase-9 (in an ATP-dependent manner) to form a complex known as the apoptosome (83;127). Formation of the apoptosome results in the cleavage and activation of caspase-9, which in turn leads to the cleavage and activation of caspase-3, a central executioner caspase.(73) Caspase-3 then cleaves and activates downstream caspases and other important proteins, which are directly or indirectly responsible for the systematic dismantling of the apoptotic cell and finally culminating in cell death by apoptosis. (see Section B.3)

2.3. FADD-independent signalling:

Several pieces of evidence demonstrated the involvement of caspase-independent pathways in the modulation of the apoptotic signal.(120;122;124) One of these pathways is the JNK pathway.(129) The involvement of this pathway in apoptosis was initially characterized in UV irradiation and TNF-α treatment. (129-131) Fas-mediated apoptosis was shown to require JNK activation in 293 and L920, but not in HeLa cells (123). On the other hand, a SEK-1 (upstream activator kinase of JNK) mutant was found to sensitize thymocytes to

Fas-induced apoptosis (132). This suggested that the involvement of the JNK pathway in Fas-induced apoptosis was cell specific, which signifies that the biological relevance of its implication requires further investigation.

The JNK pathway was linked to the Fas-R through the discovery of the DAXX protein in 1997 using an interaction trap system in yeast (123). DAXX associates with the DD of Fas-R, although it lacks a DD of its own. In addition, Yang et al. have shown that the simultaneous overexpression of Fas-R and DAXX activated the JNK pathway and enhanced Fas-induced cell death by apoptosis. This effect of DAXX was shown to be mediated by the MAP kinase kinase kinase ASK-1 (Apoptosis Signal regulating Kinase-1) (121;133).

D. ANTI-APOPTOTIC MECHANISMS

1. GROWTH FACTORS AND PROTECTION AGAINST APOPTOSIS

Not all Fas-bearing cells are susceptible to Fas-induced apoptosis. Indeed, depending on the extracellular environment and the intracellular context in which the apoptotic signal is received, the cell may or may not undergo apoptosis. Growth factors are known for their ability to antagonize apoptosis. Growth factors such as NGF, HGF, EGF, and IL-3 have been shown to protect various types of cells (e.g. astrocytes, PC12, hepatocytes, HepG2, lymphocytes, fibroblasts, etc.) against a variety of apoptotic inducers including physical ones (UV, γ-radiations), chemical ones (*cis*platinum, 5-Fluoracil), and cell-surface death receptors (Fas, TNF) (46;134-136). This protection has been mainly associated with the increase in the expression of the anti-apoptotic members of the BCL-2 family (e.g. BCL-2 and BCL-x_L) (137;138), as well as the inactivation of pro-apoptotic proteins (e.g. Bad, caspase-9), the latter following their phosphorylation by the serine/threonine kinase Akt (see below). (62;139;140)

Cells possess anti-apoptotic mechanisms to prevent the unintentional activation of the apoptotic program. Several of these mechanisms were discovered due to their abnormal expression (lack of or over expression) in diseases such as cancers and viral infections. Many types of cancer have developed mechanisms to avoid apoptosis. These include overexpression of anti-apoptotic proteins of the BCL-2 family, downregulation and

prevention of the recruitment of initiator caspases, overexpression of IAP or expression of decoy receptors (intact extracellular domain but truncated intracellular domain) (36).

In parallel, several viruses have adapted anti-apoptotic mechanisms to delay and even prevent the onset of the apoptotic program in infected cells in order to give them the time to replicate. Herpes virus (141), Baculovirus (30;142), and Polyomavirus Middle T antigen (143) are some examples of these viruses.

Here will be discussed some of the key inhibitory factors of apoptotic signalling, including the BCL-2 family, IAP, FLIP and Akt. (see Figure 2)

2. ANTI-APOPTOTIC PROTEINS OF THE BCL-2 FAMILY

The original protein BCL-2 was discovered in 1985 by Tsujimoto et al. (144) in follicular B cell lymphoma, hence its name B-cell lymphoma-2 (BCL-2). In this type of cancer, the BCL-2 gene is translocated from chromosome 18 (where it normally resides) to chromosome 14 where it becomes juxtaposed with the immunoglobulin heavy-chain (IgH) locus. Such translocation brings this anti-apoptotic gene under the influence of a strong promoter whose normal job is to drive high levels of Ig gene expression in B-cells. This results in the loss of normal regulation of the BCL-2 gene, and in the production of inappropriately high levels of BCL-2 protein in germinal center B-cells. This contributes to the neoplastic B-cell expansion by preventing cell turnover (by inhibiting apoptosis) rather than accelerating rates of cell division. BCL-2 is thus the first example of a human proto-

oncogen that functions through effects on cell death rather than cell cycle mechanisms (85). Furthermore, its expression increases the survival of cells deprived of trophic factors such as growth factors (GF) and cytokines. In addition, the anti-apoptotic effect of several GF has been associated with the increase in the expression levels of BCL-2 or BCL-x_L proteins (86;137).

These proteins have been localized in the membranes of mitochondria, nucleus and endoplasmic reticulum. (67) BCL-x_L was the first member of this family to be crystallized. This revealed a structure similar to the pore-forming bacterial toxins (colicins and diphteria) (91;145). As mentioned above, proteins of the BCL-2 family can form homo- and heterodimers.(85;146;147) The balance between cell survival and death seems to be regulated by the proportion of anti- or pro-apoptotic dimers formed (146;147) where by overexpression of BCL-2 would favours the formation of BCL-2/BCL-2 dimers. The high proportion of anti-apoptotic dimers prevents the release of cytochrome *c* and the subsequent transmission of the apoptotic signal (7;68;85;86).

Several hypotheses regarding the anti-apoptotic action of BCL-2 and BCL-x_L proteins have been put forth, including regulation of the mitochondrial megachannel (148;149)and anti-oxidant function (67;86;150;151). However, their exact mechanism of action is not yet known.

3. PHOSPHATIDYLINOSITOL 3-OH KINASE/ Akt PATHWAY

The PI 3-K is one of the phosphorylation pathways activated by GF (see Section E.3.2). Several studies have recently implicated this pathway, and especially the downstream serine/threonine kinase Akt, in the protection against apoptosis. Indeed, inhibition of the PI 3-K pathway by wortmannin or LY294002 sensitizes cells to apoptosis (152). In parallel, wortmannin treatment was found to enhance Fas- and TNF-α-induced caspase-3 activation.(153) This effect was shown to result from two phosphorylation events upstream of casapse-3 activation: 1) phosphorylation of the pro-apoptotic protein Bad (62;154-156); and 2) phosphorylation of procaspase-9.

As mentioned above, Bad can heterodimerize with BCL-2 and BCL-x_L to antagonize their anti-apoptotic effect. GF, such as IL-3 and NGF, specifically induce the phosphorylation of Bad on serine 136 (pBad-136) through Akt (62;156;157). This favours the association of pBad-136 with 14-3-3 proteins instead of BCL-2 or BCL-x_L therefore sequestering Bad in the cytoplasm and preventing it from translocating to the mitochondria.(139) Thereby, this phosphorylation promotes cell survival by allowing the unhindered action of anti-apoptotic proteins of the BCL-2 family (139;155;156).

In parallel, it was demonstrated that serine phosphorylation of pro-caspase-9 (ser196) prevents its autocleavage and therefore its activation (158). In fact, cytochrome *c*-induced proteolytic processing of procaspase-9 was defective in cytosolic extracts from cells expressing constitutively active Akt (140;158;159). Therefore, the activation of the

PI 3-K/Akt-dependent phosphorylation inactivates the important apoptotic mitochondrial pathway. These two combined phosphorylation events contribute to the protective effect of PI 3-K/Akt activation against apoptosis.

4. FLICE-INHIBITORY PROTEIN (FLIP)

The formation of the DISC complex in cell-surface death receptor signalling, such as Fas and TNF-α, can be regulated by a family of proteins called FLIP (FLICE-inhibitory protein). This family of inhibitors was originally identified in oncogenic viruses, including Kaposi's sarcoma-associated human Herpes virus-8 (HHV-8) (160) and molluscipox virus (141;161). Furthermore, high levels of FLIP were detected in melanomas where it promotes escape from T-cell induced apoptosis (160). In parallel, some studies have suggested that T-cell sensitivity toward Fas-induced apoptosis was correlated with the decrease in FLIP mRNA expression levels (162). Therefore, FLIP seems to inhibit apoptosis, and especially the activation of procaspase-8. FLIP contains two DED that mediate FLIP binding to the prodomain of caspase-8 and -10. This prevents the recruitment of procaspase molecules to these receptors and their subsequent activation(162;163). There are different forms of FLIP. The longest form (FLIPL) has, in addition to the two DED, the equivalent of an inactive caspase domain.

5. INHIBITOR OF APOPTOSIS PROTEINS (IAP)

Recently, key intrinsic inhibitors of caspases called IAP have been identified and they represent important regulatory factors in apoptotic signalling. They were originally identified as viral products that can inhibit the defensive apoptotic response of the host cell to allow more time for the virus to replicate (164). Proteins in this family (such as XIAP, HIAP1, cIAP2, NIAP and survivin) possess one or more baculovirus repeats (BIR), a characteristic cysteine-rich domain of about 70 amino acids. They also contain a carboxy-terminal RING zinc-finger that can act as ubiquitin ligase (165;166).

In general, IAPs are thought to function at the caspase activation step in the cell death pathway. They bind to the inactive, prodomain-containing caspase (zymogen) and prevent it from being processed into the active enzyme.(167;168) The BIR domains of XIAP and HIAP1 have been shown to bind and inhibit caspase-3, -7, and -9 (167;169;170). Even the smallest member of the IAP family, survivin, which contains only a single BIR, is also capable of inhibiting caspase-3 (171). Despite the sequence similarities between the BIR domains of XIAP, they exhibit different affinities in term of binding and inhibiting caspases. Indeed, BIR2 specifically inhibits caspase-3 and -7 while BIR3 inhibits caspase-9 only (170;172-174). Furthermore, it has been shown that XIAP can be cleaved into BIR1-2 and BIR3-RING zinc-finger fragments by activated caspases in vitro and in Fas-treated cells (172). Exogenous expression of a BIR3-RING fragment potently suppresses Bax-induced apoptosis. This pathway, which proceeds through the release of cytochrome *c* from

the mitochondria, and the formation of the apoptosome complex, culminates in the recruitment and activation of caspase-9 (172).

As for the ubiquitin ligase ability of XIAP and cIAP1, it appears to be responsible for auto-ubiquitination and self-degradation of these IAPs through the proteosome, in response to certain apoptotic stimuli (166). The RING finger may also function as a negative regulator of other apoptotic components, since the RING finger of cIAP1 mediates the ubiquitination of caspase-3 and -7 (165). This suggests that IAPs initially bind and inhibit caspases, then subsequently ubiquitinate and trigger the degradation of the IAP-caspase complex.

Mammalian IAPs could be important in diseases since the cIAP2 gene is often translocated in mucosa-associated lymphoid tissue lymphomas (175) and ML-IAP/Livin is often abnormally expressed in melanoma cell lines. (176). In addition, elevated expression of survivin has been observed in some cancers (177).

E. EPIDERMAL GROWTH FACTOR (EGF)

1. STRUCTURE OF THE EGF RECEPTOR

The EGF receptor (EGF-R) is a 170 kDa transmembrane glycoprotein with intrinsic enzymatic activity. It is constituted of three distinct structural elements (178-183) :

a) ***Extracellular N-terminus domain:*** It possess two cysteine-rich regions that form the binding site for specific ligands such as EGF and Tumour growth factor-α (TGFα);

b) ***Transmembrane domain:*** it is responsible for anchoring the receptor in the membrane;

c) ***Cytoplasmic C-terminus domain:*** it is comprised of three regions

   i) *Juxtamembrane region :* It contains negative-feedback phosphorylation sites for Protein Kinase C (PKC; Thr 654) and Mitogen activated Protein Kinases (MAPK; Thr 669).

   ii) *Tyrosine kinase region (TK):* This region, which contains the ATP binding site (Lysine 721), catalyses the transfer of the γ-phosphate of ATP to a tyrosine residue of the receptor and also to that of some other intracellular

proteins. The TK region is highly conserved between the different receptors Tyrosine Kinase (RTK).

iii) *Carboxy-terminal region :* it comprises 5 tyrosine residues (Tyr 1173, 1148, 1086, 1068, 992) that are the autophosphorylation sites of the EGF-R. These phosphorylated residues serve as docking sites for Src-homolgy-2 (SH2)- and phosphotyrosine homology (PTH)- containing proteins (see below) (181;184).

2. ACTIVATION OF EGF-R :

The monomeric EGF-R is inactive: it has a weak affinity toward its ligand and a low rate of TK activity. The binding of EGF to EGF-R induces a conformational change of the extracellular domain of EGF-R, which favours the dimerization of two ligand-monomers complexes. This dimerization stabilizes the interactions between the cytoplasmic domains, increases EGF-R affinity toward the ligand and amplifies its TK activity. (185) The exact mechanism by which EGF-R dimerization induces the activation of the TK activity is not yet elucidated. However, the current conceptual model postulates that the carboxy-terminal region of the receptor, in the basal non-phosphorylated state, functions as an auto-inhibitory substrate of the TK activity of the EGF-R. The conformational change induced by EGF binding places the C-terminus in proximity of the TK domain of the other monomer, leading to its phosphorylation on specific tyrosine residues (Tyr 1173, 1148, 1086, 1068, 992). This, in turn, relieves the inhibitory effect that the C-terminus exerts on the TK activity of the EGF-R. Various intracellular substrates can then access their binding sites

either in the catalytic domain (to be phosphorylated) or in the C-terminus (to interact with the phosphorylated tyrosine residues) to activate different downstream signalling pathways (179-181).

3. EGF-R SIGNAL TRANSDUCTION PATHWAYS

Signal transduction from the TKR is based on protein-protein interactions. The phosphorylation of the EGF-R on specific tyrosine residues creates binding sites for proteins with SH2 and PTH domains. These are non-enzymatic protein-protein binding domains with very high affinity to specific sequences determined by the context of amino acids surrounding the phosphorylated tyrosine (186). They are found in several intracellular proteins of different structure and function. Some of them are enzymes (like phospholipace C-γ [PLCγ] (186;187) and PI 3-K(188)) which are phosphorylated by the catalytic activity of the receptor and therefore activated, while others are adaptor proteins (ex. Grb2 et Shc) that recruits cytoplasmic molecules to the plasma membrane, thus connecting the RTK to the intracellular signalling pathways (181).(see Figure 4)

3.1. Mitogen-Activated Protein Kinases Pathway (MAPK):

This pathway is composed of a cascade of serine/threonine protein kinases, which once activated by phosphorylation, phosphorylate other proteins to modulate their activity (189). The activation of this pathway starts by the recruitment of Grb2, a SH2-containing protein,

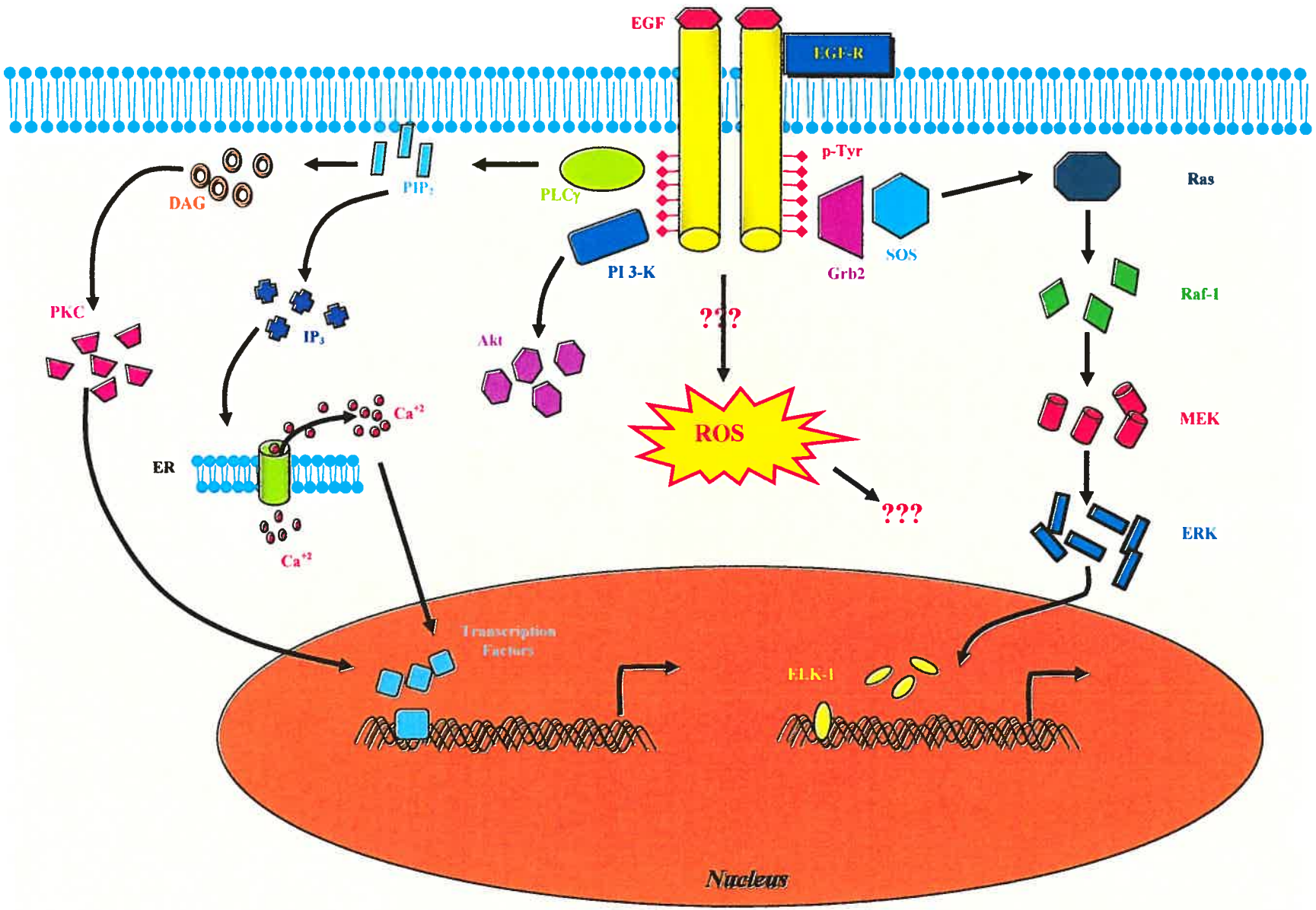
to the region of phosphorylated Tyr 1068 on the EGF-R. With it, Grb2 translocates SOS (Son of Sevenless), a guanyl nucleotide exchanger responsible for the activation of Ras by

replacing its guanyl dis-phosphate (GDP) by guanyl tris-phosphate (GTP). GTP-bound Ras recruits the serine/threonine kinase Raf-1 to the membrane and activates it. The latter, in turn, phosphorylate MAPK kinase 1 (MEK1) on serine 218 and 222, which activates it. MEK1 is a double specificity protein kinase that phosphorylates p42 and p44, two isoforms of the ERK family (Extracellular signal Regulated Kinase), on Thr 183 and Tyr 185 in order to activate them.

p42/p44 are serine/threonine kinases responsible for the phosphorylation of a wide array of cytoplasmic substrates like p90RSK kinase, cytoskeleton components and even the EGF-R itself (Thr 669 : retro-negative control). Some of the activated ERK are translocated to the nucleus where they phosphorylate transcription factors, such as c-Myc and Elk (189), to stimulate their transcriptional activity. These transcription factors induce the expression of genes controlling DNA synthesis as well as cell cycle re-entry (ex. Cyclin D1 and Dihydrofolate reductase) thus culminating in cellular proliferation. (61)

Figure 6 ***EGF receptor signalling pathways***

Ligation of EGF to its receptor induces the dimerization, activation of the intrinsic tyrosine kinase activity of the receptor and the autophosphorylation of the receptor. This creates docking site for different proteins. Grb2 binds to the phosphorylated tyrosine residues and recruits SOS to the membrane in order to activate Ras. This activates the Mitogen-associated protein kinase (MAPK) pathway starting with Raf-1, MEK then ERK. Phospholipase C γ (PLCγ) is recruited as well to phosphorylated EGF-R where it becomes activated. It hydrolyzes phosphatidylinositol 4,5 bis-phosphoate (PIP_2) into di-acyl glycerol (DAG; which activates PKC) and Inositol 1,4,5 tris-phosphate (IP_3, which releases Ca^{+2} from the endoplasmic reticulum). Phosphatidylinositol 3-kinase (PI 3-K) is a tyrosine kinase recruited to dimerized and phosphorylated EGF-R to get phosphorylated and activated. This results in the activation of the serine/threonine kinase Akt, a known anti-apoptotic pathway. A recent new pathway is emerging involving reactive oxygen species (ROS). However, the mechanisms responsible for their production as well as the mechanisms of their action are not yet elucidated.


EGF
EGF-R
p-Tyr
Ras
DAG
PIP2
PLCγ
PI 3-K
Grb2
SOS
Raf-1
PKC
IP3
Akt
???
MEK
Ca+2
ROS
ER
???
ERK
Transcription Factors
ELK-1
Nucleus

3.2. Phosphatidyl Inositol 3-OH Kinase Pathway (PI 3-K):

The PI 3-K is a family of enzymes responsible for the phosphorylation of phosphoinosides (PI) on their D-3 position (188). The products generated can then modulate the activity of multiple cellular effectors, such as PKC, PKB/Akt and PI 3-K itself, thus mediating the pleiotrophic effects of this pathway (159;190;191). PI 3-K is composed of two subunits designated according to their respective molecular weight: ***i)*** p85 : is the regulatory subunit. It contains several protein-protein interaction domains such as SH2, SH3 and PH, which play a very important role in the assimilation of different signalling pathways; ***ii)*** p110 : is the catalytic subunit.

When EGF-R is phosphorylated, PI 3-K is rapidly recruited to the plasma membrane by the SH2 domains contained in the p85 subunit where it becomes phosphorylated on tyrosine residues by the TK of the EGF-R. The activated kinase then phosphorylates PI 3,4-di-phosphate (PIP_2) to generate PI-3,4,5-tri-phosphate (PIP_3), which recruits Akt to the membrane. This interaction induces a conformational change of Akt thus exposing Thr 308 and Ser 473 and rendering them accessible to be phosphorylated by 3-phosphoinosides-dependent protein kinase-1. These phosphorylations activate Akt (192).

3.3. Phospholipase Cγ Pathway (PLCγ):

Phospholipases are enzymes (e.g. PLA1,2, PLC et PLD) involved in the metabolism of phospholipids and the generation of lipid second messengers (181;186). PLC specifically

hydrolyze the glycerophosphate bond of phosphatidylinositol 4,5- bis-phosphate (PIP_2). The result of this hydrolysis is the formation of two second messengers, but with very short lifespan : ***i)*** 1,2 di-acyl glycerol (DAG) that activates protein kinase C (PKC), a serine/threonine kinase protein, which phosphorylates various proteins to modulate their activity (e.g. Na^+/H^+ exchanger, Raf-1, EGF-R); ***ii)*** Inositol 1,4,5, tris-phosphate (IP_3), which induces the release of Ca^{+2} from endoplasmic reticulum into the cytoplasm through its interaction with the IP_3 channel-receptor. Ca^{+2} is one of most important second messengers used by cell-surface receptors, which stimulates the activity of Ca^{+2}-calmodulin kinase and therefore regulates the activity of several enzymes (e.g. PLC).

Several isoforms of PLC exist (all Ca^{+2}-dependant), grouped into three families (β, γ, δ). Only members of the γ family have SH2 domains that allow them to bind to TKR and therefore to be phosphorylated and activated by them (186).

3.4. Reactive oxygen species (ROS):

Recently, ROS have emerged as potential messengers of GF signalling. However the targets as well as the enzymes that generate them are not yet known (detailed in section F 4.4).

F. INTRACELLULAR REDOX STATE

Reactive oxygen species (ROS) have been of interest for many years in all areas of biology. Originally, ROS were recognized as being instrumental for mammalian host defence. Indeed, Baldrige and Gerard found in 1933 (193) that ROS are generated by the NADPH (nicotaminde adenine dinucleotide phosphate, reduced form) oxidase complex of specialized phagocytic cells (neutrophils and macrophages) as cytotoxic agents to fight invading pathogenic agents, a process known as the oxidative burst (194). However, over the past several decades, studies have clearly involved ROS, and the resulting state called oxidative stress, in the development of several pathologies such as atherosclerosis, neurodegenerative diseases (Parkinson and amyotrophic lateral sclerosis), stroke, diabetes, cancer, ischemia/reperfusion, alcoholic liver disease, hepatocellular carcinoma as well as acute and chronic inflammatory diseases (arthritis, colitis, pancreatitis, chronic hepatitis) (195-197). Moreover, ROS have been involved in the regulation of apoptosis, although their exact role is still controversial.(196;198;199)

On the other hand, recent work has uncovered an exciting role for ROS: that of key signalling molecules. Indeed, several mammalians cell types have been shown to produce small amounts of ROS.(194) This weak generation of ROS was shown to be important for a variety of cellular functions, including cell growth, protein synthesis, neuromodulation and ion transport. (194;198) In addition, accumulating evidence indicates a vital role for ROS in mediating cellular responses by various extracellular ligands.(168;194;200;201)

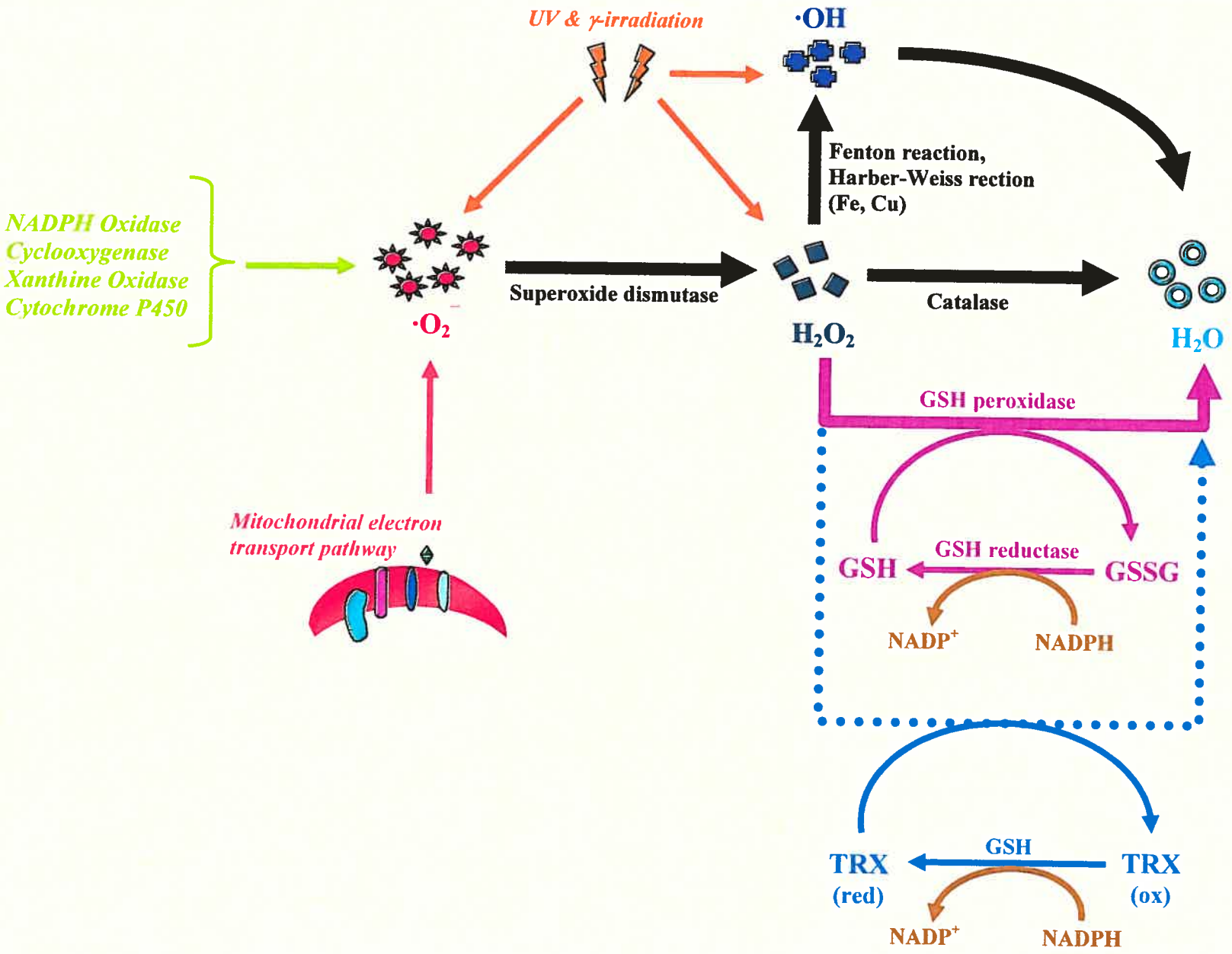
Therefore, understanding the nature, source and targets of ROS as well as the stimuli that trigger their production is of important benefit for the development of new therapeutic strategies.

1. REACTIVE OXYGEN SPECIES (ROS)

For organisms living in an aerobic environment, exposure to ROS is continuous and unavoidable. Indeed, molecular oxygen (O_2) is essential for the survival of all aerobic organisms. Aerobic energy metabolism is dependent on oxidative phosphorylation, a process by which the oxido-reductive energy of the mitochondrial electron transport (a multicomponent NADH dehydrogenase enzymatic complex) is converted to the high-energy phosphate bond of ATP. Cytochrome *c* oxidase, the terminal enzymatic component of this mitochondrial enzymatic complex, catalyzes the reduction of O_2 to H_2O. Partially reduced and highly reactive forms of O_2 (free radicals with oxygen-based unpaired electron collectively called ROS) may be formed during these electron transfer reactions, as shown in Figure 5.(194;195;202) They include superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) formed by one- or two- electron reductions of O_2, respectively. In the presence of transition metal ions (e.g. Fe, Cu, Co), the even more reactive hydroxyl radical ($OH^{\bullet}$) can be formed by Fenton or Haber-Weiss reactions (194;203). There are other types of free radicals called reactive nitrogen species (RNS) : nitrogen-based metabolites with unpaired electron such as nitric oxide ($NO^{\bullet}$).(204)

Figure 7 ***Sources of reactive oxygen species (ROS) and their mechanisms of elimination***

The most commonly formed ROS formed by NADPH oxidase, xanthine oxidase, cytochrome P450 as well as mitochondrial electron transport pathway is superoxide anion ($\cdot O_2^-$). UV and γ-irraddiation can produced $\cdot O_2^-$ in addition to hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH). Several enzymes act on ROS in order to neutralize them. Superoxide dismutase convert $\cdot O_2^-$ into H_2O_2 where catalases and peroxidases (glutahione [GSH] and thioredoxin [TRX] peroxidase) then reduce it to water. In that process, reduced glutathione (GSH) and $TRX_{(red)}$ are oxidized into oxidized glutathione (GSSG) and $TRX_{(ox)}$. Nicotimide adenine denucleotide phosphate (NADPH) is used by GSSG and TRX reductases (not shown) to replenish the reduced forms of these scavenger molecules.


UV & γ-irradiation
·OH
Fenton reaction,
Harber-Weiss rection
(Fe, Cu)
NADPH Oxidase
Cyclooxygenase
Xanthine Oxidase
Cytochrome P450
·O2−
Superoxide dismutase
H2O2
Catalase
H2O
GSH peroxidase
Mitochondrial electron
transport pathway
GSH reductase
GSH
GSSG
NADP+
NADPH
GSH
TRX
(red)
TRX
(ox)
NADP+
NADPH

ROS and RNS can be generated in almost all cell type by multiple sources including mitochondrial electron transport system, NAD(P)H oxidase, xanthine oxidase, microsomal cytochrome P450 (e.g. CYP 2E1;(196)), nitric oxide synthase (type I, I and III) and cyclooxygenase (196;204). Mitochondria are a major source of ROS under physiological conditions, because 2-3% of the O_2 consumed is converted to $O_2^{\bullet -}$ (Figure 5).(196) This production is increased in ischemia/reperfusion injury and in TNF-α–treated cells, which results in ROS accumulation if the anti-oxidant system is impaired or overwhelmed (196)(see section F.3).

2. CYTOTOXIC EFFECTS OF ROS

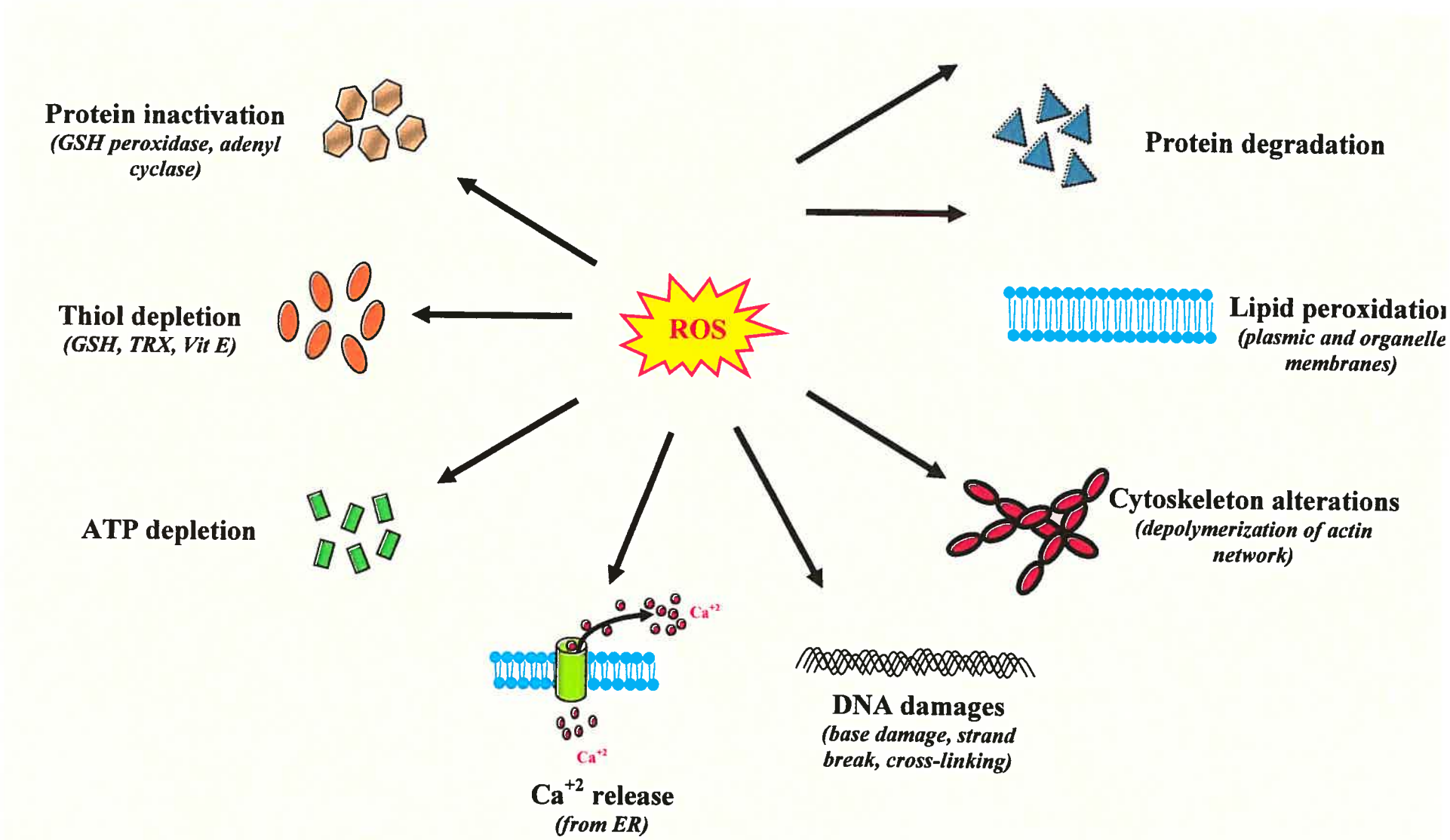
ROS can cause severe cellular damages since they are highly reactive toward intracellular macromolecules (i.e. protein, lipid and DNA), as summarized in Figure 6. Indeed, high concentrations of H_2O_2 and $O_2^{\bullet -}$ cause DNA damage such as base damage (depurination or depyrimidination), single-brand breaks, double-strand breaks, cross-linking between DNA and chromosomal aberrations (194;205;206). These damages may lead to increased risk of mutagenesis if not repaired. Furthermore, extensive oxidative modification of proteins cause a permanent loss of function from irreversible oxidation of functionally important amino-acids, the formation of protein-protein cross-linkages, fragmentation of the polypeptide chain and finally proteolytic degradation of the damaged protein (205). ROS have indeed been shown to inactivate vital proteins such as adenyl cyclase, glutathione (GSH) peroxidase, aldehyde dehydrogenase, glutamine synthase, catalase and creatine

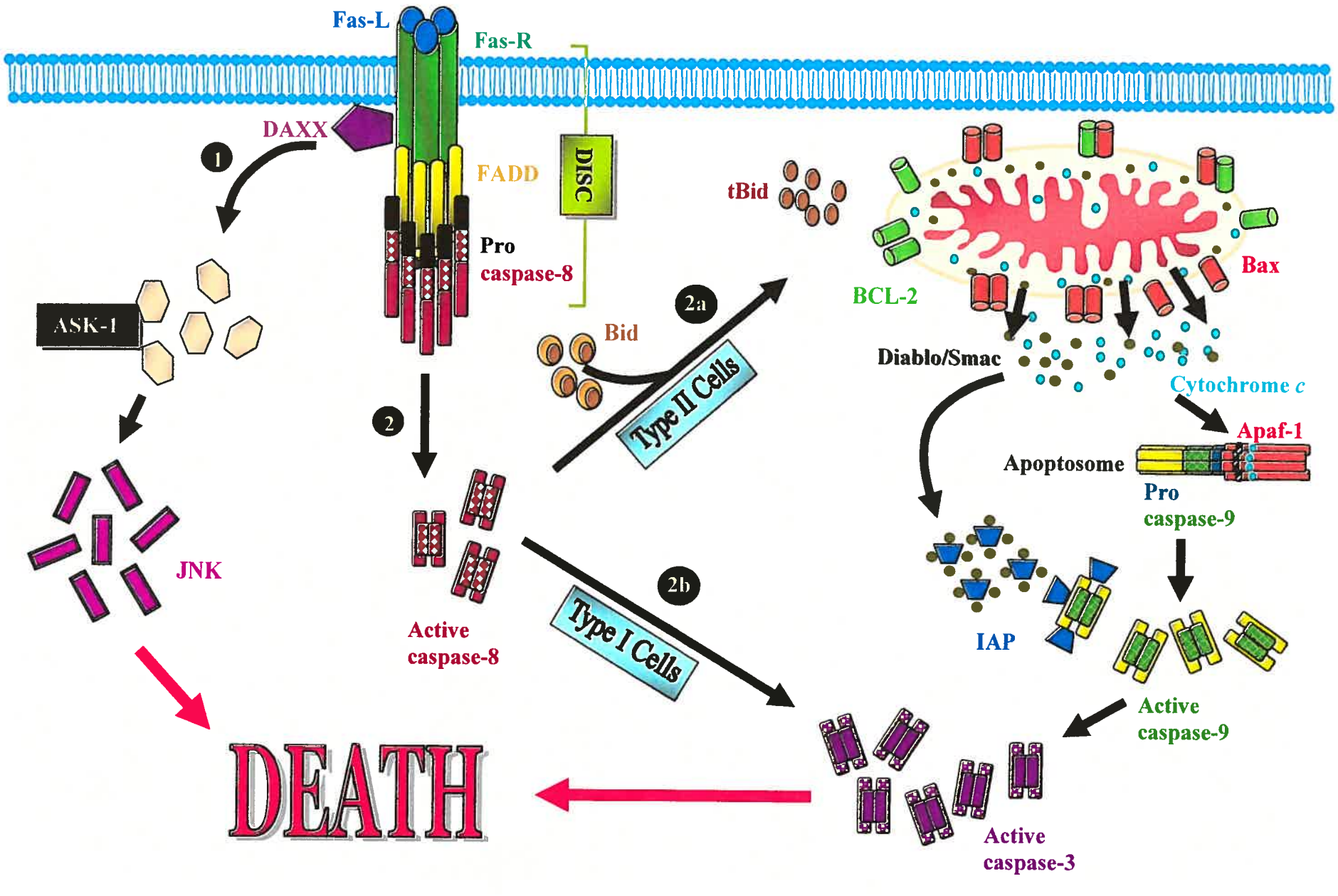
phosphokinase (194;207;208). In addition, ROS can cause an alteration of microfilaments via depolymerization of the actin network resulting in morphological changes of the plasma membrane (blebbing) (209). This occurs through irreversible oxidation of thiol-containing amino acids such as methionine (for example oxidation of methionine into methionine sulfones) and cysteine (210). Membrane lipid peroxidation is another cellular damage caused by ROS exposure. Indeed, reaction of polyunsaturated fatty acids, especially C20:4 and 22:6, with ROS results in the formation of peroxy radicals, hydroperoxides, aldehydes and malonaldehydes, which impair membrane integrity and produce a wide range of biological effects leading to cell injury (194;211). As a result, cytosolic Ca^{2+} is increased (thus altering the activity of Ca^{+2}-depending enzymes), ATP is depleted, NADPH and GSH are oxidized.(194;205;212)

These lesions are severe and can lead to cell death mainly by necrosis if not prevented or repaired. Hence, it is necessary for cells to tightly control their content in ROS. In order to do so, cells have developed several anti-oxidant (or reductant) mechanisms that maintain the intracellular redox environment in a highly reduced state.

Figure 8 ***ROS cytotoxic effects***

ROS are highly reactive to intracellular macromolecules. They can induce DNA base damage, DNA strand breaks and cross linking between chromosomes. In addition ROS induce lipid peroxidation, which impairs lipid signalling and compromises membrane integrity. Proteins are also severely affected by oxidation (degradation, inactivation and alteration of structure). Consequently, intracellular homeostasis is altered (e.g. increase in Ca^{+2} cytoplasmic concentrations, thiol and ATP depletion).


Protein inactivation
(GSH peroxidase, adenyl cyclase)
Protein degradation
Thiol depletion
(GSH, TRX, Vit E)
ROS
Lipid peroxidation
(plasmic and organelle membranes)
ATP depletion
Cytoskeleton alterations
(depolymerization of actin network)
Ca+2
Ca+2
Ca+2 release
(from ER)
DNA damages
(base damage, strand break, cross-linking)


Fas-L
Fas-R
DAXX
FADD
DISC
Pro
caspase-8
1
ASK-1
JNK
2
Active
caspase-8
Bid
2a
Type II Cells
tBid
BCL-2
Bax
Diablo/Smac
Cytochrome c
Apaf-1
Apoptosome
Pro
caspase-9
IAP
Active
caspase-9
2b
Type I Cells
Active
caspase-3
DEATH

3. INTRACELLULAR ANTI-OXIDANT MECHANISMS AND REDOX STATE

The anti-oxidant system consists of scavengers (such as glutathione, vitamins C and E, thioredoxin) and enzymes (e.g. superoxide dismutase [SOD], GSH peroxidase, catalase) to neutralize ROS before they can exert damage. (see Figure 5)

3.1. Scavengers

Scavengers are reductant substances that get oxidized in order to reduce ROS and oxidized macromolecules. They include α-tocopherol (vitamin E), Ascorbate (vitamin C), uric acid and thiols (such as glutathione and thioredoxin [TRX]). α-tocopherol is a membrane anti-oxidant due to its liposolubility. It is capable of preventing the formation of lipid peroxides in cellular membranes. Ascorbate plays an important role in restoring the α-tocopherol reduced form. In addition, ascorbate is capable of scavenging $O_2^{\bullet -}$.(196)

Thiols are sulfhydryl (SH) containing molecules that can readily give their proton (H^+) to reduce ROS or ROS-attacked molecules while they become oxidized themselves. TRX is a small multifunctional protein that has a redox-sensitive dithiol. TRX reduce critical protein sulfhydryl residues to regulate protein folding, receptor assembly, transcription factor activity as well as reducing some ROS, such as H_2O_2. (198;202) However, the most important intracellular anti-oxidants is the thiol-containing peptide glutathione. It plays

several important functions including detoxification of xenobiotics, reduction of ROS and of oxidized proteins (see Section F.3.3).

3.2. Enzymes

In general, the most efficient way to eliminate undesirable toxic species, is by means of degradation. Therefore, cells possess anti-oxidant enzymes including SOD, catalases and GSH peroxides to annihilate ROS.

SODs are a family of metalloenzymes that converts $O_2^{\bullet -}$ to H_2O_2 and O_2. It is an important ubiquitous enzyme localized in the cytoplasm, the mitochondria, the peroxisomes as well as the extracellular space. (194;196;207). Interestingly, overexpression of SOD has been shown to sensitize cells to oxidative stress. This can be overcome by increasing the expression of catalases or peroxidases suggesting that this higher sensitivity is due to H_2O_2 accumulation. (213)

Catalases and peroxidases (GSH or TRX -dependent) decompose H_2O_2 to H_2O and O_2. They are heme-containing proteins that use NADPH as the reducing factor. Catalases are mainly localized in peroxisomes while peroxidases are found in the cytosol, mitochondria and extracellular space. However, both types of enzyme have identical mechanism of action. For example, GSH peroxidase oxidizes reduced GSH to form oxidized glutathione (GSSG) to reduce H_2O_2 as well as lipid peroxides. Therefore, these enzymes play an

important role in maintaining the structure and the function of biological membranes (196;198).

3.3. GSH system

The tripeptide GSH (L-γ-glutamyl-L-cysteinyl-glycine) is ubiquitous in animals, plants, and microorganisms. GSH often reaches millimolar levels inside cells, which makes it one of the most highly concentrated intracellular anti-oxidants (214-216). This is especially true in the liver where its concentration reaches 10 mM (214). GSH is synthesized from precursor amino acids (L-glutamine, L-cysteine and L-glycine) in the cytosol of virtually all cells by the sequential action of two enzymes (197;215;217;218). First, the binding of cysteine and glutamate is catalyzed by γ-glutamylcysteine synthetase (GCS) to form γ-glutamylcysteine. This reaction is the rate limiting step of GSH synthesis and it depends on the availability of L-cysteine (215;217;218). In addition, the activity of GCS is regulated by the gene expression of its subunits, by S-nitrosylation, phosphorylation and oxidation (219-221). On the other hand, GSH acts by feedback competitive inhibition on GCS to regulate its own synthesis (222;223). Noteworthy, the liver is distinctive from other cells in regard to GSH biosynthesis in the unique ability of hepatocytes to convert methionine to cysteine (through the transsulfuration pathway) (216). The second step in GSH synthesis, catalyzed by GSH synthetase (GS), adds glycine to γ-glutamylcysteine to form GSH in an ATP-dependent manner (215;217).

The γ bond between the first two amino acids (instead of the typical alpha bond) renders GSH resistant to the degradation by standard intracellular proteases. The only enzyme capable of degrading GSH is γ-glutamyl transpeptidase, an enzyme located at the outer surface of the cell (215;217;224). This means that GSH can not be lost by catalysis inside the cell. Of note, the preformed GSH cannot enter the cell per se: it must be synthesized inside cells. Therefore, GSH availability can be increased by supplementation with GSH amino acids constituents (and especially L-cysteine) or by using GSH esters (e.g. GSH monoethyl ester, GSH diethyl ester). Indeed, GSH esters are capable of passing through the plasma membrane. Once inside, intracellular non-specific esterases liberate GSH into the cytoplasm (225).

Almost 90 % of cellular GSH is contained in the cytosol, 10 % in the mitochondria and a small percentage in the endoplasmic reticulum (214). The mitochondria cannot synthesize GSH. Therefore, a GSH transporter on the mitochondrial membrane ensures the uptake of GSH into the mitochondria. This transporter in specifically inhibited by ethanol thus explaining the preferential depletion of mitochondrial GSH in alcohol-treated hepatocytes. (216;226) Cytosolic GSH in the rat liver turns over rapidly with a half life of 2-3 hours. However, since mitochondria are normally under high oxidative stress, they avidly retain their GSH with a half-life of about 30 hours (197;227).

GSH serves several vital functions including:

1) Detoxification of xenobiotics and their metabolites where GSH S-transferases (phase II metabolic enzymes) conjugates GSH to liposoluble compounds thus rendering them more hydrosoluble and easily extractable from the cell and, in the case, of hepatocytes into the bile. However, GSH conjugation irreversibly consumes intracellular GSH (216).

2) Protection of cellular macromolecules against ROS. GSH is used by ***i)*** GSH peroxidases to detoxify peroxides (H_2O_2 as well as lipid peroxides). ***ii)*** by thiol-transferases to prevent the oxidation of the –SH moieties of proteins or to reduce the oxidized disulfide bonds induced by oxidative stress (215;217).

3) Recycling of other anti-oxidants that have become oxidized such as ascorbate and α-tocopherol (196).

To accomplish its anti-oxidant function, thiol-reduced GSH undergoes thiol disulfide exchange, thus forming GSSG (215). In healthy cells, GSH is present mainly in its reduced form and GSSG rarely exceeds 10% of total cellular glutathione (GSx). Under normal conditions, GSSG is eliminated by its recycling to GSH. This reaction is catalyzed by GSSG reductase, an enzyme that requires NADPH as a source of electrons (215;218;228). Therefore, NADPH, coming mainly form the pentose phosphate cycle, is the predominant source of GSH reducing power. However, under oxidative stress GSSG is also actively excreted out of the cell (214).

Given the widespread involvement of GSH in several important functions, it is not surprising that GSH depletion has been associated with several pathologies, including Parkinson disease, alcoholic liver disease, cirrhosis, hepatitis and atherosclerosis (197).

The redox state is the result of the balance between generation of ROS and their elimination by anti-oxidant mechanisms. Oxidative stress occurs when ROS and oxidants in general accumulate in the cell due to overproduction of ROS or exhaustion of anti-oxidant mechanisms. This state varies in severity and therefore has different consequences on cell function and fate. Indeed, mild oxidative stress has been reported to play an important role in cell signalling at the receptor level as well as in intracellular transduction pathways (See Section F.4). Somewhat more severe oxidative stress has been shown to inhibit cell death by apoptosis, although this is still subject to much debate (as detailed in Section F.5). However, it is well documented that extreme oxidative stress disrupts vital cell functions leading to cell death by necrosis (see Section F.2). (215;218)

4. REDOX SIGNALLING

Growing evidence show that cellular redox state plays an important role in cell signalling (redox signalling). Indeed, a large number of signalling pathways appear to be regulated by ROS, although the exact signalling molecules targeted by ROS are not very clear. ROS can mediate signalling by two mechanisms: 1) Alterations in the intracellular redox state; and 2) oxidative modification of proteins.

4.1. Alterations in intracellular redox state

As mentionned above, thiols (such as GSH and TRX) are important regulators of the cellular state redox due to their anti-oxidant power. Accumulating evidence suggest that, in addition to their anti-oxidant functions, thiols participate in cell signalling processes (195). GSH has been reported to regulate redox signalling by the change in the total level of GSH (229;230) as well as in the ratio of the reduced to the oxidized forms (231;232). Indeed, TGF-β-induced growth inhibition of vascular endothelial cells is accompanied by GSH depletion (233). Moreover, GSH:GSSG ratio seems to regulate the DNA binding of the sp-1 transcription factor (232). TRX has also been shown to regulate the activity of some proteins by binding them. For example, TRX has been shown to bind to the amino terminal of ASK-1 and thus to inhibit its activity (234). ROS induce the dissociation of the TRX/ASK-1 complex thus liberating the kinase, which oligomerizes and becomes activated (235).

4.2. Oxidative modifications of proteins

ROS can alter protein structure and function by modifying critical amino acids residues in or near the active site of enzymes. This oxidative modification of amino acids can occur by several mechanisms: ***i)*** The best described modification is oxidation of the -SH group of cysteine residues to sulfenic (-SOH), sulfinic ($-SO_2H$) derivative or their S-glutathionylation (-SSG). Such modification may alter the activity of the enzyme if the cysteine is located in its active site (e.g. protein tyrosine phosphatase 1B (236), caspases (77)) or the transcription factor if it is located within its DNA binding region [e.g. c-Jun(237)]). ***ii)*** Two or more cysteine residues within the same protein can be oxidized to form an intramolecular disulfide bridge, thereby inducing a conformational change of the protein and altering its activity. This is a well characterized mechanism for oxidant regulation of the activity of several transcription factors (such as p53 (238), OxyR (239)) .(195)

Protein dimerization is another mechanism mediating oxidant regulation of protein activity. Indeed, intermolecular disulfide bonds formed by the oxidation of cysteine groups of two identical or different proteins lead to the homodimerization or heterodimerization of proteins (e.g. GSH S-treansferase-π (240), ASK-1(241)). H_2O_2 or peroxidase-induced cross-linking of tyrosine residues on separate proteins has been shown to participate in the dimerization and oligomerization of these proteins (e.g. synthesis of thyroxine(242)).

Lastly, interaction of ROS with metal-ion clusters in metalloenzymes (e.g. Fe-S) leads to their oxidation in a Fenton-like reaction thus marking these proteins for ubiquitination and degradation (243;244). Therefore, modifying the stability of protein by redox-induced alterations in their levels might be another manner to regulate protein functions by oxidants.

4.3. Signalling pathways targeted by ROS

Extracellular stimuli transduce signals through a variety of cellular signalling pathways including signalling molecules such as protein tyrosine kinases (PTK), serine/threonine kinases, phospholipases and Ca^{+2}. When cells are stimulated with ROS, signals are transferred through the same signalling pathways as those triggered by growth factors.

Kinases and phosphatases : Several studies have shown that oxidants such as H_2O_2, vanadate and UV-irradiation activate RTK and PTK, protein serine/threonine kinase (195;198;202). For example, intraperitoneal injection of mice with pervanadate resulted in the tyrosine phosphorylation of several proteins including receptors for insulin, EGF and HGF as well as PLCγ and insulin receptor substrate-1 (IRS-1). In addition, H_2O_2 treatment was shown to activate MAPK cascades (JNK, p38 and ERK pathways) (198;202), Akt and PKC. However, evidence for direct activation of these kinases is still lacking. In fact, treatment of some PTK with H_2O_2 did not show any increase in enzymatic activity (202). On the other hand, protein tyrosine phosphatases (such as protein tyrosine phosphatase-1B) and serine/threonine phosphatases (such as protein phosphatase 1 and 2A (245)) are

reported to be inhibited by oxidants since all phosphatases have a reactive cysteine in their catalytic site (246). This suggests that ROS-induced activation of protein kinases is the result of inhibition of their dephosphorylation by phosphatases (e.g. PDGF receptor, EGF receptor and Fyn) (195;202).

Ca^{+2} signalling : Ca^{+2} is widely used as a second messenger that regulates a variety of biological processes including muscle contraction, neurotransmission, gene transcription and cell growth. Oxidants, such as H_2O_2 and $O_2^{\bullet -}$, have been shown to increase intracellular Ca^{+2} through inhibition of the Ca^{+2}-ATPase of sarcoplasmic reticulum, increase of membrane permeability as a result of peroxidation of membrane lipids; and increase in the probability of the opening of sarcoplasmic reticulum Ca^{+2}-channels (247).

Transcription factors : Several studies have shown that oxidants are major determinants of gene expression. Indeed, several transcription factors contain cysteine residues localized in their DNA binding domain, which are essential for the recognition of the binding site with DNA bases. The oxidation of these cysteines results in the inhibition of the binding of the transcription factor (such as NF-κB, AP-1) to DNA (248). However, H_2O_2 and TNF-α have been shown to induce the activation of the same transcription factors (e.g. NF-κB). This apparent contradiction was explained when the phosphorylation and the subsequent degradation of I-κB, an inhibitory protein that retains NF-κB in the cytoplasm, was observed following oxidant treatment. This results in the release of NF-κB from the inhibitory complex (NF-κB / I-κB) followed by its translocation to the nucleus (249).

Therefore, the activity of NF-κB seems to be under dual redox regulation, whereby anti-oxidants directly inactivate NF-κB (in the nucleus) while they suppress its upstream inhibition (in the cytoplasm). (202;248)

4.4. Ligand-induced ROS production

A variety of cytokines and growth factors have been reported to generate ROS. Indeed, TNF-α and interferon-γ (INF-γ) were among the first cytokines reported to generate ROS. For example, TNF-α–induced expression of adhesion molecules, IL-8 expression and production of chemokines have been shown to be mediated through ROS-dependent mechanisms (195). In addition, growth factors that bind RTK have also been shown to generate intracellular ROS rapidly after the activation of their receptors for a short period (a burst) and in a coordinate manner (200). Sundaresan et al. (201) have demonstrated that PDGF-induced tyrosine phosphorylation, MAPK activation, DNA synthesis and chemotaxis require transient increase in intracellular H_2O_2 concentrations. Similarly, EGF-enhanced tumorigenicty, metastatic capacity, and *in vitro* invasive capacity have been shown to be partially related to EGF-induced H_2O_2 production (250). Other receptors, such as receptor serine/threonine kinases (e.g. TGF-β) and G protein-coupled receptors (e.g. Angiotensin II receptor) have also been reported to generate ROS following binding with their ligand and to mediate some of their biological effect (195).

5. REDOX REGULATION OF APOPTOSIS

As mentioned above, accumulation of ROS leads to a state of oxidative stress where severe intracellular injuries occur and cause to cell death by necrosis. In similar manner, weak oxidative stress has been shown to induce apoptosis or to mediate the apoptotic signal. However, contradictory findings are emerging regarding this issue. Indeed, accumulating evidence shows that, on the contrary, oxidants can inhibit apoptosis.

5.1. Oxidative stress sensitizes cells toward apoptosis

An increase in intracellular ROS levels has been shown in several studies to lead to cell death by apoptosis. For example, UV radiation, γ-irradiation and TNF-α, known for their ability to stimulate intracellular ROS generation, as well as H_2O_2 can induce cell death by apoptosis in many cell systems (198). In addition, ROS triggered the expression of *c*-myc, a protein involved in the mediation of the apoptotic signal of several stimuli (251). Similarly, anti-oxidant depletion or decrease of anti-oxidant enzymes (e.g. catalases, SOD, GSH peroxidase) sensitize cells to apoptosis, while cell replenishment with anti-oxidants (GSH, TRX) or overexpression of GSH peroxidase abrogate it. (202;252;253) In fact, extrusion of anti-oxidant such as GSH was reported to be an early and requisite event in the mediation of the apoptotic signal. (254-255)

However, careful review of the literature reveals that depletion of GSH or ROS exposure have different effects on apoptosis depending on their intensity, location and timing. For example, a recent study from Fernandez-Checa's group has demonstrated that cell exposure to 3-hydroxy-4-pentenoate, which specifically depletes mitochondrial GSH, sensitizes hepatocytes to TNF-α- and Fas-induced apoptosis (256). In a similar manner, there is higher sensitivity toward apoptosis in alcoholic liver disease (45), ethanol being known to preferentially deplete mitochondrial GSH (226).

Of note, the expression of the anti-apoptotic protein BCL-2 was shown to be associated with relocalization of GSH to the nucleus. (151) Since BCL-2 is localized to the mitochondria and the nucleus, it could be postulated that BCL-2 relocates GSH to where it could effectively protect against the devastating damages of oxidative stress.

5.2. ROS protect cells against apoptosis

In spite of these findings regarding the role of ROS in apoptotic induction and execution, several studies have shown that ROS are not implicated in apoptosis or can even protect against it. For example, hypoxia should protect against apoptosis, since ROS generation should be greatly attenuated in a near-anaerobic atmosphere. However, it has been shown that, while hypoxia protectes against oxidant-induced apoptosis (257), it does not abrogate staurosporine-, Fas- or camptothecin-induced apoptosis. This suggests that some apoptotic signalling occurs independently of ROS generation

In addition, the groups of Wendel (75;76) and Jaeschke (258) have reported that *in vivo* depletion of GSH by phorone or acetaminophen treatment results in the abrogation of apoptosis induced by Fas and TNF-α. Furthermore, $O_2^{\bullet -}$ has been reported to inhibit Fas-induced apoptosis (259). These studies have postulated that redox modulation of caspases is the cause of this loss of apoptotic sensitivity. Indeed, Henzte et al. have shown that abrogation of the Fas signal by GSH depletion was not due to a decreased recruitment of caspase-8 to the DISC complex at the Fas-R, but was rather mediated by direct inactivation of caspase-8.

Hence, the involvement of the redox state in the mediation of the apoptotic signal is not yet clear. Therefore, further studies are needed to elucidate the involvement of the redox state in the sensitivity of cells toward apoptosis.

CHAPTER 2

OBJECTIVES AND HYPOTHESIS

A. RATIONAL

- GSH is a very abundant non-protein intracellular thiol and the liver contains high levels of this thiol.
- Redox state has been involved in the regulation of apoptosis; however whether it facilitates or abrogates apoptosis is still a subject for debate.
- *In vivo*, liver cells are very sensitive toward Fas-induced apoptosis while hepatocytes in culture are relatively resistant toward apoptosis.
- Growth factors (GF) protect against apoptosis; they activate tyrosine-kinase receptors, which once phosphorylated, recruit and activate several signalling pathways.
- Many biological activities of GF necessitate the tyrosine kinase activity (TK) of their receptors. However, others were shown to occur independently of the TK activity of GF.
- GF have been shown to induce the generation of ROS rapidly after the activation of their receptors.

B. HYPOTHESES

- Oxidative redox state is a negative regulator of Fas-induced apoptosis in mouse hepatocytes.
- EGF can modulate intracellular redox state to mediate protection against Fas-induced apoptosis
- EGF anti-apoptotic activity is mediated by the TK activity of its receptor.

C. OBJECTIVES

- Evaluate the effect of cell isolation and culture procedures on intracellular stocks of GSH in mouse hepatocytes.
- Establish the relationship between GSH levels and cell sensitivity toward Fas-induced apoptosis in culture.
- Determine the involvement of the TK activity of the EGF-R in the anti-apoptotic effect of EGF in mouse hepatocytes.
- Determine the effect of EGF on the GSH system and the redox state of the cell.
- Examine the relevance of EGF modulation of the redox state to its anti-apoptotic effect in mouse hepatocytes.

CHAPTER 3

EXPERIMENTAL RESULTS

3.1 ARTICLE NO. 1

RESISTANCE TO FAS INDUCED APOPTOSIS IN HEPATOCYTES: ROLE OF GSH DEPLETION BY CELL ISOLATION AND CULTURE

BY

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ABSTRACT

The involvement of reduction/oxidation (redox) state in cell sensitivity to apoptosis has been suggested by several studies in which induction of apoptosis was shown to require oxidative stress or GSH extrusion. On the other hand, biochemical studies of caspases revealed that their activation necessitates a reduced cysteine in their active site : This is ensured by maintaining intact intracellular glutathione status during apoptotic induction as reported by *in vivo* studies. Therefore, we investigated the relationship between intracellular glutathione levels and the sensitivity of mouse hepatocytes in culture to Fas-induced apoptosis as well as potential mechanisms responsible for this sensitivity. We found that total and reduced glutathione levels are decreased by half following cell isolation procedure and further decline by 25% during cell culture for 2h in normal Williams' E medium. Cell culture in medium supplemented with cysteine and methionine maintains glutathione at a level similar to that measured just after cell isolation. Results show that the capacity of Fas to activate caspase-8 and to induce apoptosis requires important intracellular glutathione levels and high GSH/GSx ratio. In conclusion, the present study shows that intracellular glutathione plays an important role in maintaining the apoptotic machinery functional and thus capable of transmitting the apoptotic signal.

Key words : glutathione, redox, Bid, BCL-x_L, caspase-8, apoptosis.

INTRODUCTION

Oxidative stress is defined as the manifestations of cell or tissue following exposure to excess oxidants.(20) Reactive oxygen species (ROS) [i], such as $O_2^{\bullet -}$, $OH^{\bullet}$ and H_2O_2, are the principal species of intracellular oxidants. They are generated as by-products of electron transport through the mitochondrial respiratory chain as well as by γ-ray and ultraviolet light irradiations. (20;27) ROS are highly reactive towards intracellular macromolecules (DNA, proteins and lipids) causing severe lesions that can lead to cell death by either necrosis or apoptosis, depending on the intensity of the oxidative stimuli.(24;40;41)

Hence, it is necessary for cells to tightly control their content in ROS. In order to do so. cells have developed several anti-oxidant (or reductant) mechanisms that maintain the intracellular redox environment in a highly reduced state. These mechanisms range from scavengers (e.g. glutathione, vitamins C and E) to enzymes that neutralize ROS (e.g. superoxide dismutase, catalases and glutathione peroxidase) before they could exert any damage.(20;27) Glutathione, a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) synthesized in the cytoplasm, is the most abundant intracellular non-protein thiol involved in anti-oxidant elimination. It is used by different enzymes to reduce not only ROS, but oxidized macromolecules as well.(20;28) Several oxidative injuries have been associated with glutathione depletion.(1;9;19;20) That is why glutathione replenishment by N-acetylcysteine administration has been proposed as a therapeutic strategy for oxidative stress injuries.(9)

Apoptosis can be triggered by different stimuli, including cell surface receptors (e.g. TNF-α and Fas (10;26;33)), γ-irradiation (30), staurosporine (39) and others. These stimuli converge onto common intracellular effectors such as caspases, endonucleases and pro-apoptotic proteins of the BCL-2 family (eg. Bad, Bid, Bax). The Fas apoptotic pathway was recently elucidated. Binding of Fas ligand to Fas receptor (Fas-R) results in receptor trimerization leading to the recruitment of the adapter protein FADD to the death domain of Fas-R. (11) The death effector domain of FADD can then interact with a similar domain of procaspase-8, which results in the oligomerization of the latter. Activation of caspase-8 through auto-cleavage leads to a series of downstream events, including Bid cleavage, cytochrome c release from the mitochondria and caspase 3 activation, finally culminating in cell death by apoptosis. (43) Ogasawara *et al.* have reported in 1993 that intraperitoneal administration of anti-Fas antibodies causes severe liver damage by apoptosis within 1-2h leading to rapid animal demise within 6h.(36) On the other hand, it is well documented that induction of apoptosis in cultured hepatocytes is less successful. (23;34)

As mentioned above, it was recently reported that apoptosis could be triggered by weak oxidative stimuli. In these studies, anti-oxidant depletion sensitized cells to apoptosis while their replenishment abrogated it.(19;24;41) Moreover, anti-oxidant extrusion (such as glutathione) was reported as an early and requisite event in the mediation of the apoptotic signal.(13;42) However, this remains controversial. Recent reports have shown that, on the contrary, oxidants prevented cell death by apoptosis. For example, caspases, which play a pivotal role in apoptosis, are themselves redox-sensitive. They require a reduced cysteine in

their active site in order to function.(25;35) In parallel, Hentze *et al.* have reported that intact intracellular glutathione status is essential for receptor-mediated caspase activation and apoptosis *in vivo*.(16;17)

Therefore, in light of the importance of glutathione for Fas-induced apoptosis *in vivo*, we investigated the possible relationship between glutathione status and the resistance of cultured mouse hepatocyte to Fas-induced apoptosis, as well as the potential mechanisms involved in this resistance. We found glutathione status to be important for the ability of Fas to induce apoptosis in culture. This resistance in culture appears to result from a lower capability to induce the proteolytic activity of caspase-8.

EXPERIMENTAL PROCEDURES

All animals received humane care according to the guidelines of the Canadian Council on Animal Care. Experimental protocols were approved by the Comité institutionnel de protection des animaux of the CHUM-Hôpital Saint-Luc.

Hepatocyte isolation and culture

Hepatocytes were isolated from the liver of fed male BALB/c mice (22-25 g) using the 2-step collagenase perfusion method described previously (32). Cells were seeded onto plastic petri dishes (26,000 cells/cm^2) in medium supplemented with 10% fetal bovine serum (GIBCO BRL, Toronto, ON, Canada) and allowed 2h to attach. Cysteine (the limiting amino acid for glutathione synthesis; Cys) and its precursor methionine (Met) were used to modulate glutathione intracellular levels. Therefore, we used two different culture media : 1) normal Williams' E medium (GIBCO BRL) containing 40 mg/L Cys and 15 mg/L Met (**Medium N**), and 2) Williams' E medium supplemented with Cys (90 mg/L) and Met (55 mg/L; **Medium C+M**). After attachment, the serum-containing medium was removed, and cells were incubated with serum-free medium for the indicated times in each experimental series. Apoptosis was induced in experimental groups with mouse anti-Fas Jo2 antibody (Research Diagonistics Inc, Flanders, NJ, USA) at a concentration of 100 ng/ml, unless stated otherwise, either directly after attachment in medium N and C+M or after 6h of cell incubation in medium C+M.

Determination of GSx and GSH levels

Culture conditions : In order to determine the effect of hepatocyte isolation procedure on total (GSx) and reduced (GSH) glutathione, we measured GSx and GSH levels at different stages of the isolation procedure. First, to measure glutathione levels in total liver, a section of the right lobe was homogenized in 0.25 M sucrose solution (20 % w/v). Cells ($2x10^6$) were also collected after liver perfusion with Hepes and collagenase solutions as well as after hepatocyte purification, and washed once in cold phosphate-buffered saline (PBS) before being resuspended in 300 µl of 0.25 M sucrose solution. To evaluate glutathione levels over time in culture, hepatocytes were attached in either medium N or C+M in the presence of 10% FBS for 2h. Then the serum-containing media was removed and cells were cultured with medium C+M for 6 h. At the end of the experiment, cells were scraped off, pelleted by centrifugation and resuspended in 300 µl 0.25 M sucrose solution. Samples were stored at –80°C until determination of GSx and GSH levels.

GSx and GSH determination : GSx and GSH levels were measured as described previously (31) with some modifications. Briefly, samples were boiled for 10 min then centrifuged at 12 000 g for 15 min at room temperature. The pellet was discarded. A portion of the supernatant (100 µl) was treated with glutathione reductase (1.2 U; Sigma; Oakville, ON, Canada) and NADPH (1.2 mM; Sigma) for 10 min at room temperature. The reaction was stopped by precipitating proteins with 4 % sulfosalicilic acid (1:1; Sigma). GSH was measured directly in the supernatant after protein precipitation. Thiol concentration was determined by adding 5,5'-dithiobis-2-nitrobenzoic acid

(DNTB; 0.01 M; Sigma) to the sample at a 9:1 dilution and measuring the absorbance at 412 nm. Protein concentration in each sample was determined according to Bradford (5).

Morphological determination of apoptosis

After attachment, cells were incubated with medium N or C+M alone or in the presence of anti-Fas antibodies. After 6h, the medium was removed, cells were fixed with 5% formaldehyde solution (Anachemia Science, Lachine, Qc, CA) and then stained with Hoechst 33258 (250 ng/ml, Sigma) to quantify apoptosis as described previously.(32) The percentage of apoptotic cells is expressed as the ratio of apoptotic nuclei versus the total number of nuclei (normal + apoptotic). We evaluated 400 nuclei for each petri dish.

Biochemical determination of cell death

Cells cultured in medium C+M were induced to undergo apoptosis with anti-Fas antibodies directly after attachment or 6h after attachment. To determine cell death, we measured AST and ALT levels released in the medium after 24h in culture as detailed elsewhere.(32) AST or ALT levels for each sample was calculated as the ratio of AST or ALT present in the medium versus the sum of the levels of AST or ALT released in the medium and that present in the homogenate of adherent cells. Results are presented as the ratio of AST or ALT levels released in Fas-treated cultures to those released in untreated cultures.

Measurement of caspase-8 activity

Culture conditions : In order to determine the effect of medium on Fas-induced caspase-8 activity, cells were treated, after attachment, with anti-Fas antibodies (250 ng/ml) for 3h in

medium N or C+M. In parallel, the effect of time in culture was evaluated on cultures incubated with anti-Fas antibodies (for 3h) either directly after attachment or 6h later in medium C+M. Cells were then scraped off in PBS and collected by centrifugation. Cells were lysed for 15 min on ice in lysis buffer (10 mM Hepes [pH 7.4], 5 mM $MgCl_2$, 42 mM KCl, 0.1 mM EDTA, 0.1 % 3- [(3-cholamidopropyl) dimethylammonio] - 2 - hydroxy – 1 -propanesulonate (CHAPSO; Calbiochem; San Diego, CA, USA), 0.1 % Triton X-100, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor and 100 µM benzamidine). Lysates were centrifuged at 13 000g for 10 min and the supernatant stored at –80°C. Human activated recombinant caspase-8 (Calbiochem) was used to determine the glutathione-dependent modulation of caspase-8 activity. In order to do so, activated caspase-8 (30 U/well) was mixed with assay buffer containing 5mM GSx with different ratios of GSH/GSx (100 % to 0 %).

Measurement of the proteolytic activity : The fluorometric Ac-IETD-AMC cleavage assay was performed in microtiter 96-well plates. Reaction mixture contained 40 µl lysates (200 µg protein), 50 µl assay buffer 2x (100 mM Hepes [pH 7.2], 200 mM NaCl, 2 mM EDTA, 20 % sucrose, 0.2 % CHAPSO, 20 mM DTT). After an initial 10 min incubation at 37°C, the reaction was started by adding 10 µl of caspase-8 fluorescent substrate Ac-IETD-AMC (100 µM; Biosource International, Camarillo, CA, USA). The cleavage activity of caspase-8 was evaluated over a period of 30 min using 380 nm and 460 nm as excitation and emission wavelengths respectively in a SPECTRAmax GEMINI microplate

spectrofluorometer (Molecular Devices, Suunyvale, CA, USA). The maximal substrate cleavage rate (Vmax/sec) was calculated by SOFTmax® Pro software (Molecular Devices). The activity of caspase-8, presented in units, was derived from a calibration curve relating Vmax/sec to increasing units of human activated recombinant caspase-8.

Immunoblotting

Cell lysis : We determined the expression level of procaspase-8, Bid and BCl-x_L proteins after 0, 0.5, 1 and 3h post-cell attachment. At the end of the experiment, the medium was removed, cells were then scraped off and pelleted by centrifugation. Cells were subsequently disrupted by sonication (Sonic & Materials, Danbury, CT, USA) in the presence of lysis buffer (PBS, pH 7.4; 1 % Igepal CA-630; 0.5 % deoxycholic acid sodium salt; 0.1 % SDS; 5 mM EDTA; 10 µg/ml leupeptin, 10 µg/ml aprotinin; 10 µg/ml soybean trypsin inhibitor and 100 µM benzamidine).

Western Blot (38) **:** Proteins samples (125 µg) of each experimental condition were separated by electrophoresis on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Equal protein loading was assessed by staining the membranes with Ponceau S (Sigma). Blots were probed with primary antibodies for 2h, then with secondary antibodies for 1h, both at room temperature with gentle agitation. The expression of procaspase-8 (57 KDa), Bid (23 KDa), BCL-x_L (29 KDa) and actin (42 KDa) proteins were detected respectively with rabbit anti-human caspase-8 (1µg/ml; R&D Systems; Minneapolis, MN, USA), goat anti-human/mouse Bid (1µg/ml; R&D Systems), mouse monoclonal anti-BCL-x antibody

(0.5 µg/ml; Transduction Laboratories, Lexington, KY, USA) and anti-mouse actin monoclonal IgM ascites (1: 2000; Oncogene Research Products; Cambridge, MA, USA). Peroxidase-conjugated anti-mouse IgG (1: 10 000; BD Pharmingen; Mississauga, ON, CA), peroxidase-conjugated anti-rabbit IgG (1: 10 000; BD Pharmingen; Mississauga, ON, CA), peroxidase-conjugated anti-goat IgG (1:5000; Santa Cruz Biotechnology) and Peroxidase-conjugated anti-mouse IgM (1: 10 000; Oncogene Research Products) activities were revealed using Western Blot Chemiluminescence Reagent Plus (NEN™ Life Science Products, Boston, MA, USA). Blots were then scanned and bands were quantified by densitometry.

Statistical analysis

All data represent the values of at least 5 experiments, each from different cell isolation. Differences between groups were analyzed by one-way analysis of variance (ANOVA) for repeated measures, unless stated otherwise. The difference between treatment and time was analyzed by two-way ANOVA for repeated measures. A p value below 0.05 was considered significant.

RESULTS

Cell isolation and culture decrease liver GSx levels

As mentioned above, glutathione is a very abundant intracellular thiol in the liver. GSx concentration amounted to 2.04±0.33 nmol/μg protein in total liver (Figure 1). Levels were greatly diminished during the cell isolation procedure. Indeed, GSx levels were decreased by 46 % following liver digestion with collagenase (1.11±0.09 nmol /μg protein; $p<0.01$), but were not significantly reduced by hepatocyte purification (1.01±0.09 nmol /μg protein; ns). When cells were cultured for 2h in commercial Williams' E medium (medium N), GSx levels were further diminished by 46 % (0.55±0.04 nmol/μg protein; $p<0.01$) as compared to just before culture. This signifies that, after 2h of attachment, cells retained only 27 % of their original content in GSx ($p<0.001$).

Cys and Met supplementation stabilizes GSx levels in cultured hepatocytes

In order to modulate GSx synthesis, we added Cys and Met to the culture medium at a final concentration of 90 mg/L and 55 mg/L respectively (medium C+M). These concentrations were chosen by performing a dose-response curve where we determined the effect of different concentrations of these two amino acids on intracellular GSx levels. As shown in Figure 1, cell culture in this enriched medium prevented the loss of GSx that was observed in medium N during the 2h attachment (0.99±0.17 vs 0.55±0.043 nmol/μg protein for medium C+M vs N; $p<0.01$).

Of note, addition of greater concentrations (up to 120 mg/L Cys and 75 mg/L Met) of these two amino acids to the culture medium during cell attachment resulted in 50 % restoration of GSx that was lost during liver digestion. However, it was more rapidly evacuated from the cells in culture in comparison to medium C+M (results not shown). Thus, our experimental conditions were optimal in preserving GSx in cultured mouse hepatocytes.

The ability of Fas to induce apoptosis is greater in cells cultured in medium C+M

Our untreated cultures displayed very low rates of apoptosis (<1 %) with no significant difference between the two media (data not shown). When hepatocytes were incubated with an agonistic Fas-R antibody, a significant apoptotic response was observed in both media. Of note, the level of necrosis, as measured by Trypan Blue exclusion, was not significantly different between untreated and Fas-treated cultures or between the two media (data not shown). Interestingly however, the level of apoptotic cells, as determined by condensed chromatin fluorescence, was more than twice as great in medium C+M (10.0 %±0.9) as in medium N (4.4 %±0.2; $p<0.05$; Figure 2A). Similarly, Fas-induced AST release was higher in medium C+M (127.6 % ± 3.1) as compared to medium N (114.6 % ± 4.0; $p<0.001$; Figure 2B). In addition, Fas-induced release of ALT was significantly higher in medium C+M (209.6 %±9.3) in comparison to medium N (186.0 % ± 10.4; $p<0.05$; Figure 2C).

The expression levels of procaspase-8, Bid and BCL-xL proteins are similar in both media over time in culture

Apoptotic induction depends on the ratio of anti- and pro-apoptotic regulating proteins. Caspase-8, Bid and BCL-x_L are some of the key proteins implicated in the regulation of Fas pathway in hepatocytes. Therefore, we measured the level of expression of procaspase-8 (57 KDa), Bid (23 KDa) and BCL-x_L (29 KDa) in both media for up to 3h post-attachment by western blot analysis. Figure 3 shows that protein expression of procaspase-8 (Figure 3A), Bid (Figure 3B) and BCL-x_L (Figure 3C) in medium N was not significantly different from that in medium C+M (ns) and this at each time point tested. This suggests that the difference in the ability of Fas to induced cell death is not due to different expression levels of these key apoptosis-regulating proteins.

Fas capacity to activate caspase-8 proteolytic activity is greater in medium C+M

Since the expression level of procaspase-8 was similar in cultures incubated with either media N or C+M, we next examined the protease activity of caspase-8 in untreated cultures or in cells treated with anti-Fas antibodies for 3h directly after cell attachment in both medium N and C+M (Figure 4). In untreated cultures, levels of caspase-8 activity in both medium N (1.16±0.45 U) as well as C+M (1.41±0.25 U; NS) were low. After 3h of Fas receptor stimulation, the activity increased to 8.70±1.25 U and 13.60±0.79 U in medium N and C+M respectively ($p<0.001$), with the difference between the two media being significant ($p<0.05$).

GSH/GSx ratio declines over time in culture

Normally, GSH/GSx ratio is kept stable in cells by different mechanisms including GSSG reduction and GSSG active extrusion. This ratio was at 83.0 %±7.3 in total liver and was not significantly modified during liver perfusion, hepatocyte purification or cell attachment for 2h (results not shown). However, GSH/GSx ratio was observed to decrease in our cultures over time. Indeed, hepatocellular content in GSx was decreased by 25 % after 6h culture with medium C+M as compared to just after 2h attachment ($p<0.05$; Figure 5A). Nonetheless, GSx levels were 60 % higher in medium C+M than in medium N at 6h ($p<0.05$, data not shown). On the other hand, GSH levels were decreased from 27.65 ± 2.61 nmol / million hepatocytes to 16.95 ± 3.31 nmol / million hepatocytes ($p<0.05$), which represents a 39 % reduction (Figure 5A). This translates into a significant decline of GSH/GSx ratio from 82.0 % ± 2.6 at 2h attachment to 65.3 % ± 5.9 at 6h ($p<0.05$; Figure 5B). While a similar decrease of GSH/GSx ratio was observed in cells cultured in medium N (data not shown), it remained significantly higher in medium C+M (74.0 % ± 2.1) as compared to medium N (67.0 % ± 2.6; $p<0.05$) when measured at 3h post-attachment.

GSH/GSx ratio decline is associated with a decrease in Fas-induced caspase-8 activation and cell death

It is well documented that profound GSH loss could promote oxidative stress which subsequently leads to cell death. However, since we observed lower levels of Fas-induced apoptosis when GSx levels were depleted, we investigated if the observed decrease in

GSH/GSx ratio (17 % drop) over time in culture was also able to reduce the transmission of Fas signal. Therefore, apoptosis was induced after 6h of culture, and caspase-8 activity as well as cell death were then assessed. As illustrated in Figure 6A, there was an 18 % decline in the proteolytic activity of caspase-8 from 13.60 U±0.79 (at 2h attachment) to 11.09 U±0.71 (after 6h culture; $p<0.05$). This decline occurred concomitantly with a decrease in the capacity of Fas to induce cell death (Figure 6B and C). Indeed, AST released from cultures incubated with Fas antibodies after 6h of attachment was 19 % less (113.3 % ± 4.0; Figure 6B) than those incubated shortly after attachment (after 30 min; 140.0 % ± 6.0; $p<0.01$). Similarly, ALT release was decreased by 23 % in a time-dependant manner as shown in Figure 6C (30 min: 235.4 %±10.6 vs 6h: 204.5 %±10.2; $p<0.01$).

In order to determine which form of GSx is associated with the observed decrease in the capacity of Fas to induce cell death over time in culture, we established a correlation between the levels of GSH and GSH/GSx ratio on one hand and ALT levels on the other. To do so, we conducted separate sets of experiments where cells were incubated with anti-Fas antibodies after different periods of culture in medium C+M following attachment, while other cells from the same animal served to measure GSH and GSx levels at the same time points. There was a significant correlation between ALT levels released and intracellular GSH levels ($R^2 = 0.476$; $p<0.05$). However, the correlation was much more important between GSH/GSx ratio and ALT levels ($R^2 = 0.846$; $p<0.0001$). This suggests that relative accumulation of GSSG in cells is associated with a reduction in the capacity of Fas to induce apoptosis over time in culture.

Recombinant Caspase-8 activity is decreased when GSH/GSx ratio is diminished in vitro

We next investigated GSx-dependent modulation of caspase-8 by determining the effect of varying GSH/GSx ratios on the proteolytic activity of the activated recombinant human form of this enzyme (Figure 7). Caspase-8 activity was not significantly different between 100% and 75% GSH/GSx ratio (20.61 U±1.55 and 19.72 U±0.70 respectively, ns). Conversely, the activity of the protease significantly decreased when this ratio declined below 75 % (R^2= 0.93; $p<0.01$). When 100% GSSG (i.e. 0% GSH/GSx ratio) was attained, there was no caspase activity detected.

DISCUSSION

The involvement of oxidative stress in cell death by necrosis is well established. Indeed, many studies have demonstrated the devastating effects of oxidants on intracellular macromolecules. For example, ROS have been shown to inactivate vital proteins (such as glutathione peroxidase and adenyl cyclase), cause DNA strand break and membrane lipid peroxydation.(20;27) As a result, cytosolic Ca^{2+} is increased, ATP is depleted, NADPH and GSH are oxidized and membrane integrity and signaling are impaired.(37) All these lesions are very harmful if not repaired. In a prolonged exposure to oxidants, cells exhaust their anti-oxidant mechanisms and their repair systems are overwhelmed leading to cumulative lesions and therefore to necrosis. (20;37)

In apoptosis, however, the question is still under debate. On one hand, depletion of reductant or anti-oxidant reserve was found to sensitize otherwise unresponsive cells to apoptosis. This was observed in several cell lines, including Jurkat (42), HepG2 (13) and cholangiocytes (6). In these studies, depletion of glutathione accentuated the apoptotic response, while high intracellular glutathione levels abrogated it. In addition, low amounts of oxidants, such as H_2O_2, were found to induce apoptosis in HL-60 cells. (24). On the other hand, growing evidence demonstrates that oxidants may actually prevent apoptosis. For example, NO-induced apoptosis of macrophages (4), Fas-induced apoptosis of T-cells (14) and cytokine-mediated apoptosis of hepatocytes *in vivo* (18) were found to depend on sufficient intracellular glutathione levels. The redox sensitivity of caspases was

hypothesized to be responsible for the observed protection because of decreased glutathione levels. In parallel, hepatocyte treatment with $O_2^{\bullet -}$ was shown to prevent Fas-induced apoptosis.(8) Therefore, it still remains unclear whether oxidants are pro- or anti-apoptotic. The aim of this study was to determine the involvement of the major intracellular anti-oxidant system, glutathione, in the sensitivity of primary hepatocyte cultures to Fas-induced apoptosis.

Since glutathione is known to be very abundant in the liver (9), we wanted to evaluate the effect of the cell isolation and culture procedure on GSx levels before determining the effect of glutathione on apoptosis in culture. Our data show important GSx depletion as a result of cell isolation and culture. Indeed, isolated hepatocytes cultured in commercial Williams' E medium (medium N) for 2h retained only 27 % of their original content of GSx. Medium supplementation with Cys and Met, while not fully restoring GSx levels to those in total liver, prevented GSx loss due to cell culture. In addition, this enriched medium maintained GSx levels more stable during extended cell culture as opposed to medium N since these levels remained twice higher in medium C+M than in medium N after 6h of culture in serum-free conditions (data not shown).

GSx represents the sum of cellular content in GSH and GSSG. Since GSH/GSx ratio was identical in cells cultured in either media (after 2h attachment), the difference in GSx levels most likely signifies that both GSH and GSSG levels diminished. Several studies have reported that cells can lose GSH by active extrusion and passive diffusion in addition to its utilization as a metabolic cofactor. (9;9) In fact, GSH efflux from hepatocytes is the first

step in the degradation of hepatic glutathione (9). Therefore, glutathione levels can drop drastically if not compensated. In parallel, the supply of the three amino acids that constitute glutathione is limited in the context of cell culture. Hence, glutathione synthesis is expected to decrease over time in culture. Concerning GSSG, cells convert it to GSH under normal redox state (over 80 % of GSx being in the form of GSH) and increase its extrusion under oxidative stress.(9) Therefore, normal culture conditions will result in a net loss of GSH and GSSG, i.e. GSx. When Cys and Met were added to the culture medium (medium C+M), Cys supply was increased both directly and indirectly, Met being a precursor of Cys. This ensures continued glutathione synthesis. In addition, the presence of Met on the external surface of the hepatocyte has been shown to inhibit GSH efflux from isolated hepatocytes (2;3). Therefore, the stabilization of GSx levels in cell cultures by medium C+M probably reflects the maintenance of glutathione synthesis and the attenuation of GSH efflux.

On the other hand, GSH/GSx ratio, which remained stable during cell isolation and attachment, declined (17 % drop) over extended period of culture in a similar manner in medium N and C+M. This drop is indicative of relative GSSG accumulation in the cells. Therefore, it seems that the processes or mechanisms responsible for GSSG elimination (reduction to GSH or active GSSG extrusion) lose their efficacy in time. Normally, the rapid reduction of GSSG to GSH is catalyzed by GSSG reductase, which requires NADPH as a cofactor (1;28) It could be postulated that alteration of NADPH availability may affect

GSH regeneration and consequently promote GSSG accumulation in the cell. Such factors could participate in the observed decrease of GSH/GSx ratio as a function of culture time.

After establishing the changes in GSx and GSH/GSx ratio in isolated hepatocytes in culture, we next evaluated the capacity of Fas to induce apoptosis in mouse hepatocytes cultured in medium N versus C+M. Our results show that cells cultured in medium C+M were more sensitive to Fas-induced apoptosis than congeners kept in medium N. Indeed, following Fas receptor stimulation, there was a higher proportion of apoptotic cells and increased AST and ALT release from cells cultured in medium C+M in comparison to those cultured in medium N. This higher sensitivity towards Fas-induced apoptosis was not due to differential expression levels of procaspase-8, Bid or BCL-x_L proteins, some of the key proteins regulating the Fas apoptotic pathway. Rather it seems to result from the higher Fas-induced proteolytic activity of caspase-8 observed in cells cultured in medium C+M as compared to medium N. In particular, it seems that equivalent proportion of activated caspases exhibit a more robust catalytic capacity in high GSH/GSx conditions. Indeed, our *in vitro* study on fixed amounts of recombinant activated caspase-8 clearly demonstrates the negative effect of oxidant (GSSG) on the proteolytic activity of this enzyme. In parallel, we observed a higher GSH/GSx ratio in medium C+M in comparison to medium N. This reinforces the hypothesis that lowering cells' buffering capacity, by GSH depletion and/or GSSG accumulation, reduces cell sensitivity towards Fas-induced apoptosis.

As mentioned above, oxidative stress is closely associated with cell death and necrosis. Consequently, replenishment of anti-oxidants, including glutathione, has been repeatedly

shown to protect against cell death. Hence, it seems contradictory to find that better preservation of glutathione levels is associated with increased sensitivity towards apoptosis. However, it is important to point that the liver is naturally sensitive to apoptosis. Indeed, Fas is capable of inducing massive and rapid apoptosis *in vivo* even in the presence of high intracellular concentrations of glutathione.(17;36) This means that apoptotic signal transduction and effectors are functional in a reducing environment. In parallel, it is well documented that hepatocytes in culture acquire resistance against apoptosis.(23;34) Since there was a drastic decrease of glutathione levels during cell isolation and culture, this suggests that the decrease in glutathione levels is responsible for the culture-acquired resistance to apoptosis. This hypothesis is further supported by the strong positive correlation between GSH/GSx ratio and Fas-induced apoptosis. In addition, caspase-8 proteolytic activity, from both human recombinant and cell lysate sources, directly and significantly correlated with GSH/GSx ratio. A similar relationship was reported by Hentze *et al.* for recombinant activated caspase 3. (17)

Conclusions similar to the present work have been reached in studies conducted by the groups of Wendel (16;17), Jaeschke (22) and Pessayre (15). These studies showed that *in vivo* depletion of glutathione, whether by acetaminophen or phorone administrations, protected the liver against receptor-induced apoptosis. Collectively, these data therefore lend support to the concept that GSH depletion and/or relative GSSG accumulation, which lower the cell's capacity to buffer against endogenous oxidants, reduce cell sensitivity towards receptor-mediated apoptosis. Furthermore, results from the present study offer

diminished caspase-8 activity, the apex of Fas/TNFα receptors apoptotic signal, as an explanation for the acquisition of resistance towards Fas-induced apoptosis by hepatocytes in culture as compared to *in vivo* conditions.

It is noteworthy that GSH depletion reported in this study occurred very rapidly. This has an importance at two levels. Firstly, it is well documented that GSH levels play a major role in mitochondrial function. In fact, mitochondrial GSH/GSSG ratio is a known modulator of the mega-channel complex, responsible for the mitochondrial transmembrane potential ($\Delta\psi_m$)(7;12;21). Therefore, GSH depletion should affect mitochondrial function and subsequently effectors of the apoptotic pathway. However, mitochondria retain GSH far more efficiently than the cytoplasm. Indeed, depletion of GSH from the cytoplasm may occur very rapidly ($t_{1/2}$ = 2h, see Figure 1 and (29)). On the other hand, it takes more than 30h for the mitochondria to loose half its GSH content ($t_{1/2}$ =30h), even conditions of extreme GSH depletion (29). Therefore, because of the rapid loss of GSH in our experimental conditions (during isolation procedure and short-term culture), there is potentially not enough time for the mitochondrial pool of GSH to be affected. This leads us to postulate that the effect of GSH on Fas-apoptotic pathway is, in fact, exerted on cytosolic components already present at the time of apoptotic induction. Thus, the rapid loss of GSH inhibits some of the key elements in the apoptotic machinery leading to protection against apoptosis.

Secondly, Haouzi *et al.* (15) have recently demonstrated that there is a difference in the sensitivity of the liver towards apoptosis depending on the length of liver exposure to GSH

depletion. Indeed, the authors have reported that long-term GSH depletion accentuated apoptosis while acute depletion prevented it. It seems that other mechanisms enter into effect in order to compensate for the loss of cell sensitivity towards apoptosis due to the rapid loss of GSH. Some of these compensatory mechanisms being increased p53 and Bax protein expression, which are well known pro-apoptotic modulators. Our results therefore provide an explanation for the acute effects of GSH depletion on the sensitivity of hepatocytes towards apoptosis. The activity of caspase-8 in situations of chronic GSH depletion would deserve to be evaluated.

CONCLUSION

In conclusion, our results clearly demonstrate that glutathione depletion by the isolation procedure and cell culture is greatly associated with the culture-acquired resistance to apoptosis. This decrease is related to lower proteolytic activity of caspase-8, which is at the apex of the Fas apoptotic pathway. Therefore, our results confirm the importance of stabilization of intracellular GSx levels for normal cellular function, one of these being the ability of cells to respond to death signals.

FOOTNOTES

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FIGURE LEGENDS

Figure 1 ***Effect of cell isolation and culture on total glutathione (GSx) levels***

GSx levels were measured, as described in Experimental Procedures, in total liver (right lobe), after liver perfusion with collagenase, in purified hepatocytes and after 2h attachment in serum-containing medium N (commercial Williams' E medium) or medium C+M (Williams' E medium supplemented with Cys and Met). The data are presented as nmol of GSx / µg of proteins collected after sample preparation for GSH determination. Values are depicted as mean ± SEM from 5 different experiments. ** : $p<0.01$; *** : $p<0.001$ as compared to total liver; #: $p<0.01$.

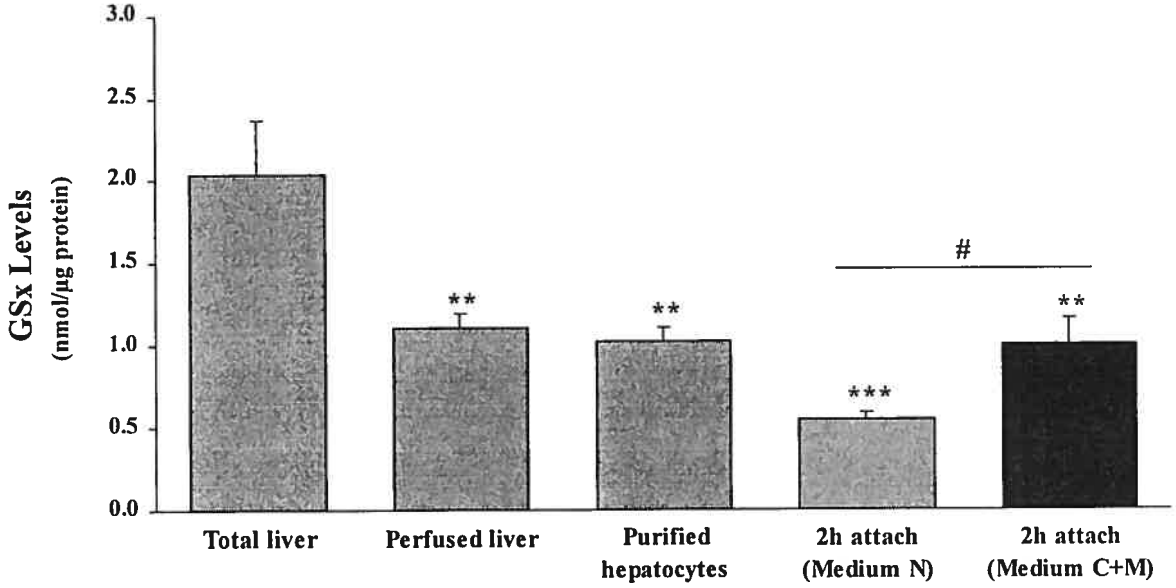
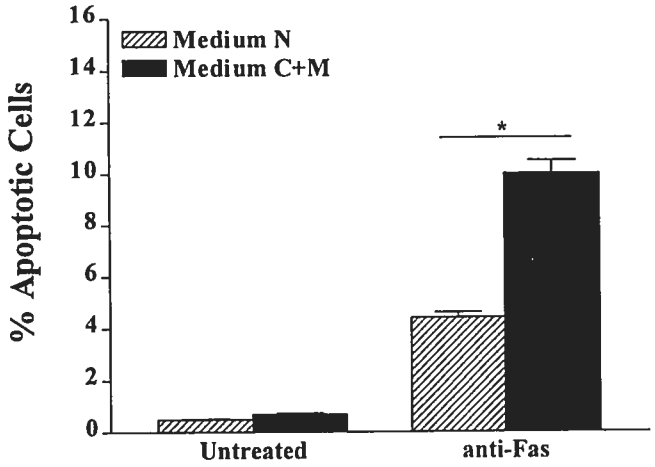

GSx Levels
(nmol/µg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
***
**
#
Total liver
Perfused liver
Purified hepatocytes
2h attach (Medium N)
2h attach (Medium C+M)

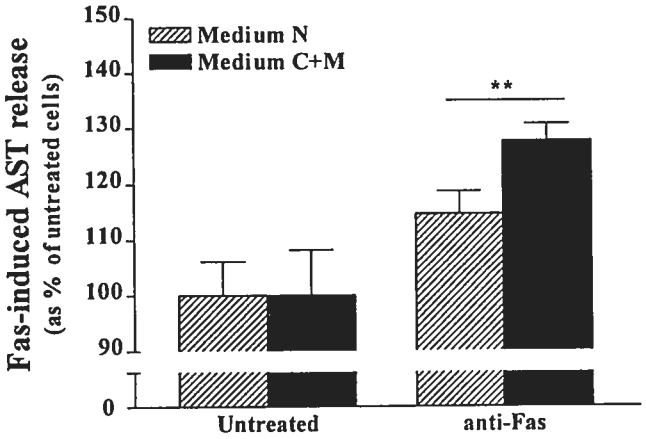
Figure 2 ***Effect of medium N and C+M on Fas-induced cell death***

After attachment, cells were treated with medium alone (untreated) or anti-Fas antibodies in medium N or C+M. Cell death, as described in Experimental Procedures, was assessed by morphologic criteria using Hoechst 33258 after 6h (panel A; $n = 5$) or biochemical analysis by quantitative determination of Fas-induced release of AST and ALT (presented as % of untreated cells) in the medium after 24h (panels B and C respectively; $n = 8$). Results are presented as mean ± SEM. Differences between groups (panel B) were analyzed by paired t-test * : $p<0.05$; ** : $p<0.01$.

A


% Apoptotic Cells
16
14
12
10
8
6
4
2
0
Medium N
Medium C+M
*
Untreated
anti-Fas

B


Fas-induced AST release
(as % of untreated cells)
150
140
130
120
110
100
90
0
Medium N
Medium C+M
**
Untreated
anti-Fas

C

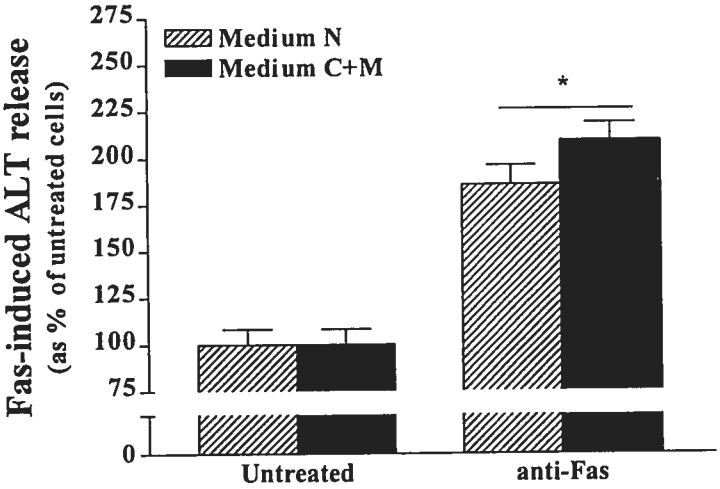

Fas-induced ALT release
(as % of untreated cells)
275
250
225
200
175
150
125
100
75
0
Medium N
Medium C+M
*
Untreated
anti-Fas

Figure 3 ***Effect of medium N and C+M on the expression levels of procapsase-8, Bid and BCL-x_L proteins***

Hepatocytes were collected after 0, 0.5, 1 and 3h post-attachment in either medium N or C+M. Samples (n = 5) were analyzed on 15 % polyacrylamide gel. Membranes were then blotted for procaspase-8 (57 KDa; panel A), Bid (23 KDa; panel B) and BCL-x_L (29 KDa; panel C) proteins with the appropriate antibody as described in Experimental Procedures. Actin (42 KDa) was used as control for equal protein loading. The data are presented as mean ± SEM. Representative blots are shown.

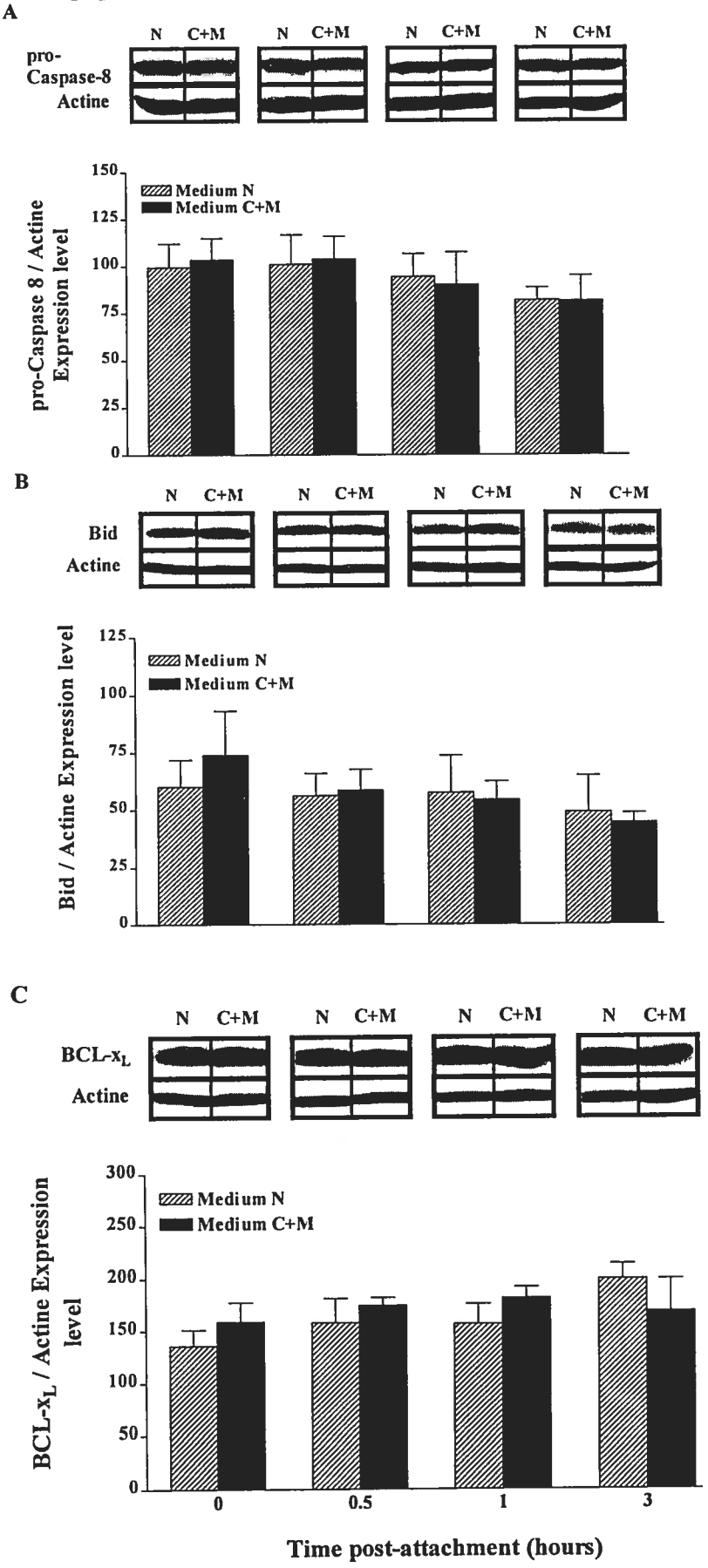

A
N C+M N C+M N C+M N C+M
pro-Caspase-8
Actine
pro-Caspase 8 / Actine Expression level
Medium N
Medium C+M
150
125
100
75
50
25
0
B
N C+M N C+M N C+M N C+M
Bid
Actine
Bid / Actine Expression level
Medium N
Medium C+M
125
100
75
50
25
0
C
N C+M N C+M N C+M N C+M
BCL-xL
Actine
BCL-xL / Actine Expression level
Medium N
Medium C+M
300
250
200
150
100
50
0
0
0.5
1
3
Time post-attachment (hours)

Figure 4 ***Effect of medium N and C+M on Fas-induced caspase-8 activation***

After attachment, cells were incubated with medium alone (untreated) or with anti-Fas antibodies [250 ng/ml] in medium N or C+M for 3h. Cells were then scraped off and caspase-8 proteolytic activity was measured in these samples (200 µg proteins; n = 6) using a fluorometric-substrate (IETD-AMC) based assay, as described in Experimental Procedures. Results are presented as mean of activated units of caspase-8 ± SEM. ** : $p<0.01$.

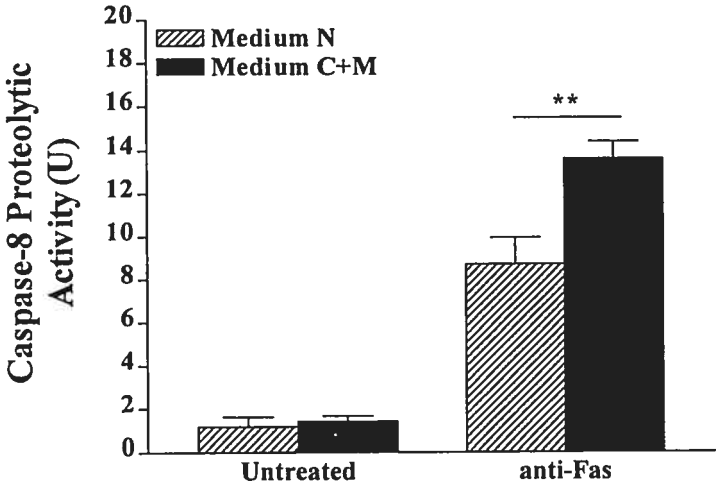
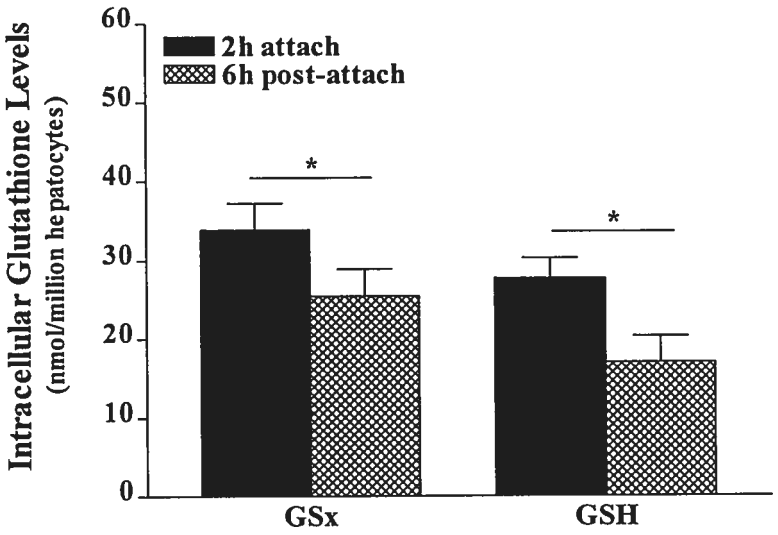

Caspase-8 Proteolytic Activity (U)
20
18
16
14
12
10
8
6
4
2
0
Medium N
Medium C+M
**
Untreated
anti-Fas

Figure 5 ***Effect of extended time in culture on GSx, and GSH levels as well as GSH/GSx ratio in cells cultured in medium C+M***

The levels of GSx and GSH (panel A) and GSH/GSx ratio (panel B) were measured either directly after or 6h after attachment in cells cultured in medium C+M. Data obtained (mean ± SEM) from 5 experiments are presented. * : $p<0.05$.

A


Intracellular Glutathione Levels
(nmol/million hepatocytes)
60
50
40
30
20
10
0
2h attach
6h post-attach
*
*
GSx
GSH

B

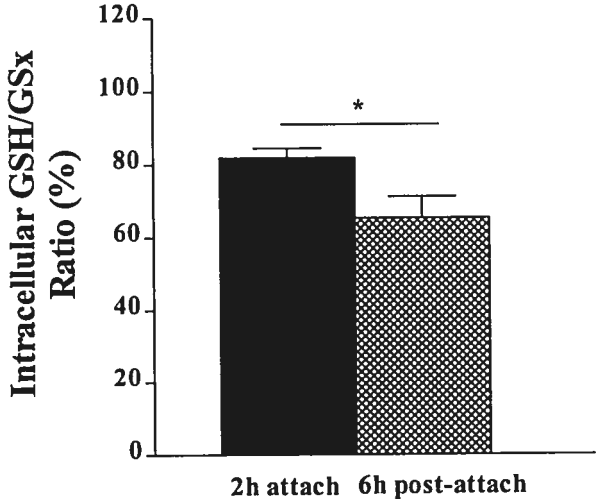

Intracellular GSH/GSx
Ratio (%)
120
100
80
60
40
20
0
*
2h attach
6h post-attach

Figure 6 ***Effect of time in culture on Fas ability to induce caspase-8 activation and apoptosis in medium C+M***

(A) Cells cultured in medium C+M were treated with medium alone (untreated) or anti-Fas antibodies [250 ng/ml] either directly after attachment or 6h later. 3h post-treatment, samples were collected to measure caspase-8 activity as described in Experimental Procedures. Results are presented as mean of activated units of caspase-8 ± SEM (n = 6). Quantitative determination of AST ***(B)*** and ALT ***(C)*** released (performed after 24h of culture) was used to evaluate the capacity of Fas to induce cell death in hepatocytes treated with anti-Fas either shortly (30 min) after attachment or 6h later. Results (mean ± SEM) from 8 experiments are presented. Differences between groups (panel B & C) were analyzed by paired t-test. ** : $p<0.01$.

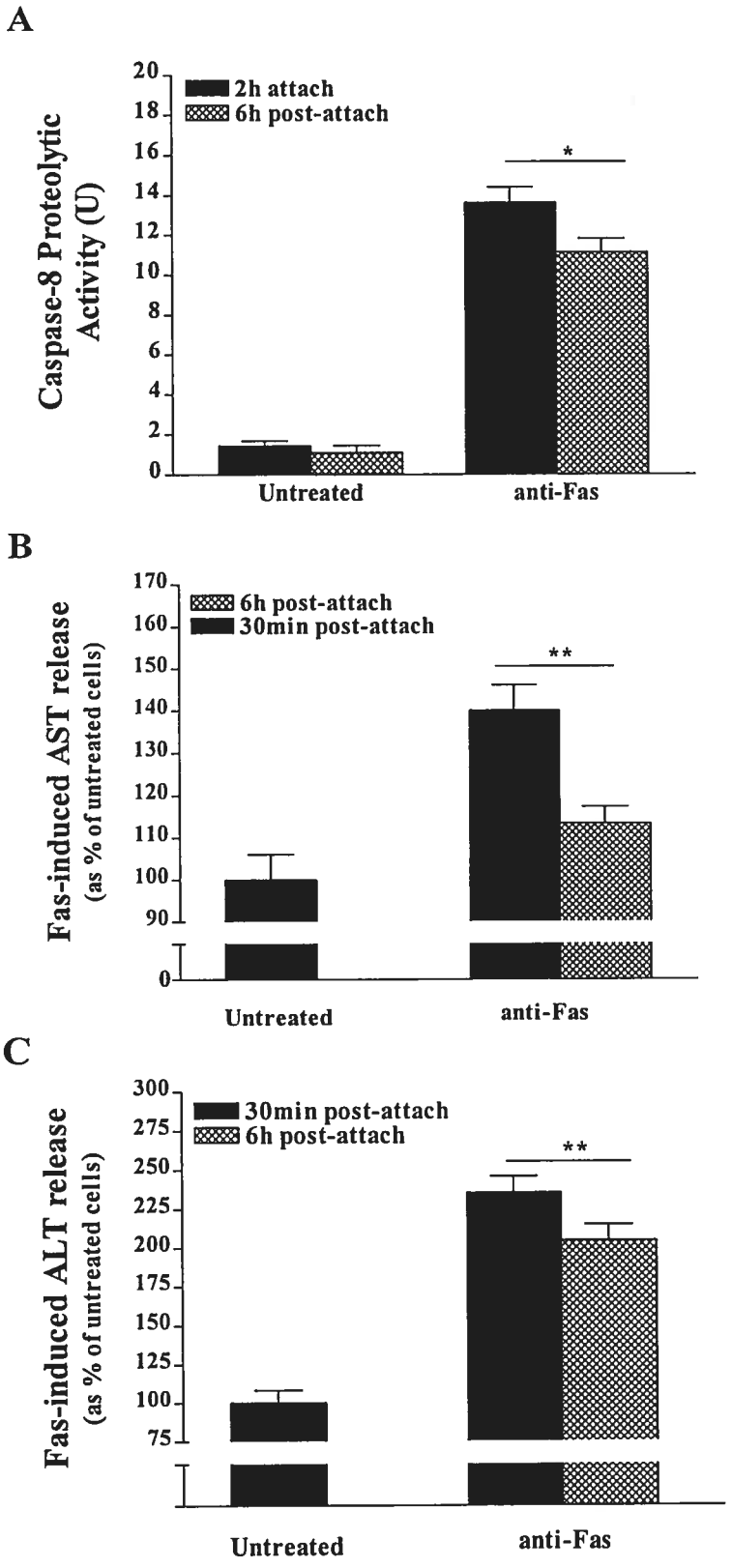
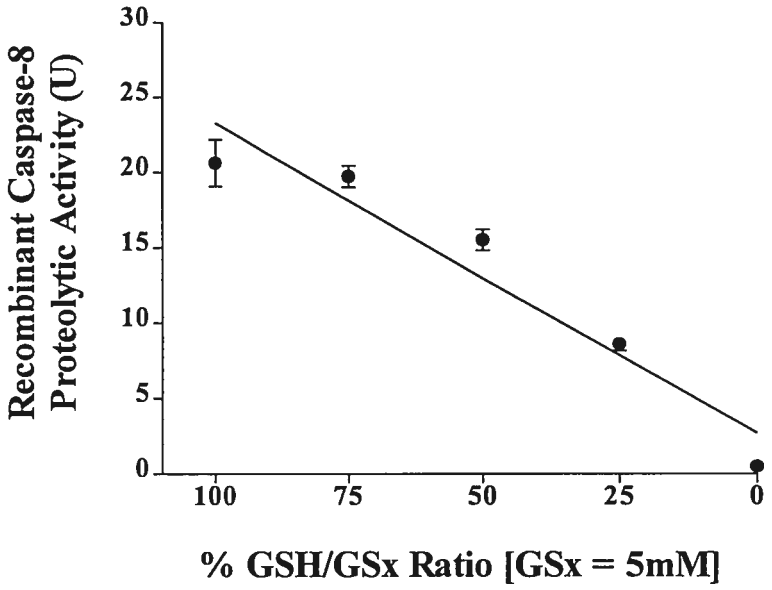

A
Caspase-8 Proteolytic Activity (U)
2h attach
6h post-attach
*
Untreated
anti-Fas
B
Fas-induced AST release
(as % of untreated cells)
6h post-attach
30min post-attach
**
Untreated
anti-Fas
C
Fas-induced ALT release
(as % of untreated cells)
30min post-attach
6h post-attach
**
Untreated
anti-Fas

Figure 7 ***Modulation of the protease activity of human recombinant activated caspase-8 by GSH/GSx ratio***

Human recombinant activated caspase-8 (30U/well) was mixed with assay buffer containing 5mM GSx with varying ratios of GSH/GSx. The protease activity was measured using a fluorometric-substrate (IETD-AMC) based assay. Data (n = 6) are presented as mean of activated units of caspase-8 ± SEM.


Recombinant Caspase-8
Proteolytic Activity (U)
30
25
20
15
10
5
0
100
75
50
25
0
% GSH/GSx Ratio [GSx = 5mM]

3.2 ARTICLE NO. 2

ROLE OF EGF RECEPTOR TYROSINE ACTIVITY IN ANTI-APOPTOTIC EFFECT OF EGF ON MOUSE HEPATOCYTES

BY

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ABSTRACT

The apoptotic Fas pathway is potentially involved in the pathogenesis of liver diseases. Growth factors, such as epidermal growth factor (EGF), can protect cells against apoptosis induced by a variety of stimuli, including Fas receptor (Fas-R) stimulation. However, the underlying mechanisms of their protection have yet to be determined. We investigated the involvement of EGF receptor (EGF-R) tyrosine kinase (TK) activity in the anti-apoptotic effect of EGF on primary mouse hepatocyte cultures. Cells undergoing apoptosis following treatment with anti-Fas antibody were protected by EGF treatment. This protection was significantly but partially decreased when cells were treated with two specific inhibitors of the TK activity of EGF-R. Evaluation of the efficacy of these compounds indicated that they were able to abolish EGF-R autophosphorylation and post-receptor events such as activation of mitogen-activated protein kinases and the phosphatidylinositol 3-kinase pathways as well as increases in BCL-x_L mRNA and protein levels. This leads us to postulate that EGF exerts its anti-apoptotic action partially through the TK activity of EGF-R. In addition, our results suggest that BCL-x_L protein upregulation caused by EGF is linked to the TK activity of its receptor.

Key words : Apoptosis, Fas, BCL-x_L, receptor autophosphorylation, inhibitors of receptor tyrosine kinases.

underlying mechanisms of this effect remain poorly understood (22;35;44;67). Several studies have correlated the anti-apoptotic effect of GF with increased expression of anti-apoptotic BCL-2-like proteins (2;24;35). However, it still remains unclear whether these proteins represent the major component of GF protection or whether other mechanisms exist.

GF signaling is mediated through enzymatic receptors that possess intrinsic tyrosine kinase (TK) activity. In response to the binding of their ligands, these receptors become oligomerized and then phosphorylated on specific tyrosine residues by their own catalytic activity (4;30;54). This phosphorylation leads to the activation of different intracellular pathways, such as mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase (PI 3-K), which mediate the biological effects of GF (17;39). For several GF, a number of studies have demonstrated that both the oligomerization (15;25;28;30) and the autophosphorylation (29;38;43) of their receptors are necessary for the transmission of their signal. On the other hand, it was reported recently that mutation of the TK domain of *c-met* (the receptor of HGF) did not abrogate the anti-apoptotic effect of HGF (5).

Therefore, we investigated the involvement of EGF-R TK activity in the protection against Fas-induced apoptosis afforded by EGF to primary cultures of mouse hepatocytes. To achieve this aim, we used specific inhibitors of the TK activity of the EGF receptor (EGF-R), such as PD168393 (18) and Tyrphostin AG1478 (37). We report that the anti-apoptotic effect of EGF requires, at least in part, the function of EGF-R catalytic activity. Moreover,

INTRODUCTION

The phenomenon of cell death by apoptosis is essential not only for the proper development of multiple systems and organs during embryogenesis (for example, through the elimination of self-reacting lymphocytes and the formation of digits) but also for the maintenance of adult tissue homeostasis (34;54;66). Given the widespread involvement of apoptosis, its potential role in several pathologies, including degenerative and autoimmune diseases, is not surprising (42;66). The pathogenesis of several liver diseases, such as hepatocellular carcinoma and alcoholic liver disease, specifically implicates abnormal apoptotic regulation (20;21;27;46;49). In addition, several studies have reported a marked increase in apoptosis in viral infections like hepatitis C (3) and B (59).

One of the apoptotic-inducing mechanisms that has been especially linked to human liver diseases is the cell-surface death receptor Fas (Fas-R), since it is constitutively expressed on hepatocytes (10;16;21;45). Fas-R induces apoptosis by activating intracellular effectors such as caspases (19;31) and endonucleases (6;12) and by recruiting pro-apoptotic proteins of the BCL-2 family (32;47). This family is divided into pro- (e.g. Bax, Bad, Bik) and anti- (e.g. BCL-2, BCL-x_L, BAG-1) apoptotic proteins that, respectively, facilitate and inhibit apoptosis (51;65).

It is well established that growth factors (GF), such as epidermal growth factor (EGF), nerve growth factor (NGF) and hepatocyte growth factor (HGF), are capable of protecting various cell types against apoptosis induced by different apoptotic agents, although the

this activity is responsible for the upregulation of BCL-x_L protein observed following EGF treatment.

MATERIALS AND METHODS

All animals received humane care according to the guidelines of the Canadian Council on Animal Care. Experimental protocols were approved by the Comité institutionnel de protection des animaux of the CHUM-Hôpital Saint-Luc.

Hepatocyte isolation and culture

Hepatocytes were isolated from the liver of fed male BALB/c mice (22-25 g) using the 2-step collagenase perfusion method described by Guguen et al. (23) with some modifications. After the induction of anesthesia with sodium pentobarbital (400 mg/kg intraperitoneally), the peritoneal cavity was opened, and the liver was perfused *in situ* via the portal vein for 4 min at 37°C with calcium-free Hepes buffer and for 7 min with Hepes buffer containing 45 mg % (w/v) Collagenase D (Boehringer-Mannheim, Laval, QC, Canada) and 135 mg % (w/v) $CaCl_2$. The perfusion rate was set at 5 ml/min for both solutions. The cells were used only if cell viability, as determined by trypan blue exclusion, was higher than 80%. The cells were seeded onto plastic petri dishes (26,000 cells/cm^2) in Williams' E medium (GIBCO BRL, Toronto, ON, Canada) supplemented with 10% fetal bovine serum (GIBCO BRL) and allowed 90 min to attach. The serum-containing medium was then removed, and the cells were subjected to different culture conditions in serum-free media. In control groups, cells were incubated with medium alone for the indicated time of the experiment. Apoptosis was induced in experimental groups with mouse anti-Fas Jo2 antibody (250 ng/ml, Pharmingen, Mississauga, CA, USA). The anti-apoptotic effect of

EGF was studied by simultaneously incubating the cells with 50 ng/ml of EGF (Sigma, Oakville, ON, Canada) and the anti-Fas antibody.

Determination of PD168393 and Tyrphostin AG1478 working concentrations

The TK activity of EGF-R was inactivated by the use of 2 specific inhibitors : ***a)*** PD168393 (10 μM, Cedarlane Laboratories; Hornby, ON, Canada) and ***b)*** Tyrphostin AG1478 (25 μM, Sigma). As PD168393 is an irreversible inhibitor {236}, its working concentration was determined by a dose-response curve where cells were incubated with EGF alone or in the presence of different concentrations of this inhibitor for 10 min, then assayed for EGF-R phosphorylation (see below): total inhibition was obtained at 10 μM (data not shown).

On the other hand, Tyrphostin AG1478 is a reversible inhibitor. Consequently its working concentration must insure complete inhibition over long-term incubation. Therefore, in addition to the dose-response curve, we conducted time-course experiments in which cells were treated with EGF in the presence or the absence of different concentrations of Tyrphostin AG1478 for 10 min, 1 h, 4 h and 8 h. The dose-response curve of Tyrphostin AG1478 indicated that complete inhibition of EGF-R autophosphorylation was obtained at 2.5 μM (data not shown). However, time-course experiments revealed that the inhibition produced by this concentration was not continuous over time (Figure 1). Therefore, to obtain complete and continuous inhibition of the TK activity of EGF-R over long periods of incubation, it was necessary to use 25 μM Tyrphostin AG1478. No evidence of cell toxicity was found at this concentration.

Morphological determination of apoptosis

After 24 h in culture, the medium was removed, and petri dishes were washed once with phosphate-buffered saline (PBS). The cells were fixed with 3% paraformaldehyde solution (pH 7.4, Sigma) for 20 min at room temperature, and then washed with PBS. To quantify apoptosis, hepatocyte nuclei were stained with Hoechst 33258 (250 ng/ml, Sigma) for 15 min, petri dishes were then washed with distilled water and left to dry at room temperature in the dark. Hoechst 33258 fluorescence was visualized under a microscope (BX50F, Olympus Optical Co., Japan) equipped with ultraviolet epifluorescence using excitation and emission filters of 355 and 465 nm respectively. When stained with this dye, normal hepatocyte nuclei appear homogenous and intact, as opposed to apoptotic nuclei which are condensed, fragmented and very bright (63). The percentage of apoptotic cells is expressed as the ratio of apoptotic nuclei versus the total number of nuclei (normal + apoptotic). 400 nuclei were evaluated for each petri dish.

Biochemical determination of cell death

Alanine aminotransferase (ALT) is an enzyme normally present in the cytosol of hepatocytes. In response to cell damage (necrosis or late-stage apoptosis), ALT is released from the cells. Therefore, to determine cell death, we measured ALT levels released in the medium after 24 h in culture. In order to do this, the medium was collected to measure ALT activity. The adherent cells were then scraped off in ice-cold PBS. Both solutions were sonicated then quantitative determination of ALT activity was performed by the Hôpital Saint-Luc Biochemistry Department with an automatic multi-analyser. ALT levels

for each sample was calculated as the ratio of ALT present in the medium versus the sum of the levels of ALT released in the medium and that present in the cell homogenate.

Immunoblotting

Cell lysis : Based on the results of time-course experiments, it was determined that EGF-R and MAPK phosphorylation assays be performed after 1-h incubation. AKT phosphorylation peak, on the other hand, was at 5 min. The expression of BCl-x_L protein was evaluated after 24 h in culture. At the end of the experiment, the medium was removed and the cells were washed once with ice-cold PBS. Cells were then scraped off and pelleted by centrifugation. Cells were subsequently disrupted by sonication (Sonic & Materials, Danbury, CT, USA) in the presence of lysis buffer [25 mM MOPS, pH 7.2; 60 mM β-Glycerophosphate; 15 mM 4-Nitrophenylphosphate; 1 mM Phenylphosphate; 1 mM Sodium Orthovanadate; 2 mM Dithiothreitol (DTT); 1 mM NaF; 15 mM EGTA; 15 mM $MgCl_2$; 10 µg/ml leupeptin, 10 µg/ml aprotinin; 10 µg/ml soybean trypsin inhibitor and 100 µM benzamidine]. Protein concentration was determined according to Bradford (7).

Western Blot (53) **:** Proteins samples (75 µg) were separated by electrophoresis on 7% (EGF-R and Akt) or 12% (MAPK and BCL-x_L) sodium dodecyl sulfate (SDS)-polyacrylamide gels, then transferred electrophoretically overnight to HYBOND-ECL nitrocellulose membranes (Amersham Pharmacia Biotechnology, Baie D'Urfée, QC, Canada). After transfer, equal protein loading was assessed by staining the membranes with Ponceau S (Sigma). Blots were probed with primary antibodies for 2 h, then with secondary antibodies for 1 h, both at room temperature with gentle agitation. To determine the levels

of Akt, of MAPK and of EGF-R phosphorylation, blots were incubated respectively with rabbit polyclonal anti-phospho Ser^{473} Akt (1:1000; New England Biolabs, Mississauga, ON, Canada), mouse monoclonal anti-phospho ERK (1 μg/ml; E-4, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-phosphotyrosine (1 μg/ml; PY20, Santa Cruz Biotechnology) antibodies. BCL-x_L protein expression was detected with mouse monoclonal anti-BCL-x antibody (Transduction Laboratories, Lexington, KY, USA) used at a concentration of 1.5 μg/ml. The activity of secondary anti-mouse IgG antibodies (1:2000) coupled to alkaline phosphatase (Santa Cruz Biotechnology) was revealed by 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl-phosphate reagents (Boehringer- Mannheim). Peroxidase-conjugated anti-rabbit IgG (1:5000) activity was revealed using Western Blot Chemiluminescence Reagent Plus (NEN™ Life Science Products, Boston, MA, USA). Blots were then scanned and bands were quantified by densitometry.

$H_3{}^{32}PO_4$ incorporation into mouse hepatocytes in culture :

Cells were attached onto 35 mm petri dishes (26,000 cells/cm^2) for 90 min as described above. After cell attachment, the serum-containing medium was removed and cells were incubated with phosphate-free Minimal Essential Medium (GIBCO BRL) supplemented with sodium pyruvate (25 mg/L; Sigma) for 10 min at 37°C then $H_3{}^{32}PO_4$ was added (30 μCi/petri; ICN Biochemicals, Irvine, CA, USA) for 1h-incubation at 37°C. The medium was removed and cells were washed once with Williams' E medium (37°C). The cells were incubated with 1 ml lysis buffer on ice for 30 min Cerenkov radiation was

quantitated in 200 µl of this homogenate using a β radiation counter. Proteins were precipitated from this homogenate by addition of 10 % sulfosalicilic acid (1:1) then resuspended in distilled water before protein concentration was determined according to Bradford.

Reverse-transcriptase polymerase chain reaction (RT-PCR):

RNA preparation : Samples for RT-PCR were prepared from cell cultures treated with medium alone or EGF with or without PD168393 for 6 h. Total RNA cellular content was isolated by Trizol® LS reagent (GIBCO BRL) according to manufacturer's recommendations. Briefly, cells (26,000 cells/cm^2) were homogenized with 2.25 ml Trizol® LS reagent. The aqueous phase containing total RNA was obtained by adding chloroform to the homogenate solution in 1:5 volume then separated by centrifugation at 12 000 g at 4°C. To precipitate RNA present in this phase, isopropanyl alcohol was added (1:1) and the resulting solution was incubated for 10 min at room temperature then centrifuged at 12 000 g at 4°C. The pellet was washed once in 75 % ethanol then resuspended in diethyl pyrocarbonate (DEPC)-treated water. RNA was then purified by Rnase-free DNase-I (0.5 U/µl; Roche Diagnostics, Laval, Qc) treatment for 1 h at 37°C in a buffer containing RNase inhibitor (0.1U/µl; Roche diagnostics), 10 mM Tris (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$. The purified RNA was extracted by phenol/chloroform treatment and precipitated with 95 % ethanol and 0.3M sodium acetate. The pellet was washed once in 75 % ethanol then resuspended in DEPC-treated water. RNA yield was determined by

measuring absorbance of an aliquot of each sample in distilled water at 260 and 280 nm. RNA concentration was calculated as follows :

$$\left(\frac{O.D._{260} - O.D._{280} \text{ x dilution factor}}{0.01} \right)$$

RT-PCR : PCR primers were made against mouse BCL-x_L and 18s ribosomal protein sequences with oligonucleotide primers designed using the PRIMER program (Genetic Computer Group, Wisconsin) and synthesized by the Sheldon Center (McGill University, Montréal, Qc). The forward and reverse primers were, respectively : BCL-x_L: 5′-ATGGCAGCAGTGAAGCAAG-3′ and 5′-GCAATCCGACTCACCAATACC -3′; 18S: 5′-TACCTGGTTGATCCTGCAGTA -3′ and 5′-AATGGATCCTCGTTAAAGGATT -3′. RNA (1µg) was mixed with 10mM Tris (pH 8.3), 1 mM $MgCl_2$, 50 mM KCl, 100g/ml BSA, 100 µM dNTPs, 1 µM primers, 100U/ml RNase inhibitor, 125 U/ml AMV reverse transcriptase, 20 U/ml Taq polymerase and 20µCi/ml [α-^{32}P] dCTP for a total reaction volume of 50 µl. Reverse transcriptase reaction was conducted at 50°C for 15 min followed by 3 min at 95°C. PCR was conducted at 95°C for 30 sec, 59°C for 45 sec and 72°C for 90 sec. BCL-x_L and 18s were amplified, respectively, for 23 and 15 cycles. These amplification conditions were determined by a kinetic study for each set of primers to ensure that PCR products remain proportional to initial gene expression template. Following PCR reaction, samples were electrophoresed on 8 % polyacrilamide gels, dried and Cerenkov radiation in excised band was quantitated using β radiation counter.

Statistical analysis

All data represent the values of at least 3 experiments, each from different cell isolation. Differences between groups were analyzed by one-way analysis of variance (ANOVA) for repeated measures (unless stated otherwise). A p value below 0.05 was considered significant.

RESULTS

EGF protects primary mouse hepatocyte cultures against Fas-induced apoptosis

Our control cultures displayed minimal levels of apoptosis (< 1%), even though they remained without serum for 24 h, as illustrated in Figure 2. However, hepatocyte incubation with a monoclonal antibody specific for mouse Fas-R (Jo2 clone) induced a significant increase in the level of apoptotic cells (21.9%±3.8; $p<0.001$). The percentage of apoptotic cells was significantly lower when hepatocytes were simultaneously incubated with EGF and anti-Fas (5.0%±1.1; $p<0.001$) than with anti-Fas alone, confirming that this GF is capable of protecting mouse hepatocytes against Fas-induced apoptosis, as previously reported by our laboratory (13).

EGR-R catalytic activity was effectively inhibited by TK inhibitors

As stated above, the TK activity of GF is important for the transmission of their signal in various cell types. However, several lines of evidence suggest that the TK activity of GF receptors (GF-R) may not be essential for all GF effects. To test the involvement of EGF-R TK activity in the anti-apoptotic effect of EGF, we assessed the content of EGF-R in phosphotyrosine in the absence and presence of two specific inhibitors of EGF-R catalytic activity : PD168393 and Tyrphostin AG1478. We used anti-phosphotyrosine antibodies to detect the activated EGF-R since GF-R phosphorylates itself on tyrosine residues following ligand binding. The putative EGF-R was detected at 170 kDa as reported in the literature {278}. Its content in phosphotyrosine after 1 h of EGF addition significantly increased as

compared to control untreated cultures (23.5±3.8 vs 3.25±0.5 arbitrary units (a.u.) respectively; $p<0.001$; Figure 3) indicating increased TK activity. When cell were treated with EGF in the presence of PD168393, EGF-R displayed similar phosphotyrosine content (4.0±1.2 a.u.) as that of EGF-R in untreated cells (4.0±1.1 a.u.; ns; Figure 3), indicating effective inhibition of EGF-R TK activity by PD168393. Similar results were obtained with Tyrphostin AG1478 (25 μM; data not shown). In addition, to insure the absence of residual EGF-R TK activity, we assessed $H_3{}^{32}PO_4$ incorporation into mouse hepatocytes after 1h treatment either with EGF alone or in the presence of both EGF and either inhibitor. As shown in Table 1, both inhibitors successfully prevented the EGF-induced increase in $H_3{}^{32}PO_4$ incorporation ($p<0.01$).

EGF-R downstream signaling is blocked by TK inhibitors

Cells treated with GF display increased activation of several intracellular signaling pathways (e.g. ERK pathway of MAPK family and PI 3-K pathway) and increased synthesis of growth and survival promoting proteins (e.g. BCL-x_L). Therefore, in order to ascertain that EGF-R signal was not transmitted via the TK activity of EGF-R in the presence of TK inhibitors, we used western blot analysis to measure **1)** the phosphorylation (and therefore the activation) of two signal transduction pathways using phospho-specific antibodies against p42/p44 (ERK) and Akt (PI 3-K) proteins, and **2)** the level of expression of the anti-apoptotic protein BCL-x_L. All these experiments were performed in untreated cells and in cells treated either with or without EGF in the presence of the inhibitors.

1) ***Signal transduction pathways :*** Cell treatment with EGF increased basal phosphorylation levels of p42 ($p<0.05$; Figure 4A), and of p44 ($p<0.05$; data not shown) by 2 fold and induced the phosphorylation of Akt on Ser^{473} ($p<0.001$; Figure 4D). Cell treatment with PD168393 (Figure 4A+D) or Tyrphostin AG1478 (data not shown) abolished EGF-induced increase in p42/44 and Akt phosphorylation ($p<0.01$) strongly suggesting that EGF-R signal was not transmitted into the cell neither through the ERK nor the PI 3-K pathways in the presence of these inhibitors.

2) ***BCL-x_L mRNA and protein expressions :*** BCL-x_L protein, a known homologue of the BCL-2 anti-apoptotic protein, is constitutively expressed in hepatocytes. As already observed in our laboratory (14), the level of BCL-x_L mRNA expression in untreated mouse hepatocytes (57.75±5.4 a.u.) increased by 30 % in EGF-treated cells (74.5±7.6 a.u.; $p<0.01$; Figure 4B). As a consequence, BCL-x_L protein expression level was doubled following EGF treatment (17.1±0.2 vs 33.7±5.9 a.u. for untreated vs EGF-treated cultures respectively; $p<0.05$; Figure 4C). PD168393 slightly increased basal BCL-x_L protein cellular content in untreated cells, but this effect was not significant (22.4±1.4 vs 17.1±0.2 a.u. for PD168393-treated vs control cells respectively; ns). Cell treatment with EGF in the presence of 10 μM PD168393 totally blocked EGF-induced elevation in BCL-x_L mRNA in comparison to control conditions (57.75±4.9 vs 74.5±7.6 a.u. respectively; $p<0.01$; Figure 4B). This was confirmed by protein expression (19.4±2.6 vs 33.7±5.9 a.u. respectively; $p<0.05$; Figure 4C). Finally,

treatment with this inhibitor prevented cell spreading, a characteristic morphological change of EGF-treated cells (Figure 5).

Taken altogether, these results establish the efficacy of PD168393 and Tyrphostin AG1478 as inhibitors of EGF-R TK activity and its downstream signaling events. Therefore, these compounds represent adequate tools to study the implication of the catalytic activity of EGF-R in the anti-apoptotic effect of EGF.

Inhibition of EGF-R TK activity diminishes the anti-apoptotic effect of EGF

The level of apoptosis in anti-Fas+EGF-treated cultures was significantly increased by PD168393 addition as illustrated in Figure 6A (13.4% ±3.4 vs 44.8% ±1.0 for control vs PD168393 respectively; $p<0.05$). Interestingly, however, addition of this inhibitor did not fully abolish EGF protective capacity against Fas-induced apoptosis ($p<0.01$). This phenomenon was also observed when we measured the level of ALT released in the medium. Indeed, PD168393 addition significantly increased ALT levels in the medium of anti-Fas+EGF-treated cultures (84.2%±5.2) as compared to control anti-Fas+EGF cultures (52.1%±9.3; $p<0.01$; Figure 6B). Nonetheless, medium ALT levels of cultures treated with anti-Fas and EGF in the presence of PD168393 remained significantly lower than in cultures treated with anti-Fas alone ($p<0.05$). Similarly, EGF provided partial protection to mouse hepatocytes against Fas-induced apoptosis in the presence of Tyrphostin AG1478 (Figure 6C). It is important to point out that treatment of primary mouse hepatocyte cultures with PD168393 or Tyrphostin AG1478 did not significantly affect the level of apoptosis in untreated or anti-Fas-treated cultures (data not shown).

Taken altogether, these observations strongly suggest that, in spite of the potency and efficacy of the inhibitors that we established above, the presence of either inhibitor caused only a partial decrease in the ability of EGF to protect mouse hepatocytes against Fas-induced apoptosis.

DISCUSSION

Growth Factors (such as EGF, HGF and NGF) are essential for several cell functions, including proliferation, differentiation, and survival (1). Therefore, several investigators have focused their studies on the identification and characterization of the intracellular effectors involved in the execution of GF biological responses in various cell types. Hence, a general model regarding the mode of action of GF was elaborated (26;68) : the interaction of GF with their receptors induces the oligomerization of the latter and, consequently, activates the TK domain of the same receptor. This domain was shown to be responsible for the phosphorylation of the receptor itself on specific tyrosine residues, which then serve as docking sites for SH2-containing molecules such as Grb2 and PI 3-K. These molecules lead to the activation of several intracellular pathways (such as MAPK, PLCγ and PI 3-K) which consequently mediate the diverse biological responses of GF (17;39;40;50;62). Thus, according to this hypothesis, the TK activity of GF-R plays a central role in the transmission of the GF signal.

Nevertheless, there have been some conflicting reports regarding the importance of this activity and of the subsequent receptor autophosphorylation in the mediation of some of the biological functions of GF. Several studies have indeed reported that the TK activity of GF-R is required for the transmission of biological responses such as proliferation and gene transcription (29;38;43;68). On the other hand, evidence has accumulated to show that some GF effects may occur independently of the TK activity of their receptors. For

instance, Schreiber et al. have reported that cross-linking of cell-bound monoclonal EGF-R antibodies resulted in the clustering of the receptor and the stimulation of DNA synthesis without activation of the TK domain of this receptor (55). Therefore, the aim of the present study was to determine the importance of the EGF-R TK activity in the anti-apoptotic effect of EGF.

We first examined the effect of EGF on normal and Fas-stimulated mouse hepatocytes in primary cultures. EGF addition to Fas-treated cultures protected more than 70 % of mouse hepatocytes susceptible to Fas-induced apoptosis. This protection occurred in parallel with increased EGF-R phosphotyrosine content as well as MAPK and Akt phosphorylation. In addition, EGF treatment increased the cellular content of BCL-x_L mRNA and protein suggesting the implication of this protein in the anti-apoptotic effect of EGF.

It is important to point out that Fas-R stimulation had no influence on either baseline levels or EGF-induced levels of EGF-R, p42/p44 and Akt phosphorylation (data not shown). In addition, Fas-induced apoptosis in primary mouse hepatocytes cultures was shown by our laboratory to occur without any modulation of BCL-x_L protein expression since Fas-R stimulation had no significant effect on BCL-x_L mRNA and protein levels (14).

The involvement of the TK activity of EGF-R in the anti-apoptotic effect of EGF was then tested using two inhibitors of this activity : PD168393 and Tyrphostin AG1478. As previously reported (18;52), these compounds selectively inhibit the TK activity of the EGF-R by competing for its substrate subsite. Consistent with this fact, the findings of the

present study demonstrate that these inhibitors did not affect the basal state of any of our tested parameters. Indeed, PD168393 and Tyrphostin AG1478 addition to the culture media of untreated cultures had no significant effect on either overall $H_3\,^{32}PO_4$ incorporation into the cells (see Table 1) nor on basal MAPK and Akt phosphorylation levels (Figure 4A and D respectively). In addition, the level of apoptosis in untreated and Fas-treated cultures was not significantly affected when these inhibitors were present (data not shown). Therefore, these results clearly indicate that PD168393 and Tyrphostin AG1478 have no direct effect on other kinase activities in mouse hepatocytes. Consequently, these compounds represent adequate tools to selectively inhibit the TK activity of EGF-R. In addition, their use permitted us to utilize normal mouse hepatocytes instead of cell lines transfected with mutated receptors, and thus allowed us to test conditions close to physiological settings.

In addition to being selective, these inhibitors are efficient. Indeed, cell treatment with either compound completely abolished EGF-induced 1) EGF-R autophosphorylation, 2) p42/p44 (ERK) phosphorylation 3) Akt (PI 3-K) phosphorylation, 4) BCL-x_L protein expression, and 5) $H_3\,^{32}PO_4$ incorporation into cells. Their effect was observed irrespective of the presence of pro-apoptotic Fas-R stimulation (data not shown). Therefore, these data strongly demonstrate that, in our experimental conditions, no residual TK activity (and consequently no TK-dependent EGF-R signal) could be detected in the presence of these inhibitors.

However, in spite of these observations, we were surprised to find that EGF-induced anti-apoptotic effect, even though significantly reduced by both TK inhibitors, was not

completely abolished. Indeed, we found that the level of apoptotic cells was higher in anti-Fas+EGF-treated cultures in the presence of PD168393 (by at least 35 %) and Tyrphostin AG1478 (by 50 %) as compared to control Fas+EGF-treated cultures. This level, however, never reached that observed in Fas-treated cultures, indicating that some of EGF protective signal still passes through into the cell in the presence of these inhibitors. Therefore, our results strongly suggest that part of this protection is not related to the TK activity of EGF-R.

Several studies have shown that the TK activity is important for the anti-apoptotic effect of GF. For instance, it was demonstrated that an IGF-1 receptor lacking a functional ATP binding site provided no protection from apoptosis (48). In addition, it was reported that Tyrphostin AG1478 was able to negate the anti-apoptotic action of EGF in a human bladder carcinoma cell line : transformed cells that express high levels of EGF-R (8). Here we report that EGF anti-apoptotic signal requires, at least in part, the catalytic activity of EGF-R to be able to modulate certain cell death pathways. This modulation would probably be mediated through the well established TK-dependent signaling cascades such as MAPK and PI3-K, which either induce the activation of survival-promoting proteins (i.e. BCL-x_L protein), the down-regulation of pro-apoptotic proteins (i.e. Bid and Blk (14)) or simply their inactivation (i.e. PI3-K–mediated suppression of caspase-9 activation (33;64) or Bad protein inactivation by phosphorylation following GF treatment (9;69)).

However, contrary to the above-cited studies, we found that part of EGF anti-apoptotic response persisted independently of the TK activity of the receptor. As mentioned earlier,

there are precedents to anti-apoptotic signal transduction being independent from the catalytic activity of GF-R. Indeed, Bardelli et al. have reported that mutation of HGF-R in its catalytic domain did not affect HGF protective effect (5). More interestingly, partial involvement of the TK domain in the anti-apoptotic effect of a GF was previously reported : Dews et al. have demonstrated that mutation of IGF-1 receptor in its TK domain resulted in reduction of the anti-apoptotic action of IGF-1, without completely abolishing it (11). Here we report similar observations for EGF-R in primary mouse hepatocyte cultures.

To date, these TK-independent pathways have not been identified. However, in an attempt to understand these processes, it is important to remember that following GF binding to their receptors, another important initial event occurs; that is, receptor oligomerization. This phenomenon of receptor association and subsequent conformational changes may favor interactions between certain intracellular proteins in a manner that would link the oligomerized receptor to certain effectors of the anti-apoptotic machinery. Bardelli et al. (5) have indeed proposed that the anti-apoptotic protein BAG-1, a cytoplasmic protein of the BCL-2 family expressed in many cell types including hepatocytes (5;14;60), may play a role in these interactions. This group has shown that BAG-1 is capable of interacting with the cytoplasmic domain of GF-R through its C-terminus (5) independently of the GF-R phosphorylation state. At the same time, the BAG-1 N-terminus (which contains a ubiquitin-like domain) has been implicated in the mediation of protein-protein interactions (61). It is known that only full BAG-1 protein is capable of transmitting the anti-apoptotic signal of certain GF (61). Hence, with such functional and structural properties, BAG-1

(and other BAG-1-like proteins) may link GF-R with intracellular anti-apoptotic effectors without the need for GF-R autophosphorylation.

Another interesting point is raised by our observations. We showed that BCL-x_L protein is upregulated by EGF treatment only when TK activity of its receptor is functional and that this increase was also observed in Fas+EGF-treated cells (14). Expression of BCL-2-like proteins by gene manipulation (overexpression) has been shown by many to be sufficient to protect cells against apoptosis induced by different stimuli (2;56;57). For instance, it was reported recently that overexpression of the BCL-2 transgene was able to protect mouse hepatocytes against apoptosis induced by Fas-R stimulation (36). However, our observations suggest that it might not be the sole anti-apoptotic pathway activated by the EGF signal. Indeed, BCL-x_L levels did not increase when hepatocytes were treated with EGF-R TK inhibitors, yet part of EGF protection persisted. Some investigators have also observed that overexpression of BCL-2 and BCL-x_L proteins did not abrogate Fas/Tumor necrosis factor α (TNFα)-induced apoptosis, but protected the same cell types against radiation- (58) or glucocorticoid- (41) induced apoptosis. Therefore, protection against apoptosis can occur without the involvement of BCL-2-like proteins. In the present study, we focused on the protective effect of EGF against Fas-induced apoptosis in primary mouse hepatocytes cultures. Therefore, our results lead us to postulate that when BCL-x_L protein is increased in physiological settings in mouse hepatocytes, it represents one of several key constituents of the intracellular anti-apoptotic machinery.

CONCLUSION

In conclusion, our studies clearly showed that the protection afforded by EGF against Fas-induced apoptosis is mediated, at least in part, through intracellular mechanisms that depend on the TK activity of EGF-R (receptor autophosphorylation, MAPK and PI 3-K activation, as well as BCL-x_L protein increase). Nevertheless, our results strongly suggest that part of EGF-R signal might be transmitted into the cell independently of these mechanisms.

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FIGURE LEGENDS

Figure 1 ***Effect Kinetics of EGF-R phosphorylation in mouse hepatocytes treated with EGF in the absence and presence of Tyrphostin AG1478***

Primary mouse hepatocyte cultures were incubated with EGF [50 ng/ml] alone or in the simultaneous presence of 2.5 μM or 25 μM Tyrphostin AG1478 for 10 min, 1 h, 4 h and 8 h. The cells were then scraped off, lysed and assayed for EGF-R phosphorylation, as described in Materials and Methods.

Time	10 min.				1h				4h				8h			
EGF [50ng/ml]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AG1478[2,5?M]	-	+	-	-	-	+	-	-	+	-	+	-	+	-	+	-
AG1478[25?M]	-	-	+	-	-	-	+	-	-	+	-	+	-	+	-	+

Figure 2 ***Anti-apoptotic effect of EGF on primary mouse hepatocyte cultures subjected to Fas receptor stimulation***

After attachment, mouse hepatocytes were treated with medium alone (untreated cells), with anti-Fas [250 ng/ml], with EGF [50 ng/ml] or with anti-Fas and EGF simultaneously. After 24 h in culture, apoptosis was assessed by microscopy, as described in Materials and Methods, using Hoechst 33258. Data obtained (means ± SEM) from 7 experiments are presented. *** : $p<0.001$.

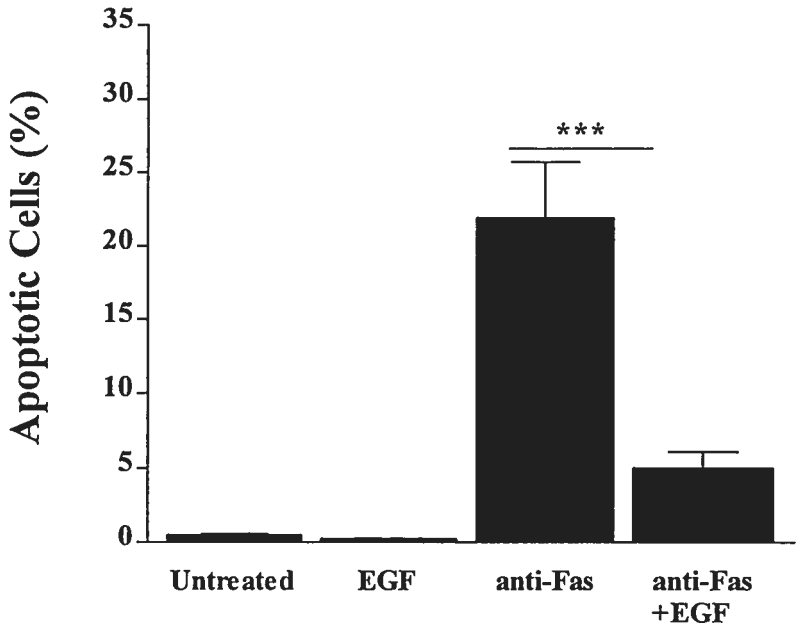

Apoptotic Cells (%)
35
30
25
20
15
10
5
0
***
Untreated
EGF
anti-Fas
anti-Fas +EGF

Figure 3 ***Effect PD168393 on EGF-R phosphorylation in mouse hepatocytes treated with EGF***

Hepatocytes were incubated for 1 h in medium alone or with EGF [50 ng/ml] in the absence or presence PD168393 [10 μM]. They were then lysed and analyzed by western blotting (7% polyacrylamide gel) using anti-phosphotyrosine antibodies. Results were obtained from 5 experiments and are presented as means ± SEM. A representative blot is shown.*** : $p<0.001$; ns : not significant.

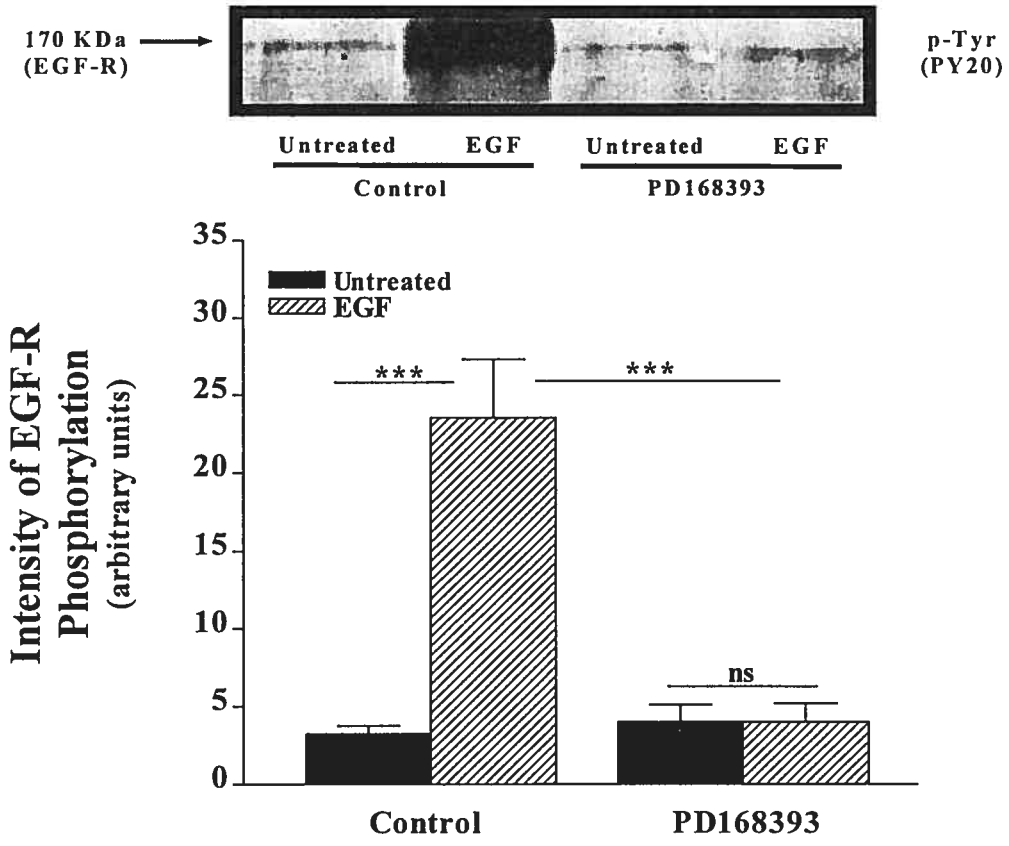

170 KDa
(EGF-R)
p-Tyr
(PY20)
Untreated
EGF
Untreated
EGF
Control
PD168393
Intensity of EGF-R Phosphorylation
(arbitrary units)
35
30
25
20
15
10
5
0
Untreated
EGF
***
***
ns
Control
PD168393

Figure 4 ***Effect of PD168393 on post-receptor events in mouse hepatocytes treated with EGF***

Untreated and EGF-treated cells were incubated in the absence and presence of PD168393 [10 μM] ***(A)*** Samples (n = 3) were incubated for 1 h with each condition then analyzed on 12 % polyacrylamide gel. Membranes were then blotted with anti-phospho MAPK antibodies. ***(B)*** Samples (n = 4), after 6 h incubation, were analyzed by RT-PCR for BCL-x_L gene using the ribosomal protein 18s as a control gene. Results are represented as a ratio of BCL-x_L / 18s. ***(C)*** Samples (n = 4) were incubated for 24 h with each condition then analyzed on 12 % polyacrylamide gel. Membranes were then blotted with anti-mouse BCL-x_L antibodies. ***(D)*** Samples (n = 3) were incubated for 5 min with each condition then analyzed on 7 % polyacrylamide gel. Membranes were then blotted with anti-phospho Ser^{473} Akt antibodies. The data are presented as means ± SEM. Representative blots are shown. * : $p<0.05$; ** : $p<0.01$; ns : non significant.

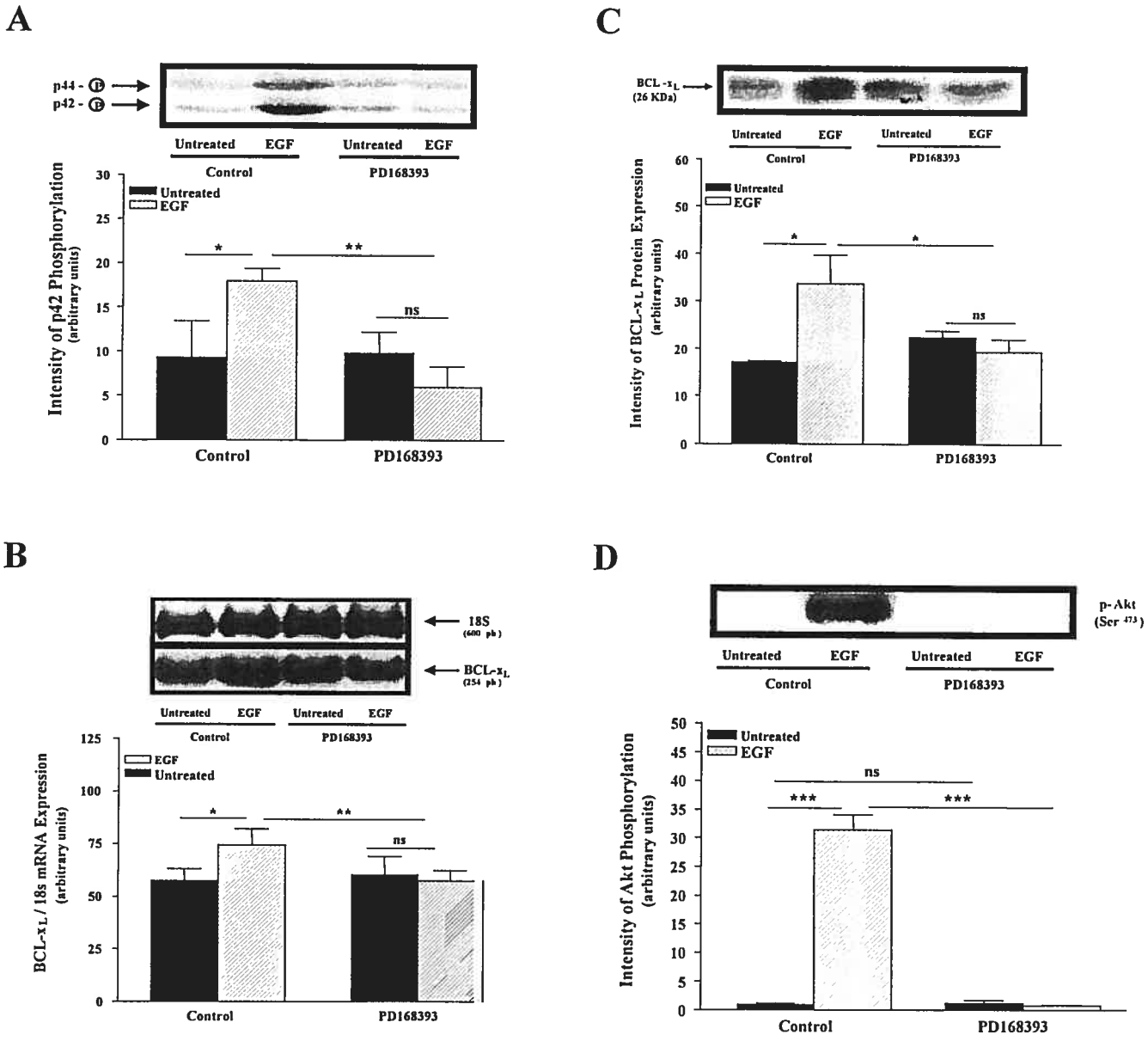

A
p44 - P
p42 - P
Untreated EGF Untreated EGF
Control PD168393
Untreated
EGF
Intensity of p42 Phosphorylation (arbitrary units)
30
25
20
15
10
5
0
*
**
ns
Control
PD168393
C
BCL-xL (26 KDa)
Untreated EGF Untreated EGF
Control PD168393
Untreated
EGF
Intensity of BCL-xL Protein Expression (arbitrary units)
60
50
40
30
20
10
0
*
*
ns
Control
PD168393
B
18S (600 pb)
BCL-xL (254 pb)
Untreated EGF Untreated EGF
Control PD168393
EGF
Untreated
BCL-xL / 18s mRNA Expression (arbitrary units)
125
100
75
50
25
0
*
**
ns
Control
PD168393
D
p-Akt (Ser 473)
Untreated EGF Untreated EGF
Control PD168393
Untreated
EGF
Intensity of Akt Phosphorylation (arbitrary units)
50
45
40
35
30
25
20
15
10
5
0
ns
***
***
Control
PD168393

Figure 5 ***Morphology of EGF-treated mouse hepatocytes in the presence and absence of PD168393***

Primary mouse hepatocyte cultures were incubated with medium (untreated) or EGF [50 ng/ml] in the presence or absence of 10 μM PD168393. After 24 h in culture, EGF-treated hepatocytes were spread and their cell surface increased, compared to untreated cells. Hepatocytes treated with EGF in the presence of PD168393 were spheroid and resembled control cells with or without PD168393. [Magnification, x100]

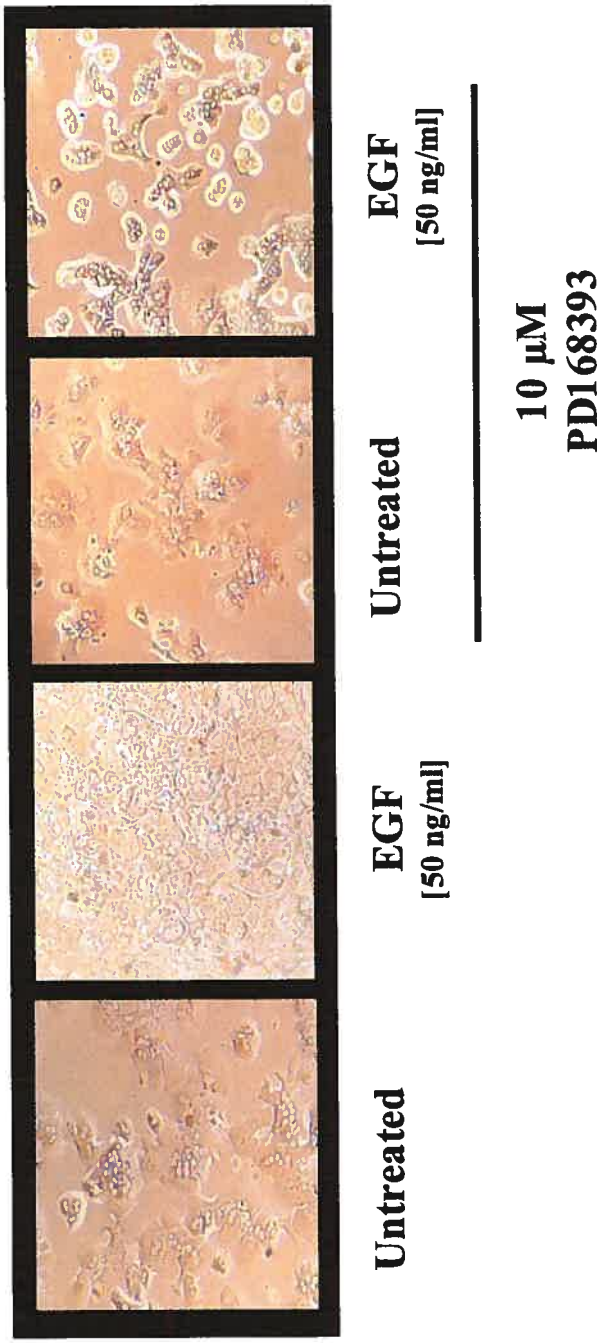

Untreated
EGF
[50 ng/ml]
Untreated
EGF
[50 ng/ml]
10 μM
PD168393

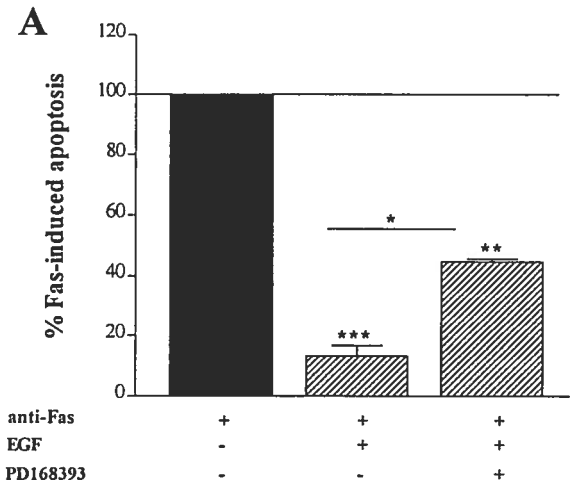
Figure 6 ***Effect of PD168393 and Tyrphostin AG1478 on the anti-apoptotic response of EGF in mouse hepatocytes***

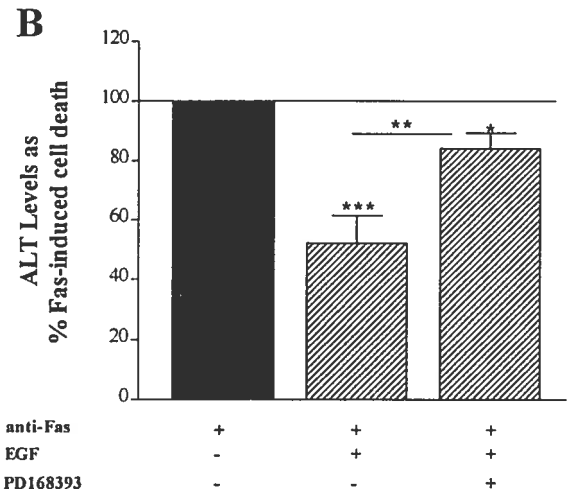
Hepatocytes were incubated with anti-Fas [250 ng/ml] alone or with EGF [50 ng/ml] in the absence or presence PD168393 [10 μM; panels **A** and **B**; n=3] or Tyrphostin AG1478 [25 μM; panel **C**; n=7] for 24 h. Results are presented as ratio

$$\left(\frac{\text{\% apoptotic cells [anti-Fas+EGF]}}{\text{\% apoptotic cells [anti-Fas]}} * 100 \right)$$

At the end of the experiment, cell death was assessed by morphologic criteria, using Hoechst 33258 (panels **A** and **C**) or by biochemical analysis by quantitative determination of ALT activity released in the medium (panel **B**). Differences between groups were analyzed by paired t-test.

* : $p<0.05$; ** : $p<0.01$; *** : $p<0.001$.


A
% Fas-induced apoptosis
120
100
80
60
40
20
0
*
**
***
anti-Fas + + +
EGF - + +
PD168393 - - +


B
ALT Levels as
% Fas-induced cell death
120
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80
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*
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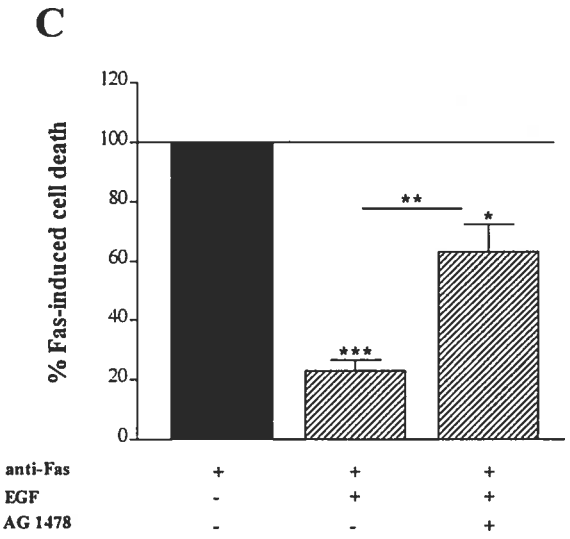
anti-Fas + + +
EGF - + +
PD168393 - - +


C
% Fas-induced cell death
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anti-Fas + + +
EGF - + +
AG 1478 - - +

Table 1 ***Effect of PD168393 and Tyrphostin AG1478 on $H_3{}^{32}PO_4$ incorporation into mouse hepatocytes treated with EGF***

	CPM / µg protein ($x10^3$) ± SEM		
	Control	**PD168393**	**Tyrphostin AG1478**
Untreated	32.4 ±2.2	30.7 ±1.0	31.5 ±1.1
EGF	40.8 ±1.3 *	31.3 ±0.3 †	32.3 ±1.6 †

* : $p<0.01$ as compared to control untreated cultures.

† : $p<0.01$ as compared to control EGF-treated cultures.

3.3 ARTICLE NO. 3

EGF MEDIATES PROTECTION AGAINST FAS-INDUCED APOPTOSIS BY DEPLETING AND OXIDIZING INTRACELLULAR GSH STOCKS

BY

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FOOTNOTE

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No. of figures: 6

No. of tables : 1

ABSTRACT

Several pieces of evidence have demonstrated the importance of reduction/oxidation (redox) signalling in biological processes, including sensitivity toward apoptosis. In parallel, it was recently reported that growth factors induce the generation of ROS. Therefore, we tested the hypothesis that the effect of EGF was mediated by changes in the redox state of hepatocytes through changes in GSH stocks. Isolated mouse hepatocytes were cultured and exposed to anti-Fas stimulation in order to induce apoptosis. Cell death by apoptosis was assessed by Hoechst 33258 staining and by measuring caspase-3 proteolysis activity. Cell treatment with EGF significantly decreased total (GSx) and reduced (GSH) glutathione levels in the presence and the absence of anti-Fas. Furthermore, glutathione reductase activity was lower in EGF-treated cultures (by 28%) as compared to untreated cultures which lead to a significant decline in GSH/GSx ratio. These effects were found to be EGF-specific. Co-stimulation of cells with anti-Fas and EGF attenuated caspase-3 activation and cell death by apoptosis by 70%. GSH monoethylester (GSHmee) significantly attenuated the effect of EGF on GSH and GSH/GSx ratio. It caused an increase in caspase-3 activation and in the percentage of apoptotic cells in anti-Fas+EGF-treated cells, thus resulting in a 53% decline in the protective effect of EGF. In conclusion, EGF induces a significant and specific depletion and oxidization of intracellular GSH, paralleled by a protection against Fas-induced apoptosis. GSH replenishment partly counteracted these effects suggesting that GSH depletion contributed to the protective effect of EGF against caspase-3 activation and cell death by apoptosis.

INTRODUCTION

The apoptotic process is tightly regulated considering the disastrous consequences that may ensue from excess or lack of apoptosis. That is why the apoptotic intracellular pathways are under intensive investigation. Protection against apoptosis is of special interest in cases of excess apoptosis such as in viral hepatitis and alcoholic liver disease (Galle 1997; Galle and Krammer 1998; Pinkoski et al. 2000). Growth Factors (GF) are well known protectors against apoptosis induced by a variety of stimuli. Some of the anti-apoptotic mechanisms activated by GF have recently been elucidated: 1) increases in the expression of the anti-apoptotic proteins of the BCL-2 family such as BCL-2, BCL-x_L and BAG-1 (Allsopp et al. 1993; Bardelli et al. 1996); 2) inactivation of Bad (a pro-apoptotic protein of the BCL-2 family(del Peso et al. 1997; Zha et al. 1996)) and procaspase-9 (Webster and Anwer 1999) through phosphorylation by protein kinase B (PKB/Akt). However, whether these mechanisms are the only ones employed by GF is not yet known.

Reactive oxygen species (ROS), generated from the mitochondria and other sources, have been traditionally regarded as toxic by-products of metabolism with potential to cause serious damage to intracellular macromolecules (such as lipids, DNA and proteins)(Kaplowitz and Tsukamoto 1996; Orrenius 1993). Therefore, cells employ different anti-oxidant mechanisms to protect the intracellular macromolecules and organelles and therefore to prevent cellular injury. The glutathione system, the most abundant soluble cellular thiol, acts as a homeostatic redox buffer(Meister 1988; Kamata

and Hirata 1999; Kaplowitz and Tsukamoto 1996). This is an important cellular parameter, since the intracellular redox state monitors the relative amounts of the oxidized and reduced species of each redox system within the cell. Oxidative stress occurs when intracellular redox homeostasis is altered. The imbalance may be due to either an overproduction of ROS or a deficiency in anti-oxidants. (Kamata and Hirata 1999; Kaplowitz and Tsukamoto 1996)

Several lines of evidence demonstrate that intracellular redox state affects cellular signalling (both at the receptor level and in intracellular pathways; reviewed in (Kamata and Hirata 1999; McCord 2000; Thannickal and Fanburg 2000b)). For example, the reversible oxidation of cysteine residues can significantly modify the cellular response to a given stimulus. Therefore, proteins (e.g. transcription factors, phosphatases and caspases) rich in reduced cysteine residues in their active site are susceptible to the intracellular redox state. Given the nature of these redox- sensitive proteins, variation of the redox state has very important consequences on a wide variety of cellular functions (cell signalling, enzyme activation, DNA synthesis, selective gene expression) and even on cell viability (Reviewed in (Powis et al. 1997; Gamaley and Klyubin 1999)).

ROS and oxidative stress have long been implicated in cell death by necrosis and recently by apoptosis as well (Orrenius 1993; Kaplowitz and Tsukamoto 1996). Although their role in necrosis is well established, their role in apoptosis is still under debate. Several studies ascribe ROS as inducers or at least mediators of apoptosis. (Zamzami et al. 1997; Sarafian and Bredesen 1994) Others, on the other hand, have demonstrated that they may in fact

function as anti-apoptotic agents (Clement and Stamenkovic 1996). This discrepancy might be explained by the intensity and localization of ROS production as well as the cellular context in which ROS production occurs. Indeed, depending on the level of H_2O_2 treatment, cellular response shifts from proliferation to apoptosis to necrosis (Kaplowitz and Tsukamoto 1996). Of note, recent findings showing that GF induce specific bursts of ROS (Thannickal et al. 2000a) might tip the balance even more toward protection since GF are known protectors against apoptosis.

Our laboratory has recently demonstrated the importance of intact glutathione status and especially the ratio of reduced (GSH)/total (GSx) glutathione on Fas-induced caspase-8 activation and consequently cell death by apoptosis (Musallam et al. 2002). Indeed, lower rates of caspase-8 activity and apoptotic bodies were found in cells containing low levels of GSH and GSH/GSx ratio. Based on these findings and the data mentioned above from the literature, we tested the hypothesis that the anti-apoptotic effect of EGF was mediated, at least in part, by changes in GSH stocks.

MATERIALS AND METHODS

All animals received humane care according to the guidelines of the Canadian Council on Animal Care. Experimental protocols were approved by the Comité institutionnel de protection des animaux of the CHUM-Hôpital Saint-Luc.

Hepatocyte isolation and culture

Hepatocytes were isolated from the liver of fed male BALB/c mice (22-25 g) using the 2-step collagenase perfusion method described previously (Musallam et al. 2001). Cells were seeded onto plastic petri dishes (26,000 cells/cm^2) in enriched Williams' E medium (cysteine : 90 mg/L and methionine : 55 mg/L) supplemented with 10% foetal bovine serum (GIBCO BRL, Toronto, ON, Canada) and allowed 2h to attach. After attachment, the serum-containing medium was removed, and cells were incubated with serum-free medium for the indicated times in each experimental series. Apoptosis was induced in experimental groups with mouse anti-Fas Jo2 antibody (Research Diagonistics Inc, Flanders, NJ, USA) at a concentration of 250 ng/ml. The anti-apoptotic effect of epidermal growth factor (EGF) was studied by simultaneously incubating cells with 50 ng/ml of EGF (Sigma, Oakville, ON, Canada) and the anti-Fas antibody. PD168393 (Cedarlane Laboratories; Hornby, ON, Canada), a specific inhibitor of the tyrosine kinase activity of EGF receptor, was used at 10 μM, a concentration previously shown to be effective in blocking EGF-R autophosphorylation (Musallam et al. 2002). Glutathione monoethylester

(GSHmee; Sigma) was used at 2.5 mM to increase the intracellular content of GSH on the basis of preliminary dose-response experiments.

Determination of GSx and GSH levels

Culture conditions : Cells were treated according to the different experimental conditions. At the end of the experiment, cells were scraped off, pelleted by centrifugation and resuspended in 300 µl 0.25 M sucrose solution. Samples were stored at –80°C until determination of GSx and GSH levels. **GSx and GSH determination :** GSx and GSH levels were measured by a colorimetric assay using DTNB as described previously (Musallam et al. 2002).

Morphological determination of apoptosis

After attachment, cells were incubated with medium alone, anti-Fas, EGF or the combination of both EGF and anti-Fas for 1h. The medium was replaced with medium alone (Control conditions) or with one containing GSHmee [2.5 mM; GSHmee conditions]. After 5h, cells were fixed with 5% formaldehyde solution (Anachemia Science, Lachine, Qc, CA) and then stained with Hoechst 33258 (250 ng/ml, Sigma) to quantify apoptosis as described previously (Musallam et al. 2001).

Measurement of Glutathione reductase (GR)

Culture conditions : Cells were treated with medium alone, anti-Fas antibodies, EGF and anti-Fas+EGF for 3h. Cells were collected and were subsequently disrupted by sonication (Sonic & Materials, Danbury, CT, USA) in the presence of Tris 50mM [pH 7.5] and 5 mM

EDTA buffer. Lysates were centrifuged at 13 000g for 10 min and the supernatant stored at –80°C. **Measurement of GR activity :** GR is responsible for the reduction of one molecule of oxidized glutathione (GSSG) into two molecules of GSH, using NADPH as source of protons. The measurement of GR activity is based on the decrease in the auto-fluorescence of NADPH at 340 nm as a result of its oxidation. Samples (250µg proteins) were diluted in 200 µl (final volume) Sample Diluent Buffer (125 mM KH_2PO_4 [pH 7.5]; 1 mM EDTA, 1 mg/ml BSA) and warmed to room temperature. One min before the start of the reaction, 400 µl of Assay Buffer (125 mM KH_2PO_4 [pH 7.5]; 2.5 mM EDTA) containing 2.5 mM GSSG was added. GSSG was not added in the blank. The reaction was started by the addition of 400µl of 0.45mg/ml NADPH. D.O. were recorded every 60 sec for 5 min at 340 nm. The slope of the regression curve for each sample was used to calculate GR activity using the following equation:

$$1\ mU = 1\ nmol\ NADPH/\ min = (A_{340}/min)/0.00622$$

Measurement of caspase-3 activity

Culture conditions : In order to determine the effect of GSHmee on the anti-apoptotic effect of EGF on caspase-3 activity, the medium of untreated, Fas-treated, EGF-treated and Fas+EGF –treated cultures was changed after 1-h incubation and replaced with medium alone or with one containing 2.5 mM GSHmee for 3h. Samples were prepared as described previously and stored at –80°C.

Measurement of the proteolytic activity : The fluorometric Ac-DEVD-AMC (100 µM; Biosource International, Camarillo, CA, USA) cleavage assay was used to measure caspases-3 activity (Musallam et al. 2002). The reaction was evaluated over a period of 30 min using 380 nm and 460 nm as excitation and emission wavelengths respectively and the maximal substrate cleavage rate (Vmax/sec) was calculated. The activity of caspase-3, presented in units, was derived from a calibration curve relating Vmax/sec to increasing units of human activated recombinant caspase-3.

Immunoblotting

Cell lysis : We determined the effect of GSHmee treatment on the level of procaspase-3 protein expression and EGF receptor (EGF-R) autophosphorylation. After cell incubation with medium alone or EGF for 1h, the culture medium was removed and cells were cultured in medium alone or with medium + GSHmee for 1h30 (for EGF-R phosphorylation) or 3h (for procaspase-3 protein expression). At the end of the experiment, cells were collected and prepared for SDS-PAGE as detailed elsewhere (Musallam et al. 2001).

Western Blot (Sambrook et al. 1989) **:** Proteins samples (125 µg) of each experimental condition were separated by electrophoresis on 10% (EGF-R) or 12% (procaspase-3) SDS-PAGE. Equal protein loading was assessed by staining the membranes with Ponceau S (Sigma). The expression of procaspase-3 (34 KDa), and actin (42 KDa) proteins were detected respectively with rabbit anti-human caspase-3 (2µg/ml; Santa Cruz

Biotechnologies; Santa Cruz, CA, USA) and anti-mouse actin monoclonal IgM (1: 2000; Oncogene Research Products; Cambridge, MA, USA). The level of EGF-R autophosphorylation was detected by mouse anti-mouse anti-phosphotyrosine (1 μg/ml; PY99, Santa Cruz Biotechnology). Peroxidase-conjugated anti-mouse and anti-rabbit IgG (1: 10 000; BD Pharmingen; Mississauga, ON, CA) as well as peroxidase-conjugated anti-mouse IgM (1: 20 000; Oncogene Research Products) activities were revealed as described previously (Musallam et al. 2001).

Statistical analysis

All data represent the values of at least 3 experiments, each from different cell isolation. Differences between groups were analyzed by one-way analysis of variance (ANOVA) for repeated measures, unless stated otherwise. The difference between treatment and time was analyzed by two-way ANOVA for repeated measures. A p value below 0.05 was considered significant.

RESULTS

EGF decreases GSx intracellular levels and GSH/GSx ratio in a time- and dose-dependent manner

Given the importance of glutathione for hepatocyte sensitivity toward apoptosis, we first evaluated the effect of cell treatment with anti-Fas and/or EGF on GSx intracellular stocks after 3h incubation. In untreated cultures, GSx levels were at 26.2 ± 2.4 nmol/10^6 hepatocytes. When anti-Fas antibodies were added, GSx levels were not significantly affected (24.6 ± 3.2 nmol/10^6 hepatocytes; Figure 1; not significant [NS]). However, GSx levels decreased following exposure to EGF alone (13.8 ± 2.4 nmol /10^6 hepatocytes; $p<0.001$) as well as in combination with anti-Fas (15.0 ± 2.6 nmol /10^6 hepatocytes; $p<0.01$). Similarly, GSH levels were decreased following EGF treatment in the presence ($p<0.001$) and in the absence of Fas ($p<0.001$; Figure 1). The drop is more pronounced in the case of GSH levels in comparison to GSx. This resulted in a decrease in GSH/GSx ratio (by 18 %) in EGF-treated cultures as compared to untreated ones (Figure 1B; $p<0.05$). This lower ratio is indicative of relative accumulation GSSG in cells.

EGF decreases the activity of GSSG reductase (GR)

Cells convert GSSG to GSH under normal redox state (over 80 % of GSx being in the form of GSH) and increase its extrusion under oxidative stress (Deleve and Kaplowitz 1990). Normally, the rapid reduction of GSSG to GSH is catalyzed by GR, which requires NADPH as a cofactor (Meister 1988; Anderson 1998). Therefore, since EGF decreased

GSH/GSx ratio, we analyzed the activity of GR following 3-h EGF treatment. The activity of this enzyme in untreated cultures was 35.8 ± 0.5 μU/μg protein (Figure 2). However, it declined by 28 % in EGF-treated cultures (25.6 ±0.2 μU/μg protein; $p<0.01$). Stimulation of Fas receptor did not significantly affect baseline or EGF-depressed GR activity. This relative inhibition of GR activity probably accounts for the observed lower GSH/GSx ratio in EGF-treated cultures.

Based on the results mentioned above, as well as on those from our previous work (Musallam et al. 2002), this suggests that EGF may transmit its anti-apoptotic effect in part by lowering GSH intracellular stocks. In order to demonstrate this, we had to first explore whether EGF-induced depletion and oxidization of intracellular GSx stocks is specific.

EGF decreases GSx intracellular levels and GSH/GSx ratio in a time- and dose-dependent manner

First, we evaluated the time course of GSx decrease following EGF treatment. As shown in Figure 3A, GSx levels in untreated cultures slightly decreased over time. We had previously observed this process and suggested that it was responsible for the relative resistance of hepatocytes in culture toward apoptosis as compared to in vivo (Musallam et al. 2002). However, when cells were treated with EGF, GSx levels decreased more steeply to reach 13.5 ± 2.1 nmol /10^6 hepatocytes 3-h post-EGF treatment. Thus, after 3h in culture, GSx is 54 % lower is EGF-treated cultures in comparison to untreated ones ($p< 0.01$). In parallel, increasing concentrations of EGF (0.5 – 50 ng/ml) resulted in a dose-dependent

decrease of GSx levels ((EC50 = 3.0 ng/ml; Figure 3C) which suggests that EGF can specifically affect GSH intracellular levels.

Similar to GSx, GSH levels decreased, but more sharply than GSx, in a time- and dose-dependent manner (data not shown). This difference led to lower GSH/GSx ratio. Indeed, Figure 3B showed that GSH/GSx ratio is significantly lower in EGF-treated cultures as compared to untreated ones, at each time point tested ($p<0.05$). GSH/GSx ratio diminished in a dose-dependent manner as well (EC50 = 2.8ng/ml; Figure 3D).

PD168393 inhibits EGF-induced GSH depletion and oxidation

In order to assess the specificity of EGF's effect on GSx and GSH stocks, GSH/GSx ratio and GR activity, we used PD168393, a specific inhibitor of the tyrosine kinase activity of the EGF receptor. We have previously tested the efficacy of this inhibitor and showed that it effectively inhibited the autophosphorylation of the EGF-R as well the downstream phosphorylation cascades (330). As illustrated in Figure 4, the use of this inhibitor abolished the observed depletion of GSx ($p<0.01$; Figure 4A) and GSH ($p<0.01$; Figure 4B) following EGF addition. Furthermore, cell treatment with PD168393 in combination with EGF reversed the decline in GR activity reported in EGF-treated cultures (35.6 ± 0.8 μU/μg protein vs 25.6 ± 0.2 μU/μg protein respectively; $p<0.01$; Figure 4C). This resulted in a return of the GSH/GSx ratio of EGF + PD168393 cultures ($71.0\% \pm 5.5$) to a similar value as that of untreated cultures in the presence ($73.0\% \pm 6.1$) or in the absence ($74.0\% \pm 3.2$) of PD168393.

GSH monoethylester treatment partially reversed EGF-induced decrease of GSx levels and GSH/GSx ratio

Since EGF seems to specifically lower GSH intracellular content and create a weak oxidative stress, the next step was to explore the involvement of this phenomenon in the anti-apoptotic effect of EGF against Fas-induced apoptosis by GSH replenishment. Normally, GSH can not enter into the cell (Anderson and Meister 1989). On the other hand, the ester form of GSH (called GSHmee) can cross the plasma membrane. Intracellular non-specific esterases then liberate GSH into the cytoplasm (Anderson and Meister 1989). Therefore, we used GSHmee to increase intracellular GSH levels. Cell treatment with GSHmee alone increased GSH intracellular content (data not shown). However, when GSHmee was added in the presence of EGF, there was no significant increase in GSH levels as compared to cultures treated with EGF alone (data not shown). This suggested that the effect of EGF is powerful enough to mask the observed increase in GSH in untreated cultures. Hence, we had to find another treatment protocol to counteract the strength of EGF effect. We have previously verified that brief exposure of cells to EGF (1h) followed by its retrieval produced similar EGF-R and MAPK phosphorylations as well as comparable protection against Fas–induced apoptosis as compared to continuous exposure to EGF for the same time period (Musallam et al., unpublished data). Therefore, after 1h-treatment, the EGF-containing medium was replaced with medium alone (EGF-treated control cultures) or one containing GSHmee (2.5 mM; EGF GSHmee-treated cultures) for 3h. These conditions were compared to cultures treated with (GSHmee untreated cultures) or without (untreated control cultures) GSHmee in the absence of EGF.

EGF in control conditions (added for 1h then removed) was able to decrease the levels of GSx and GSH as well as GSH/GSx ratio in a similar manner to conditions where it was present during the whole time of treatment (see Table 1). GSHmee addition slightly increased the levels of GSx in the absence of EGF, albeit not significantly (Table 1). However, the EGF-induced decrease of GSx levels was less prominent in the presence of GSHmee since higher levels of GSx were observed in GSHmee + EGF–treated cultures (18.4 ± 1.7 nmol/10^6 cells) as compared to control EGF–treated cultures (12.6 ± 1.0 nmol/10^6 hepatocytes; $p<0.05$). This signifies that there was a decline of GSx levels by 44 % following EGF treatment in control conditions but this decrease was only by 28% when GSHmee was added to the medium. In parallel, EGF-induced decline in GSH levels was 56% in the absence of GSHmee ($p<0.01$; Table 1) as compared to 37% decline in its presence (NS; Table 1). However, according to our previous work, the most important factor determining hepatocytes sensitivity toward apoptosis is the intracellular ratio of GSH/GSx. Therefore, we determined the effect of GSHmee addition on this parameter. EGF addition induced a significant decrease in GSH/GSx ratio from 78.2 ± 7.8 % in control untreated cultured to 57.3 ± 7.5 % in control EGF-treated cultures ($p<0.01$; Table 1). This represents a 27 % decline. When GSHmee was added, EGF only induced a 10 % decline in this ratio which was not significantly different from baseline (NS; Table 1). This means that GSHmee addition diminished by 63 % the effect of EGF on GSH/GSx ratio.

GSHmee treatment partially inhibited the EGF anti-apoptotic effect

Since GSHmee treatment significantly attenuated EGF-induced decrease of GSx and GSH levels as well as GSH/GSx ratio, we tested the effect of replenishing GSH on the anti-apoptotic effect of EGF. First, untreated cultures displayed negligible levels of apoptotic bodies (<1%; Figure 5A) and caspase-3 activity (0.33 ± 0.08 U; Figure 5B) in both media. The apoptotic response to anti-Fas as well as the protective effect of EGF could be identified in control conditions even though anti-Fas and/or EGF were removed from the medium after 1-h exposure. Indeed, cell stimulation with anti-Fas antibodies induced a significant increase in the rate of apoptotic bodies (11.0 ± 0.4 %; $p<0.001$) and caspase-3 activity (46.0 ± 2.9 U; $p<0.001$). The simultaneous addition of EGF with anti-Fas protected 70 % of mouse hepatocytes against cell death (3.3 ± 0.5 %; $p<0.001$) and attenuated caspase-3 activation by 70 % as well (13.8 ± 2.3 U; $p<0.05$). When GSHmee was added, the level of apoptotic bodies significantly increased to 18.3 ± 1.8 % ($p<0.001$; Figure 5A) accompanied with a similar increase in caspase-3 activity (71.1 ± 10.1 U; $p<0.05$; Figure 5B). However, when anti-Fas+EGF-treated cells were exposed to GSHmee, the protective effect of EGF was significantly impaired. Indeed, the level of caspase-3 activity (47.2 ± 8.5 U) and apoptotic bodies (12.1 ± 1.1 %) were higher in GSHmee Fas+EGF-treated cultures in comparison to control Fas+EGF –treated cultures ($p<0.001$). This signifies that EGF protection was halved in the presence of GSHmee (34 %) as compared to its absence (70 %; $p<0.05$). Of note, EGF-R autophosphorylation (Figure 6A) and procaspase-3 protein expression (Figure 6B) were not significantly affected by GSHmee

addition in the presence or in the absence of EGF. Therefore these results suggest that the decrease in GSH levels is important for the EGF-mediated protection against Fas-induced apoptosis in mouse hepatocytes.

DISCUSSION

GF signalling is mediated through enzymatic receptors that possess intrinsic tyrosine kinase activity. In response to the binding of their ligands, these receptors become oligomerized and then phosphorylated on specific tyrosine residues by their own catalytic activity (Schlessinger 1990; Ballotti et al. 1992; Hurwitz et al. 1991). This activates different intracellular phosphorylation pathways to transmit the biological signal of GF. The classical intracellular cascades activated by GF are phospholipase C (PLCγ), mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase (PI 3-K), which have been implicated in the mediation of several of the biological effects of GF (Force and Bonventre 1998; Margolis and Skolnik 1994).

Recent experimental data demonstrate that GF might use a new and previously unsuspected pathway to mediate their signal: ROS. Several studies have reported that GF generate ROS in a specific and coordinate manner suggesting that ROS might be used as mediators of GF signalling. For example, some GF such as EGF and PDGF, induce the rapid production of superoxide anion ($O_2^{\bullet -}$) and/or hydrogen peroxide (H_2O_2) (Thannickal et al. 2000a). Moreover, oxidants can also stimulate receptor tyrosine kinases even in the absence of their ligands as well as the downstream effectors in signal transduction pathways including Ras, PKC, MAPK and c-Jun N-terminal kinase (JNK) (Kamata and Hirata 1999). In addition, low concentrations of H_2O_2 and $O_2^{\bullet -}$ are known to stimulate growth of several cell types in vitro (Martin and Barrett 2002; Heffetz et al. 1990; Monteiro and Stern 1996; Burdon

1995). Taken together, this suggests that GF can modulate redox components to transmit their signal. Thus, the aim of this study was to determine if modulation of intracellular redox state participated in the anti-apoptotic effect of EGF against Fas-induced apoptosis on mouse hepatocytes.

First, we observed that cell exposure to EGF resulted in a time- and dose-dependent decrease in the levels of GSx and GSH. This occurred concomitantly with an attenuation of the activity of GR leading to lower GSH/GSx ratio in EGF-treated cultures in comparison to untreated control. These effects were abolished by PD168393 strongly suggesting that they are specific to EGF-R activation. The decline in GSH and GSH/GSx ratio may be due to the glutathionation of redox-sensitive proteins. Indeed, these reversible glutathionation events have been detected at the peak of ROS generation in response to GF such as EGF (Barrett et al. 1999). This may protect cellular proteins from irreversible oxidation damage by ROS. On the other hand, the EGF-induced inhibition of GR activity maintains cells in a mild oxidized redox state. Such a state was shown necessary for the full expression of the proliferate action of GF. In fact, inhibition of de novo synthesis of GSH has been shown to stimulate proliferation by GF and, in general, to potentiate the sensitivity of cells to extracellular stimuli acting via MAPK activation (Kamata et al. 1996; Bhunia et al. 1997).

In parallel, redox state has also been implicated in the control of apoptosis. Some studies have reported that Fas-induced apoptosis requires extrusion of GSH (van den Dobbelsteen et al. 1996; Ghibelli et al. 1998). Others have reported that ROS production is necessary for the induction of apoptosis. (Kamata and Hirata 1999; Slater et al. 1995; Lennon et al. 1991)

However, this is still a controversial subject. Indeed, accumulating evidence suggests that, on the contrary, oxidative stress can abrogate apoptosis (Lawson et al. 1999; Haouzi et al. 2001; Musallam et al. 2002; Clement and Stamenkovic 1996). Therefore, it was important to assess the effect of Fas on GSH levels in our cultures. Fas treatment did not significantly modify the levels of GSx and GSH, GR activity or GSH/GSx ratio. This confirms our previous finding (Musallam et al. 2002) as well as that of others (Hentze et al. 1999; Hentze et al. 2000) showing that intact GSH status and high redox state were essential for the transmission of the Fas apoptotic signal.

We next investigated the relevance of EGF-induced oxidative state to the anti-apoptotic effect of EGF against Fas-induced apoptosis. Cell treatment with GSHmee effectively increased GSx and GSH levels by 46% and 59% in cultures co-treated with EGF. This represents a 36% and 34% attenuation of the EGF-induced decrease of GSx and GSH levels. In addition, the effect of EGF on GSH/GSx ratio was 67% less in the presence of GSHmee in comparison to control conditions. This partial normalization of the reduced intracellular redox state in EGF-treated cultures was paralleled by a decrease in the protective effect of EGF against Fas-induced cell death by apoptosis. Indeed, the anti-apoptotic effect of EGF declined by 53% following GSHmee addition. This occurred concomitantly with the loss of 53% of the ability of EGF to inhibit caspase-3 activity without, however, modification of the expression of procaspase-3. Since redox state has been shown to modulate the activity of protein tyrosine kinase cascades, we confirmed that no significant alteration of EGF-R autophosphorylation was observed following GSHmee

treatment in comparison to cultures not treated with it. Therefore, GSHmee addition seems to exert its effect through modulation of GSH intracellular stocks and intracellular redox state and not by interfering with the activation of the EGF-R.

Taken together, these results confirm the negative regulation of apoptosis by oxidative stress. Indeed, the findings of this study are in accordance with our previous work as well as that of others in which depletion of GSH by various treatments resulted in the abrogation of the transmission of the apoptotic signal. Such protection is thought to proceed through redox inhibition of caspases activity (due to their need of a reduced cysteine in their active site). In fact, Hentze et al. have recently shown that GSH depletion resulted in the inhibition of the activity of caspase-8 without alteration in the recruitment of DISC complex to the Fas receptor (Hentze et al. 2002). Similarly, we have shown that increase in GSH intracellular content lead to higher activation of caspase-8 activity and cell death by apoptosis without affecting the expression of procaspase-8, Bid or BCL-x_L proteins (Musallam et al. 2002).

Other studies have reported that GSH depletion and ROS production are necessary for higher sensitivity toward apoptosis. However, careful review of the literature reveals that depletion of GSH or ROS exposure has different effects on apoptosis depending on its intensity, location and timing. For example, a recent study from Fernandez-Checa's group has demonstrated that cell exposure to 3-hydroxy-4-pentenoate, which specifically depletes mitochondrial GSH, sensitizes hepatocytes to TNFα– and Fas-induced apoptosis (Mari et al. 2002). In a similar manner, there is higher sensitivity toward apoptosis in alcoholic liver

disease, ethanol being known to preferentially deplete mitochondrial GSH (Fernandez-Checa et al. 1997). On the other hand, cytoplasmic depletion of GSH results in inhibition of apoptosis as shown by the groups of Wendel (Hentze et al. 1999; Hentze et al. 2000), Jaeschke (Lawson et al. 1999) and Pessayre (Haouzi et al. 2001) as well as our own. Of note, rapid depletion of GSH, should principally deplete the cytoplasmic pool mainly since mitochondria retain GSH far more efficiently than the cytoplasm. Therefore, it appears that if the GSH mitochondrial pool is not affected, GSH depletion inhibits apoptotic signal. Thus such GSH depletion may be used by GF to mediate their anti-apoptotic effect.

Indeed, the data of the present study strongly suggest that EGF specifically induces mild oxidative stress to mediate its biological signal of protection against apoptosis. Thus, anti-oxidants should abrogate this signal. Such findings were reported in isolated digestive gland cells where EGF-induced stimulation of glycolic enzymes was abolished by cell treatment with antioxidant *N*-acetyl-cysteine (NAC)(Canesi et al. 2000). In addition, NAC treatment in PC-12 cells resulted in lower NGF-induced MAPK activation while Buthionine Sulfoximine (a specific GSH synthesis inhibitor) had the opposite effect (Kamata et al. 1996). This suggests the concept that oxidation/reduction of the cysteine residues in the active site of proteins can be used as regulatory mechanism by external stimuli to modulate their activity under pathological as well as physiological conditions. The involvement of ROS production/redox state in a multitude of normal biological process defines a new role for ROS production in normal cell homeostasis as opposed to classical toxic damage to cellular macromolecules. This also adds a new perspective to the use of

anti-oxidants. Indeed, depending on the circumstance they can be beneficial by protecting against high levels of ROS but harmful by inhibiting GF signalling.

Finally, there appear to be multiple check points in the control of the apoptotic machinery by GF. Indeed, their pro-survival signal is divided between the activation of anti-apoptotic processes and the inhibition of pro-apoptotic ones. In the later category, redox modulation of the activity of caspases appears to be another step in the negative regulation of pro-apoptotic mechanisms by GF which acts in conjunction to the phosphorylation of Bad and procaspase-9.

CONCLUSION

In conclusion, our results clearly demonstrate that EGF specifically induces glutathione depletion, which is important for the full anti-apoptotic activity of EGF. This depletion is associated with a lower proteolytic activity of caspase-3. Therefore, our results confirm the importance of stabilization of intracellular GSx levels for the sensitivity of hepatocytes to apoptosis and show that EGF employs redox signalling to protect against Fas-induced.

ACKNOWLEDGEMENT

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Table 1 ***Effect of GSHmee on the intracellular levels of GSx, GSH and GSH/GSx ratio in mouse hepatocytes treated with EGF***

		nmol / x10^6 hepatocytes ± SEM		
		GSx	**GSH**	**GSH/GSx ratio**
Control(s)	**Untreated**	21.5 ± 1.4	17.1 ± 2.4	78.2 ± 7.8
	EGF	12.6 ± 1.0 *	7.3 ± 1.3 **	57.3 ± 7.5 **
GSHmee	**Untreated**	25.6 ± 2.3	18.2 ± 2.3	71.2 ± 5.8
	EGF	18.4 ± 1.7 * †	11.6 ± 0.5 NS †	63.8 ± 4.2 NS

NS: not significant; * : $p<0.05$; **: $p<0.01$ as compared to the respective untreated cultures (ANOVA).

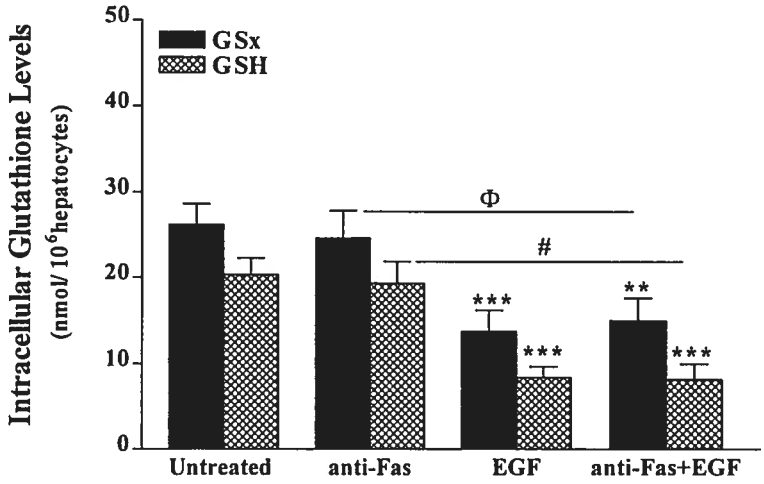
† : $p<0.05$ as compared to control EGF-treated cultures (t-paired test).

FIGURE LEGENDS

Figure 1 ***Effect of anti-Fas and/or EGF on intracellular glutathione levels and GSH/GSx ratio***

After attachment, hepatocytes were incubated with medium alone, anti-Fas [250ng/ml] and/or EGF [50ng/ml] for 3h. Cells were then collected to measure total (GSx) and reduced (GSH) glutathione levels (panel **A**) as well as GSH/GSx ratio (panel B), as described in Materials and Methods. Values are depicted as mean ± SEM from 5 different experiments. * : $p<0.05$; ** : $p<0.01$; *** : $p<0.001$ as compared to untreated culture; # : $p<0.001$; Φ : $p<0.01$ as compared to anti-Fas alone.

A


Intracellular Glutathione Levels
(nmol/ 10^6 hepatocytes)
GSx
GSH
50
40
30
20
10
0
Φ
#
***
***
**
***
Untreated
anti-Fas
EGF
anti-Fas+EGF

B

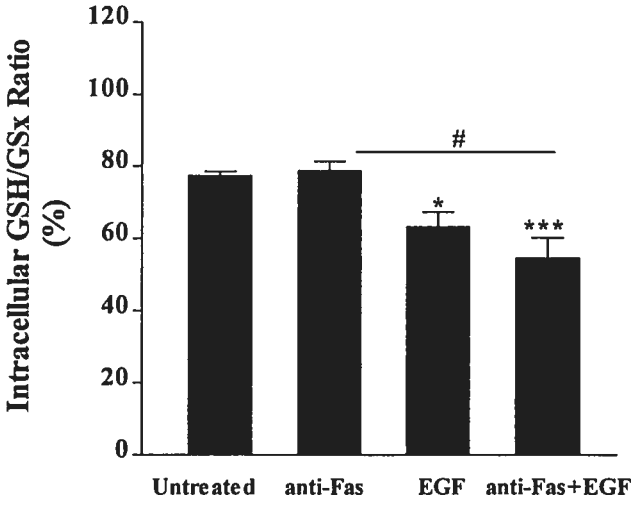

Intracellular GSH/GSx Ratio
(%)
120
100
80
60
40
20
0
#
*
***
Untreated
anti-Fas
EGF
anti-Fas+EGF

Figure 2 ***Effect of anti-Fas and/or EGF on the activity of glutathione reductase (GR)***

After 3h with anti-Fas and/or EGF, cells were collected, lysed and 250 µg proteins were used to monitor the decrease in NADPH absorbance at 340nm (which is indicative of NADPH oxidation and GSSG reduction), the slope being used to calculate the activity of GR. Results (n=3) are presented as mean ± SEM. NS : not significant; * : $p<0.05$; ** : $p<0.01$ as compared to untreated cultures.

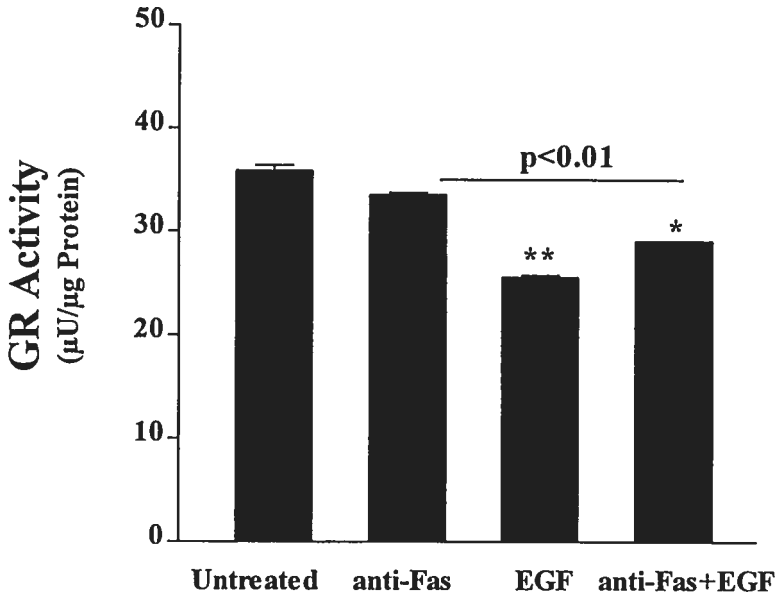

GR Activity
(μU/μg Protein)
50
40
30
20
10
0
p<0.01
**
*
Untreated
anti-Fas
EGF
anti-Fas+EGF

Figure 3 ***Time-course and dose-response curves of intracellular GSx levels and GSH/GSx ratio following exposure to EGF***

Time-course: untreated or EGF-treated cultures were collected after 30 min, 1h 3h and 6h of incubation (n = 5; Panels A and B). Dose-response curves: hepatocytes were treated with increasing concentrations of EGF (0.5-50 ng/ml) then collected after 3h (n=5; Panels C and D). GSx and GSH/GSx ratio were measured as detailed in Materials and Methods. The data are presented as mean ± SEM.*: $p<0.05$; **: $p<0.01$.

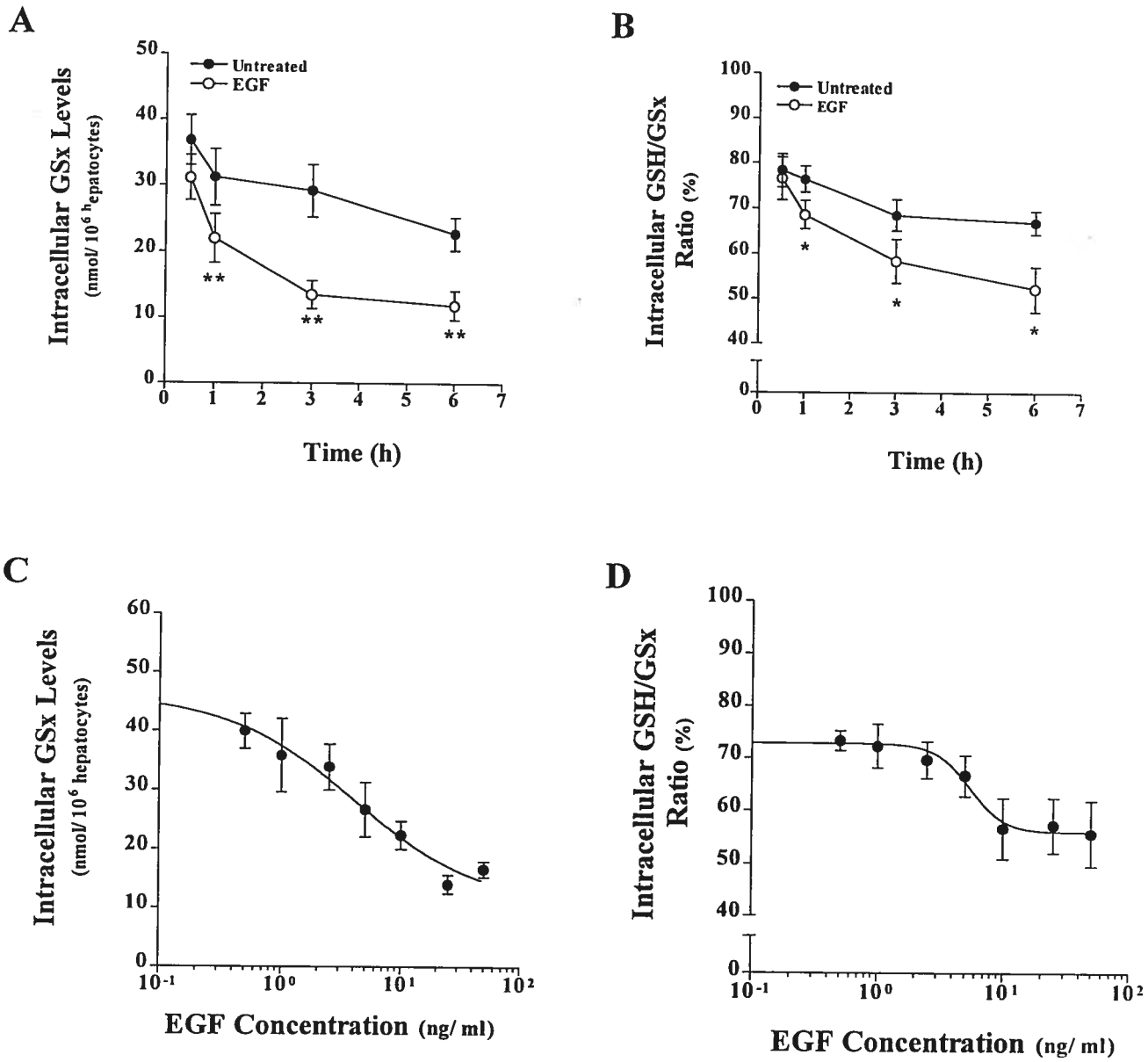

A
Intracellular GSx Levels
(nmol/ 10^6 hepatocytes)
Untreated
EGF
Time (h)
B
Intracellular GSH/GSx Ratio (%)
Untreated
EGF
Time (h)
C
Intracellular GSx Levels
(nmol/ 10^6 hepatocytes)
EGF Concentration (ng/ ml)
D
Intracellular GSH/GSx Ratio (%)
EGF Concentration (ng/ ml)

Figure 4 ***Effect of PD168393 on EGF-induced decrease of GSx, GSH levels as well as GSH/GSx ratio and GR activity***

Cells were treated with medium alone (untreated) or with EGF (50 ng/ml) in the presence of PD168393 [10μM] or in its absence (control conditions). After 3h, cells were collected as described in Materials and Methodsto measure GSx (panel A) and GSH (Panel B) levels as well as GSH/GSx ratio (Panel C) and GR activity (Panel D) using colorimetric assays. Results are presented as mean ± SEM. *: $p< 0.05$; ** : $p<0.01$ for the indicated comparisons.

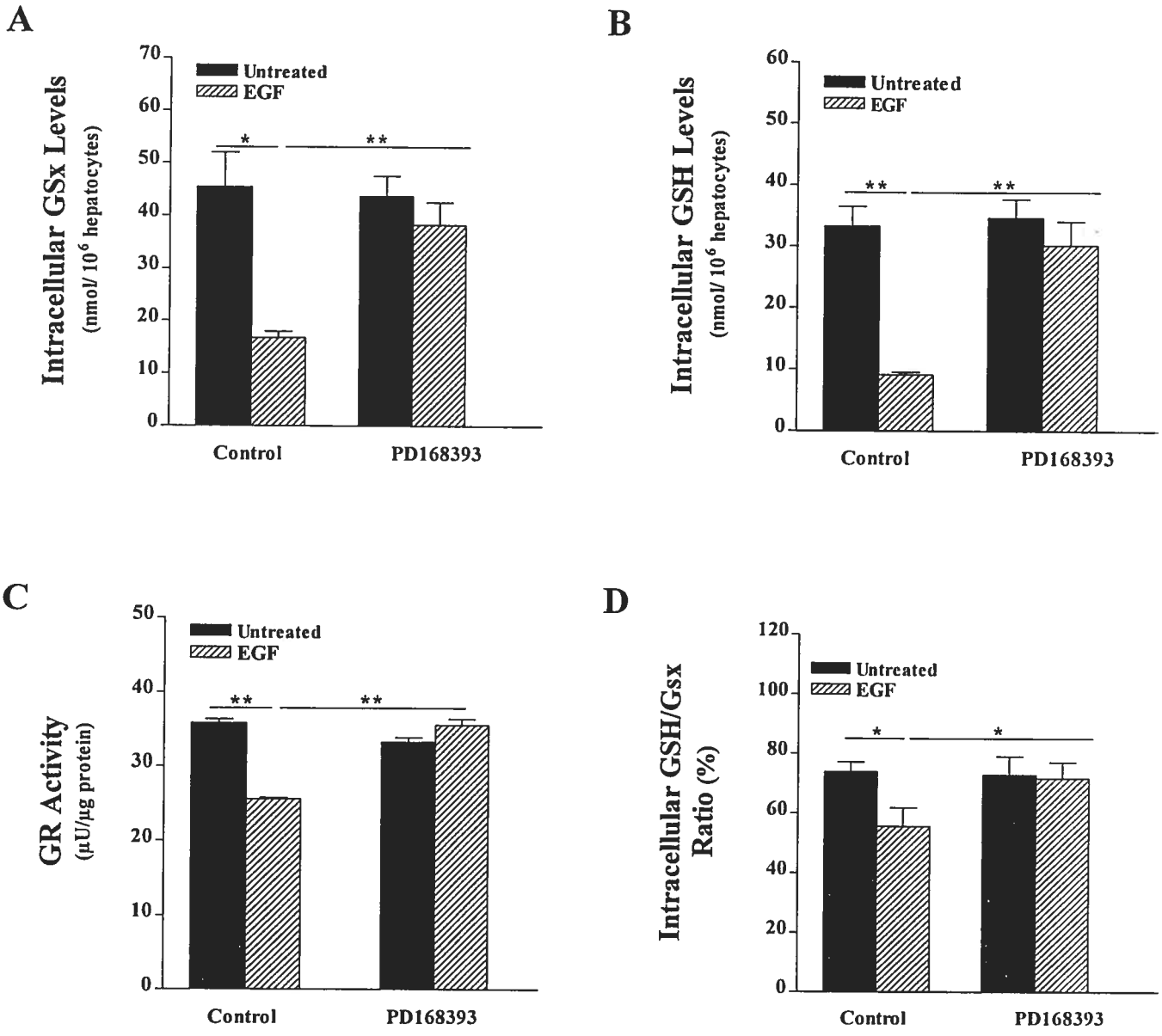

A
Intracellular GSx Levels
(nmol/ 10^6 hepatocytes)
Untreated
EGF
*
**
Control
PD168393
B
Intracellular GSH Levels
(nmol/ 10^6 hepatocytes)
Untreated
EGF
**
**
Control
PD168393
C
GR Activity
(μU/μg protein)
Untreated
EGF
**
**
Control
PD168393
D
Intracellular GSH/Gsx
Ratio (%)
Untreated
EGF
*
*
Control
PD168393

Figure 5 ***Effect of glutathione monotheyl ester (GSHmee) on protective effect of EGF against Fas-induced caspase-3 activation and cell death by apoptosis***

After attachment, hepatocytes were treated with medium alone, anti-Fas [250 ng/ml] and/or EGF [50 ng/ml] for 1h, followed by medium alone or one containing GSHmee [2.5mM]. ***A:*** Cell death by apoptosis was measured after 5h where cells were fixed then stained with Hoechst 33258 as described in Materials and Methods. Data are presented as mean ± SEM. (n = 4). ***B:*** after 3h, samples were collected to measure caspase-3 activity as described in Materials and Methods. Results are presented as mean of activated units of caspase-3 ± SEM. (n = 4). NS : not significant; * : $p<0.05$; ***: $p<0.001$ for the indicated comparisons.

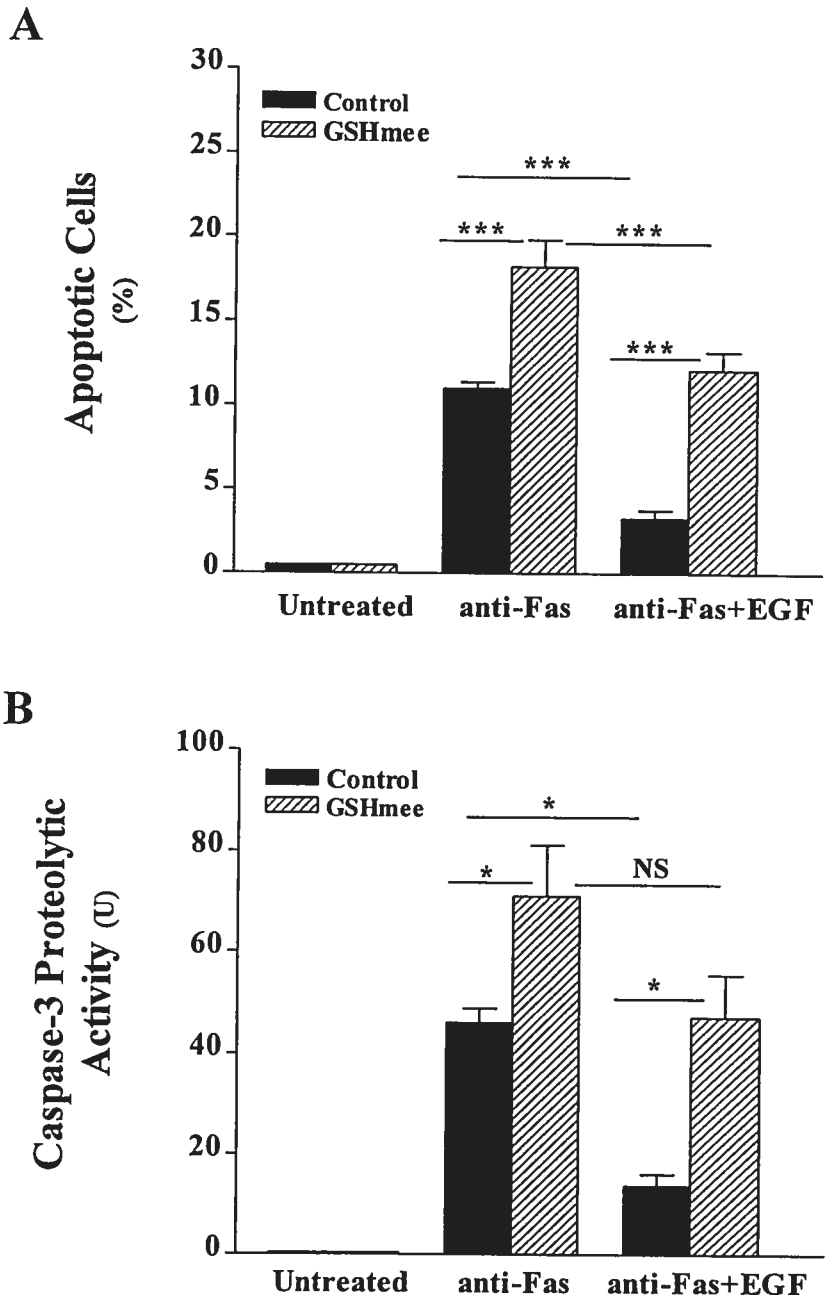
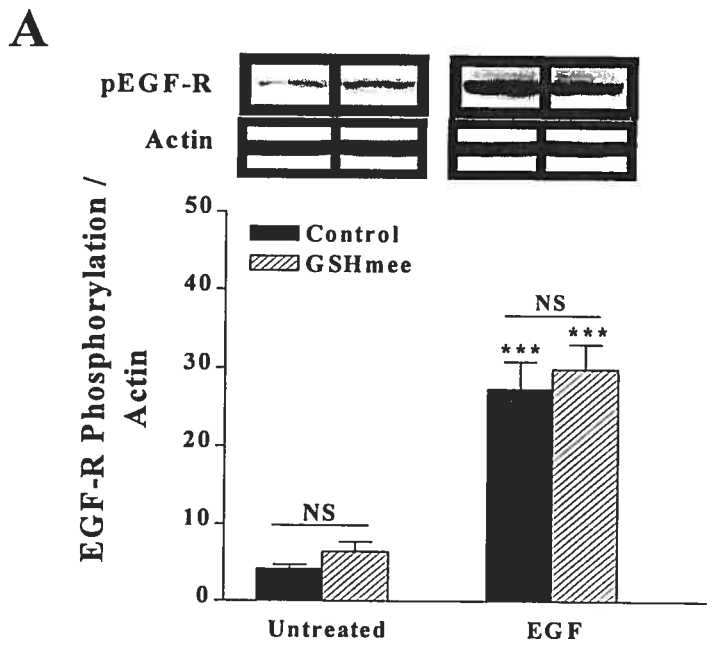
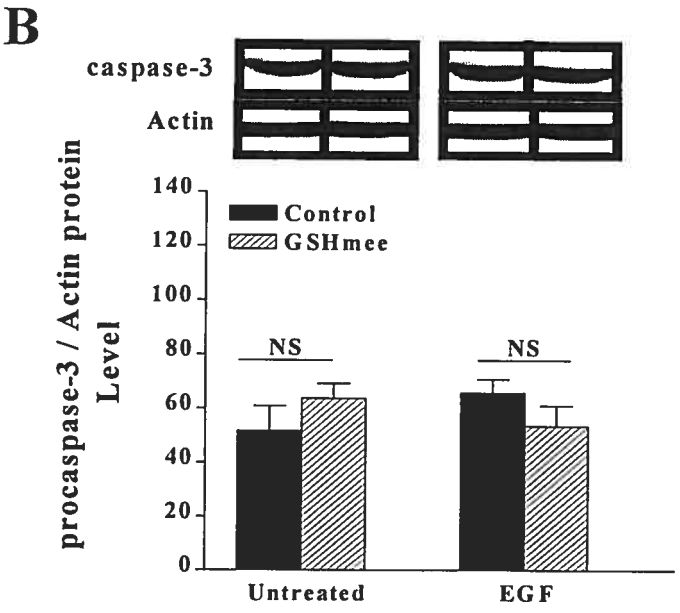

A
Apoptotic Cells (%)
30
25
20
15
10
5
0
Control
GSHmee
***
***
***
***
Untreated
anti-Fas
anti-Fas+EGF
B
Caspase-3 Proteolytic Activity (U)
100
80
60
40
20
0
Control
GSHmee
*
*
NS
*
Untreated
anti-Fas
anti-Fas+EGF

Figure 6 ***Effect of GSHmee on EGF receptor (EGF-R) autophosphorylation and procaspase-3 protein expression in EGF treated cultures***

Cells were cultured in medium (Untreated) or EGF for 1h then with medium alone (Control) or one containing GSHmee [2.5mM] for 1h30 for detection of EGF-R autophosphorylation (Panel A) and after 3h for detection of procaspase-3 protein expression (Panel B). Samples were collected and analyzed by Western blot as detailed in Materials and Methods. Results (mean ± SEM) from 4 experiments are presented. NS: not significant *** : $p<0.001$ as compared to untreated counterparts.


A
pEGF-R
Actin
EGF-R Phosphorylation / Actin
50
40
30
20
10
0
Control
GSHmee
NS
***
***
NS
Untreated
EGF


B
caspase-3
Actin
procaspase-3 / Actin protein Level
140
120
100
80
60
40
20
0
Control
GSHmee
NS
NS
Untreated
EGF

CHAPTER 4

DISCUSSION & CONCLUSION

In the present work, the mechanisms implicated in the regulation of apoptosis and especially those involved in the protection against Fas-induced apoptosis in primary mouse hepatocyte cultures were studied. We focused on the redox control of apoptosis and its relevance in the protection afforded to these cells by epidermal growth factor (EGF) as well as the involvement of the tyrosine kinase (TK) activity of the EGF receptor (EGF-R).

Redox signalling is a new concept which implies control of intracellular signalling pathway by the redox state of the cell. The primary consequence of redox signalling is a change in the oxidative state of cysteine residues localized in the catalytic domain of several key proteins. Some of these proteins are transcription factors, phosphatases, receptors(195;198) and caspases (77;260-263) . Redox signalling has been implicated in several cellular functions including host protection, cell proliferation and gene expression. (198;264)

First, we determined the involvement of the GSH system, the most abundant intracellular non-protein thiol, in the sensitivity of mouse hepatocytes in primary culture toward Fas-induced apoptosis. We found that the isolation procedure and culture resulted in significant loss of intracellular total glutathione (GSx) content. In fact, after only 2h of culture in commercial Williams' E medium (medium N), cells retained only 27% of their original stocks of GSx (total liver). Cell culture in a cysteine- and methionine- enriched medium (medium C+M) maintained GSx levels twice higher as compared to culture in N medium, and this even over an extended culture time. GSH/GSx ratio, which reflects the redox state of the cell, was unaffected by the isolation procedure. On the other hand, this ratio decreased significantly over time in culture in medium C+M as well as medium N,

although it remained significantly higher in the former (74%) as compared to the latter (67%).

Since the liver is very sensitive to apoptosis in vivo (where GSH levels are very high) as compared to in culture (where GSH levels are significantly diminished), this suggested that GSH depletion might play a role in hepatocyte sensitivity toward Fas-induced apoptosis. Indeed, cells cultured in medium C+M displayed higher caspase-8 activation and cell death by apoptosis following Fas-treatment in comparison to medium N cultures cells. There was, however, no effect on the expression of apoptosis-regulating proteins (pro-caspase-8, Bid and BCL-x_L). In addition, *in vitro* evaluation of recombinant active caspase-8 activity revealed that pro-oxidant states (i.e. decrease in GSH/GSx ratio) significantly and gradually attenuated the proteolytic activity of the caspase, finally leading to its complete inactivation when GSH/GSx ratio reached 0%. Our results thus favour the interpretation that redox modulation of the sensitivity of mouse hepatocytes to Fas-induced apoptosis is attributable principally to alteration of the activity of caspases.

Of note, stimulation of the Fas receptor did not significantly alter the redox state of mouse hepatocytes since GSx and GSH levels, GSH/GSx ratio and GSSG reductase activity were not affected by cell treatment with anti-Fas antibodies (see article 3). Moreover, one expects the apoptotic machinery to be functional in a highly reduced state, particularly in the liver where high levels of GSH are present. Taken together, these facts do not support the concept that Fas-induced apoptosis may require GSH depletion or the generation of an intracellular oxidative state to transmit its apoptotic signal in hepatocytes.

In support of this, Henzte et al have reported that *in vivo* GSH depletion using phorone abrogated Fas-induced liver apoptosis (75;76). This depletion was not due to a decrease in caspase-8 recruitment to the DISC complex, but rather by redox modulation of the proteolytic activity of the caspase (261). Biochemical studies have confirmed the redox control of all caspases. They possess a redox-sensitive cysteine in their active site, which must be reduced for the caspase to function (262;263). Indeed, Disulfiram, a potent inhibitor of caspases, acts by oxidizing the cysteine residues in the active site of the enzymes (77). Furthermore, $O_2^{\bullet -}$ was shown to abrogate Fas-induced apoptosis.(259) Similar results were also reported by the Pessayre's group who observed that a rapid depletion of GSH resulted in the protection against Fas-induced apoptosis *in vivo* (265). However, prolonged depletion resulted in the upregulation of compensatory mechanisms (such as increase in Bax expression) to regain sensitivity toward apoptosis. On the other hand, several studies have reported that treatment with oxidants or anti-oxidant depletion can accentuate apoptosis (202;252;253). In fact, it was reported that GSH extrusion was a necessary step for the induction of apoptosis (254;255). These findings seem to contradict our own findings as well as those just mentioned.

The findings of the Passaeyre's group (265) might shed light on the mechanisms responsible for the discordance concerning the involvement of oxidative stress in the control of apoptosis. As mentioned above, there are two important intracellular pools of GSH located in the cytosol and in mitochondria respectively. The latter retain GSH far more efficiently than the former (227). Studies that reported higher sensitivity toward

apoptosis involved either mitochondrial GSH depletion (by ethanol, 3-hydroxy-4-pentenoate (226) or prolonged general GSH depletion (265)) or generation of mitochondrial ROS (e.g. TNF-α-induced apoptosis (266)). It must be noted that the mitochondrial mega-channel is controlled by redox status and GSH levels. Oxidation of the mitochondrial interior space induces the opening of the mega-channel (267). This culminates in the loss of the mitochondrial transmembrane potential, which has been repeatedly shown to cause apoptosis (149;268-270). Hence, in conditions where mitochondrial GSH is depleted, apoptosis may be enhanced.

On the other hand, when the mitochondrial GSH pool is spared (for example when there is rapid and transient GSH depletion), this leads to protection against apoptosis since some of the key cytosolic effectors of the apoptotic machinery (notably caspases) are inhibited by the ensuing oxidative stress. Indeed, careful review of the literature suggests that mild oxidative stress can protect against apoptosis, as long as the mitochondria and the nucleus are spared (151;271-273). In fact BCL-2, a known anti-apoptotic protein present on the mitochondrial and nuclear membranes, has been shown to relocate GSH from the cytosol to the nucleus (151) lending support to this observation. Hence, cytosolic GSH depletion leads rather to a reduction of the sensitivity toward apoptosis.

The recent discovery that growth factors (GF), known anti-apoptotic agents, induce a coordinate generation of ROS that is required for some of their biological effects (200;274;275), led us to hypothesize that GF can negatively control apoptosis by inducing a mild intracellular oxidative stress.

Therefore, we examined the effect of EGF on the GSH system in mouse hepatocyte primary cultures. EGF was able to effectively decrease intracellular GSx and GSH levels in a time- and dose-dependent manner. However, since the decline in GSH levels was steeper than that of GSx levels, this resulted in a decrease of the GSH/GSx ratio, hence a shift toward a more oxidized redox state. Attenuation of GSSG reductase (GR) activity was detected in EGF-treated cultures as compared to untreated ones, which might explain the decline in GSH/GSx ratio. This EGF-induced mildly oxidized redox state was shown by others to be necessary for the full expression of the proliferative action of GF. In fact, inhibition of *de novo* synthesis of GSH has been shown to potentiate the sensitivity of cells to extracellular stimuli acting via MAPK activation (275;276).

In order to verify the specificity of the effect of EGF on GSH stocks, we used an inhibitor of the tyrosine kinase activity of the EGF-R called PD163893. We have shown that this compound is capable of completely abolishing the autophosphorylation of the EGF-R, the phosphorylation and activation of the MAPK and Akt pathways, the incorporation of $H_3{}^{32}PO_4$ into cells as well as the increase in BCL-x_L mRNA and protein expression observed following EGF treatment. Therefore, these findings showed that PD168393 is a specific and potent inhibitor of the TK activity of EGF-R.

Cell treatment with PD168393 completely abolished the EGF-induced decrease in GSx and GSH levels, GSH/GSx ratio as well as the attenuation of GR enzymatic activity. This suggests that EGF-induced GSH depletion and oxidation was mediated by the tyrosine kinase (TK) activity of the EGF receptor. Therefore, these results strongly indicate that

EGF specifically induces an oxidative state in primary hepatocyte cultures due to depletion of GSH stocks and by favouring GSH oxidation.

EGF has been shown by many investigators, including our group, to be able to protect against apoptosis induced by various stimuli (136;277-279). From our results, this protection against apoptosis occurs in conjunction with the establishment of a mild oxidative stress. Thus, it was necessary to investigate the relevance of the observed GSH depletion and oxidation to the anti-apoptotic effect of EGF. Cell GSH replenishment with glutathione monoethylester (GSHmee) resulted in a partial normalization of intracellular redox state in EGF-treated cultures by diminishing EGF-induced decrease in GSx and GSH levels as well as GSH/GSx ratio. This partial normalization also partly attenuated (53% decline) the protective effect of EGF against Fas-induced caspase-3 activation and cell death by apoptosis. These findings are in accordance with the concept that depletion of GSH by various treatments (e.g. cell isolation, acetaminophen, phorone) results in the abrogation of the transmission of the apoptotic signal. Such a protection is thought to proceed through redox inhibition of caspases activity (due to their need of a reduced cysteine in their active site) as reported by the Wendel group (261) and our own (280). Therefore, this suggests that the anti-apoptotic effect of EGF is mediated, at least in part, through redox modulation of key components of the apoptotic machinery.

GF have been shown to use mild oxidative stress to mediate some of their biological signals. Indeed, PDGF induces H_2O_2 generation that has been shown to be required for PDGF-induced tyrosine phosphorylation, DNA synthesis and MAPK activation (201).

Furthermore, the anti-oxidant N-acetyl-cysteine (NAC) has been reported to inhibit EGF-induced stimulation of glycolic enzymes in isolated digestive gland cells (274). Hence, the involvement of ROS production/altered redox state in a multitude of normal biological processes defines a new role for ROS production in normal cell homeostasis in addition to their classical toxic damage to cellular macromolecules. This also adds a new perspective to the use of anti-oxidants. Indeed, depending on the circumstance, they can be beneficial by protecting against high levels of ROS but harmful by inhibiting GF signalling (264).

Summarizing the mechanisms used by GF to protect against apoptosis, their pro-survival signal is divided between the activation of anti-apoptotic processes (such as the anti-apoptotic proteins of the BCL-2 family) and the inhibition of pro-apoptotic ones (e.g. Bad. caspase-9). In the latter category, redox modulation of the activity of caspases appears to be another step in the negative regulation of pro-apoptotic mechanisms by GF, which acts in conjunction with the phosphorylation of Bad and procaspase-9 by Akt. Of note, these mechanisms have all been shown to depend on the TK activity of GF receptors. Indeed, the increase in BCL-x_L levels, the activation of Akt kinase and the depletion and oxidation of GSH were all blocked by EGF-R TK inhibitors (PD168393 and Tyrphostin AG 1478). However, some data show that not all of the biological effects of GF can be mediated through TK-dependent mechanisms.

As mentioned earlier, when EGF binds to its receptor, it induces the oligomerization of the latter. The subsequent conformational change activates the TK activity of the EGF-R. This model of activation is similar to most GF that bind TK receptors. A number of studies have

demonstrated that both the oligomerization (281-284) and the autophosphorylation (285-287) of these receptors are necessary for the transmission of the signal of GF. On the other hand, evidence has accumulated to show that some GF effects may occur independently of the TK activity of their receptors. For instance, Schreiber et al. have reported that cross-linking of cell-bound monoclonal EGF-R antibodies resulted in the clustering of the receptor and the stimulation of DNA synthesis without activation of the TK domain of this receptor (288). Therefore, we investigated the involvement of the TK activity of the EGF-R in the anti-apoptotic effect of EGF. We used two inhibitors of TK activity: PD168393 and Tyrphostin AG1478. Their efficacy was confirmed by measuring EGF-R autophosphorylation, MAPK and PI 3-K activation, ^{32}P incorporation into cells, as well as of BCL-x_L mRNA and protein expression.

As mentioned above, EGF co-stimulation with anti-Fas decreased the levels of apoptotic cells as compared to cultures treated with anti-Fas only. Cell treatment with TK inhibitors resulted in the significant increase of the level of apoptotic cells in anti-Fas+EGF–treated cultures as compared to their control counterparts. However, we were surprised to find that this level (anti-Fas+EGF+TK inhibitors) never reached that observed in Fas-treated cultures (anti-Fas+TK inhibitors). This suggested that some of the EGF protective signal was still transmitted into the cell in the presence of these inhibitors. Therefore, our results strongly suggest that part of the EGF-induced protection is not related to the TK activity of EGF-R. Similar findings were reported by Dews et al. who have demonstrated that the

mutation of IGF-1 receptor in its TK domain resulted in a reduction of the anti-apoptotic action of IGF-1, without completely abolishing it (289).

To date, these TK-independent pathways have not been identified. However, in an attempt to understand these processes, it is important to remember that following GF binding to their receptors, another important initial event occurs; namely, receptor oligomerization. This phenomenon of receptor association and subsequent conformational change may favour interactions with certain intracellular proteins. For instance, oligomerization may create a link between the receptor and certain effectors of the anti-apoptotic machinery through protein-protein interactions. BAG-1, an anti-apoptotic protein of the BCL-2 family, has been shown to associate with the cytoplasmic domain of GF-R through its C-terminus (138) independently of GF-R phosphorylation state. In addition, BAG-1 has the ability to bind to heat-shock proteins and other chaperones molecules, which play an important role in protein stability. Hence, with such functional properties, BAG-1 (and other BAG-1-like proteins) may link GF-R with intracellular anti-apoptotic effectors without the need for GF-R autophosphorylation.

CONCLUSION

In conclusion, the work presented here shed some light on the involvement of the intracellular redox state in the control of apoptosis, which is still subject for much debate. Indeed, the results of these studies demonstrate that oxidative stress protects against Fas-induced apoptosis and that this may represent a novel mechanism used by growth factors to protect hepatocytes in primary cultures. These findings are in accordance with an emerging concept, which postulates that not all oxidative stress is harmful to cells. In fact, several recent lines of evidence indicate that mild oxidative stress is important for the mitogenic effect of growth factors and host defence.

However, the relevance of these findings to *in vivo* models is worth further investigating, especially since thiol depletion is a known pathogenic factor in several diseases. Indeed, the effect of growth factors on *in vivo* GSH stocks is interesting to study especially in models of acetaminophen hepatotoxicity where important question need to be answered: Does growth factors potentiate this kind of pathologies? Or again, the over all cellular context of GSH depletion plays a role in defining the end result? In addition, what is the potential effect of oxidative stress on the immune response during hepatic, or other organ, virus infection or cancer defence since the Fas system is particularly important in these immune responses?

Therefore, much work remains to be done in the quest for the understanding of the modulation of apoptosis for therapeutic purposes.

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