CHARACTERIZATION OF INTRACELLULAR SIGNALING CASCADES IN 4-HYDROXYNONENAL-INDUCED APOPTOSIS: MAP KINASES AND BCL-2 PROTEIN FAMILY

by
OZGUR KUTUK

Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Sabancı University
February 2006

CHARACTERIZATION OF INTRACELLULAR SIGNALING CASCADES IN 4-HYDROXYNONENAL-INDUCED APOPTOSIS: MAP KINASES AND BCL-2 PROTEIN FAMILY

APPROVED BY:

Prof. Dr. Hüveyda Başağa ..... (Dissertation Supervisor)

Prof. Dr. Beyazıt Çırakoğlu .....

Assoc. Prof. Dr. Batu Erman .....

Asst. Prof. Dr. Alpay Taralp .....

Asst. Prof. Dr. Hikmet Budak .....

DATE OF APPROVAL:…….

ABSTRACT

In this thesis we have studied the signaling pathways involved in HNE-induced apoptotis and the effect of resveratrol in signal transduction mechanisms leading to apoptosis in 3T3 fibroblasts when exposed to 4-hydroxynonenal (HNE).

The results demonstrate the ability of HNE to induce apoptosis and ROS formation in a dose-dependent manner. In order to get insight into the mechanisms of apoptotic response by HNE, we followed MAP kinase and caspase activation pathways; HNE induced early activation of JNK and p38 proteins but downregulated the basal activity of ERK 1/2. We were also able to demonstrate HNE-induced release of cytochrome *c* from mitochondria, caspase-9 and caspase-3 activation. Resveratrol effectively prevented HNE-induced JNK and caspase activation hence apoptosis, as well as the formation of ROS. Activation of AP-1 along with increased c-Jun and phospho-c-Jun levels could be inhibited by pretreatment of cells with resveratrol. Additionally, overexpression of dominant negative c-Jun and JNK1 in 3T3 fibroblasts prevented HNE-induced apoptosis, which indicates a role for JNK-c-Jun/AP-1 pathway. Moreover, HNE induced decreased Bcl-2 and increased Bax, Bak and Bim protein levels, which could be prevented by resveratrol.

In light of the JNK-dependent induction of c-Jun/AP-1 activation and the protective role of resveratrol, these data indicate a critical potential role for JNK in the cellular response against toxic products of lipid peroxidation. Resveratrol also prevents modulation of Bcl-2 proteins and formation of ROS by HNE. In this respect, resveratrol acting through MAP kinase and Bcl-2 protein pathways in addition to act as antioxidant-quenching reactive oxygen intermediates is a potential small molecule against apoptosis-related human pathologies.

ÖZET

Bu tezde 3T3 fibroblast hücrelerinde 4-hidroksinonenal (HNE) tarafından indüklenen apoptotik sinyal ileti yolaklarının ve resveratrol'ün bu yolakların üzerine etkisinin ortaya konulması için yapılan çalışmalar sunulmuştur.

Elde edilen sonuçlar HNE'nin doza bağımlı olarak apoptosise ve oksidatif stres oluşumuna yol açtığını göstermiştir. HNE tarafından indüklenen apoptosis meknizmaları derinlemesine incelenmiş, HNE'nin JNK ve p38 proteinlerinin aktivasyonuna ve ERK 1/2 proteininin inhibisyonuna yol açtığı ortaya konulmuştur. HNE'nin aynı zamanda mitokondriden sitokrom *c* salınımına, kazpaz-9 ve kazpaz-3 aktivasyonunu sağladığı gözlenmiştir. Resveratrol'ün HNE tarafından indüklenen JNK ve kazpaz aktivasyonunu ve oksidatif stres oluşumunu engellediği ortaya konulmuştur. AP-1 aktivasyonu, c-Jun ve fosforile-c-Jun proteinlerinin HNE ile indüklenen artışı da resveratrol tarafından engellenmiştir. Dominant negatif c-Jun ve JNK1 overekspresyonları da HNE tarafından indüklenen apoptosisi önlemiştir, böylece JNK-c-Jun/AP-1 yolağının bu süreçteki rolü ortaya konulmuştur. Ek olarak HNE Bcl-2 proteininde azalmaya ve Bax, Bak Bim proteinlerinde artmaya yol açmıştır, bu etkiler de resveratrol tarafından önlenmiştir.

JNK'a bağımlı c-Jun/AP-1 aktivasyonu ve resveratrol'ün koruyucu rolü ışığında elde edilen sonuçlar JNK'ın lipid peroksidasyon toksik ürünlerine hücresel cevapta kritik bir rol üstlendiğine işaret etmektedir. Resveratrol antioksidan özelliklerine ilaveten MAP kinazlar ve Bcl-2 proteinleri üzerinden gösterdiği etkilerle apoptosise bağımlı olarak gelişen hastalıklar için önemli bir potansiyel küçük moleküldür.

To my family,

past, present and future . . .

Shoot for the moon. Even if you miss, you will land among the stars.

Les Brown

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Prof. Dr. Hüveyda Başağa for her all encouragement, enthusiasism and for many insightful conversations during the development of the ideas in this thesis. Thank you for your skillful guidance through this challenging and arduous scientific journey from the beginning. Thank you for your enthusiasm in every single experiment, for patience with my interminable and unperfect manuscripts. For always being there for me and having time to discuss conclusions, develop new hypotheses and encourage me after unsuccessful experiments.

I am very thankful to faculty members at Biological Sciences and Bioengineering Program; Prof. Drs. Zehra Sayers, İsmail Çakmak, Selim Çetiner, Assoc. Prof. Dr. Batu Erman, Assist. Prof. Drs. Uğur Sezerman and Hikmet Budak for their inspiration and efforts during my education in Sabanci University.

I am also grateful to Assist. Prof. Dr. Alpay Taralp for reshaping my way of thinking and teaching me the power of creative thinking and taste of protein chemistry.

Thank you my friends for sharing hard times and fun times, crying and laughing, hope and desperation; Kıvanç Bilecen, Mert Şahin, Özgür Gül, Burcu Kaplan, Süphan Bakkal, Ümit Öztürk, Melis Tiryakioğlu, Pelin Akan, Mutlu Doğruel and Filiz Dede.

I am very thankful to my former and current labmates for their collaboration and support throughout my research; Mazhar Adlı, Ece Gamsız, Serkan Göktuna, Mehmet Alper Arslan, Ayşegül Verim, Elif Damla Büyüktuncer and Dilek Telci…and to all other colleagues in the lab.

I would like to thank Prof. Dr. Jonathan Ham, University College London, UK and Prof. Dr. Roger Davis, UMass Medical School, USA for providing plasmids, Prof. Dr. Giuseppe Poli, Turin University, Italy for discussions and support on the HNE project, Prof. Dr. Stanley Korsmeyer, Harvard University, USA for his fundamental papers on the mechanisms of apoptosis, Prof. Dr. Uğur Yavuzer for her motivation and encouragement in the beginning of my career, Assoc. Prof. Dr. Maria C. Shoshan for

her patience and guidance, Prof. Dr. Nefise Barlas Ulusoy for her vast scientific capacity inspiring me the taste of hardworking.

My family, M. Emin, Fatma, Özlem, thanks for your gratuitous love, for always believing in me and supporting my decisions; Kemal, Şükran, Mahmut, Meryem and other members of my family, for all your love and care; Turgut and Ömür, for inspiring me, that the limit is only sky, just if you are tough enough.

I would like to express special thanks to my comrades during sleepless nights; coffee and cigarettes.

Finally, thank you my beloved Nurgül, for love, support and never-ending patience…and beyond all, for making me happy.

Ozgur Kutuk

February 2006, Istanbul

TABLE OF CONTENTS

ABBREVIATIONS

AIF: Apoptosis inducing factor

AO: Acridine orange

AP-1: Activator protein-1

Apaf-1: Apoptosis protease activating factor-1

ASK: Apoptosis stimulating kinase

BH: Bcl-2 homology

CoxIV: Cyclooxygenase IV

C-terminus: Carboxyl terminus

CED: Cell death defective

CRE: cAMP response element

DCHF-DA: Dichlorodihydrofluorescein diacetate

DD: Death domain

DISC: Death inducing signaling complex

DIABLO: Direct inhibitor of apoptosis binding protein with low pI

DMSO: Dimethylsulfoxide

DR: Death receptor

EGFR: Endothelial growth factor receptor

Epo: Epoetin

ER: Endoplasmic reticulum

ERK: extracellular signal-regulated kinase

ET-1: Endothelin-1

FADD: Fas-associated death domain

FBS: Foetal bovine serum

GCC: Glutathione cysteine ligase

GSH: Glutathione

GST: Glutathione S-transferase

HNE: 4-Hydroxynonenal

HO: Hoechst dye

HPNE: Hydroperoxynonenal

9-(S)-HPODE: Hydroperoxyoctadecadienoic acid

IKK: inhibitor kappa kinase

IL-2: Interleukin-2

JNK: c-Jun N-terminal kinase

LDL: Low density lipoprotein

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinase

MCP-1: Monocyte chemoattractant protein-1

MEF: mouse embryonic fibroblast

MW: Molecular weight

NF-κB: Nuclear factor-kappa B

NGF: Nerve growth factor

NMR: Nuclear magnetic resonance

N-terminus: Amino terminus

Ox-LDL: oxidized low density lipoprotein

PARP: Poly-(ADP-ribose) polymerase

PDGFR: Platelet-derived growth factor receptor

PI3K: Phosphatidylinositol-3 kinase

PI: Propidium iodide

PKC: Protein kinase C

PPAR: Peroxisome proliferator-activated receptor

RIP: Receptor interacting protein

ROS: Reactive oxygen species

RTK: Receptor tyrosine kinase

SMAC: Second mitochondria-derived activator of caspases

STAT: signal transducer and activator of transcription

TCF: Ternary complex factor

TNF: Tumor necrosis factor

TRADD: TNF-R-associated death domain

TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand

TRE: TPA response element

UV: Ultraviolet

VLDL: Very low density lipoprotein

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

1 INTRODUCTION

The presence of all human societies is determined by birth, death, and disease. Throughout the history of world, illness has often been attributed to the will of the gods and faith until the rise of positivist scientific medicine along with other revolutions in 18th century. The first written materials on medicine were discovered in an ancient Egypt papyrus, dated around 1536 BC (Figure 1.1) [230]. Furthermore, the ancient Greek physician and philosopher Hippocrates developed the first methodical approach towards examination, diagnosis, treatment and prognosis in 400 BC. The Endeavour of medicine through out centuries kept going despite the ethical, religious and economical hindrance. Ignaz Philipp Semmelweis, a Hungarian-Austrian physician, managed to reduce the death rate of women related to childbirth by simply introducing "wash your hands before attending to women in childbirth" in 1847, an initial theory of antisepsis [231]. Joseph Lister, Robert Koch and Louis Pasteur, developed novel therapeutic and preventive approaches against infectious diseases, further developed this theory [232]. The new concept of "cause-disease" relationship continued to be constructed and the progress in surgery and pharmacological sciences in the 20th century had a great impact on general public health, which revolutionized the practice of medicine. Today the new era of genomics and proteomics facilitate the progress of medicine and transformation of clinical practice along with molecular and genetic characterization of disease pathogenesis. The impact of increasing resources devoted to basic research enable rapid translation of molecular findings to clinical field: bench to bedside.

Oxidative modification of biolipids has prominent consequences in the pathogenesis of human pathologies. In addition to massive *in vivo* oxidative modification and deposition of lipids, lipid peroxidation end-products may act as secondary messengers in cellular signaling pathways [1]. Regulation of cellular function takes place through a network of intracellular and extracellular signaling cascades and

the physiological or pathological outcome is defined by the duration, amplitude, intensity and nature of these signaling pathways. Oxidized low-density lipoproteins (Ox-LDLs) and other lipid peroxidation end products interfere with various signaling pathways including G-protein coupled receptors, protein kinase C and D, NF-κB and MAP kinases [1-3]. Aldehydes have been defined as one of the most significant among the various end products of the oxidative breakdown of biomembrane polyunsaturated fatty acids [4]. 4-hydroxynonenal (HNE), one of the major aldehydic products of the peroxidation of membrane w-6 polyunsaturated fatty acids, has been demonstrated to be present in the pathogenesis of many human diseases such as atherosclerosis, cancer, neurodegenerative disorders and diabetes [5-7]. Nevertheless, the molecular targets and the mechanisms of their activation by oxidized lipids, specifically by HNE, remain largely unknown. Moreover, considering the involvement of apoptosis (programmed cell death) as a common molecular process in all these diseases, it would be convenient to explore the mechanisms of 4-HNE-induced apoptosis, if any.

Figure 1.1 Ancient Egyptian medicine. Homer in the Odyssey remarked,
"*In Egypt, the men are more skilled in Medicine than any of human kind*" [230].

Apoptosis is an essential and evolutionary conserved process for normal embryogenesis, organ development, tissue homeostasis and elimination of deleterious cells from multicellular organisms (Figure 1.2). Any deregulation or aberrant activation of apoptosis can be involved in the pathogenesis of human diseases such as atherosclerosis, chronic heart failure, cancer, diabetes and neurodegenerative disorders [8,9]. Thus, the cellular and genetic integrity of a cell under stress or oncogenic stimuli should be strictly controlled by cellular signaling pathways to maintain its functionality and viability. Gene expression, post-translational protein modifications and protein-protein interaction modules mainly mediate the regulation of apoptosis at cellular level. Protein phosphorylation is a main protein modification and is executed by phosphotransferase enzymes designated as kinases. Mitogen-activated protein kinases (MAP kinases) are a group of protein serine/threonine kinases that are differentially activated in response to a variety of pro- or anti-apoptotic stimuli [10]. In combination with several other signaling pathways, they can differentially alter phosphorylation status of the transcription factors, suxh as c-Jun/AP-1 [10]. Three major types of MAP kinase cascades have been reported in mammalian cells that respond synergistically to different upstream signals: c-Jun N-terminal kinase/stress activated protein kinase (JNK), extracellular signal-regulated kinases 1/2 (ERK 1/2) and p38 [10]. It is important to recognize that these kinases are essential for many physiological functions and spatiotemporal dysregulation of these kinases has been proposed to be involved in common human pathologies. Thereby, regulation of kinase function with small molecule approach (either *de novo* synthesized or natural products) has been implicated as a promising approach for rational disease treatment.

Apoptosis is executed by caspases, which are cysteine-directed aspartate proteases, cleaving their substrates on the carboxyl side of an aspartate residue. Intrinsic apoptosis signaling pathways mainly intersect at mitochondria and following mitochondrial loss of integrity, cytochome *c* is released into the cytosol. This translocation of cytochrome *c* has been followed by formation of an apoptosome complex and activation of caspase-9, which is an essential component of apoptotic machinery [11]. Recent research advances in apoptosis research provided us a new protein family member: Bcl-2 proteins [12]. The Bcl-2 proteins consist of a protein family related through their conservation of helical sequences known as Bcl-2 homology (BH) domains. These proteins are known to modulate mitochondrial function

and regulate the release of activating factors of apoptosis, such as cytochrome c, from mitochondria and each Bcl-2 family member contain at least one of four BH-domains, BH1-BH4 [12]. Modulation of Bcl-2 proteins either at gene expression level or at post-translational level determines the pro- or anti-apoptotic response. Bcl-2 proteins, together with upstream kinases and their targets, are key checkpoints and determinants for the cellular response to stress.

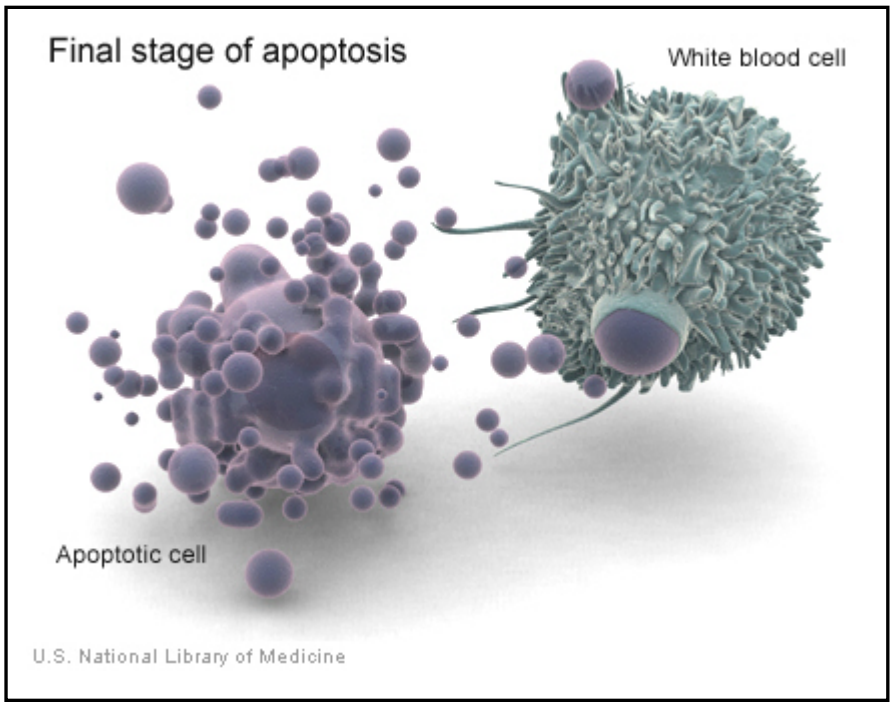


Figure 1.2 Apoptosis: an ancient Greek word used to describe the "falling off" of petals from flowers or leaves from trees (from the courtesy of U.S. National Library of Medicine, http://ghr.nlm.nih.gov, 2006)

In this study our aim was to characterize the pro-apoptotic signaling pathways involved in HNE-induced apoptosis in 3T3 fibroblasts and studying the potential protective role of resveratrol, which is a phytoestrogen widely distributed in nature and highly found in grape skin and seed, mulberries and peanuts. For this purpose, we characterized the dose and time kinetics of HNE-induced apoptosis and investigated the involvement of mitochondrial apoptosis pathway, including cytochrome *c* release in this process. Since the protective effect of resveratrol on HNE-induced apoptosis has not been studied before, we have also studied the effect of resveratrol on HNE-induced apoptosis and oxidative stress. Furthermore, HNE-induced apoptosis signaling pathways, involving MAP kinases, c-Jun/AP-1 transcription factors, caspases and Bcl-2

protein members were explored and the modulation of these pathways by resveratrol was investigated. This is the first study, which describes a complete picture of signaling pathways involved in HNE-induced apoptosis and molecular targets of resveratrol against cellular stress.

A chapter for the background follows this introductory section, which involves current perspectives of this research. This background chapter is followed by Chapter 3, which explains materials and methods utilized in this study in a detailed fashion. The results are presented in Chapter 4 along with the figures explaining experimental findings. The results are discussed in Chapter 5 in the light of current literature. The final Chapter 6 involves a brief synopsis and conclusions of this study along with future perspectives.

CHAPTER 2

2 BACKGROUND

2.1 Apoptosis

2.1.1 Historical Perspective

Apoptosis is of Greek origin, having the meaning "falling off or dropping off", in analogy to leaves falling off trees or petals. Already since the mid-nineteenth century, many observations have indicated that cell death plays a considerable role during physiological processes of multicellular organisms, particularly during embryogenesis and metamorphosis [13,14]. The first notion of programmed cell death in physiological conditions was first identified in the neuronal system of developing toad embryos [15]. The term programmed cell death was introduced in 1964, proposing that cell death during development is not of accidental process but follows a sequence of controlled steps leading to locally and temporally defined self-destruction [16]**.** Eventually, Kerr *et al.* introduced the term apoptosis to describe the morphological processes leading to controlled cellular self-destruction [17]. Considering the last thirty years of apoptosis research, the key steps for making the decision of a cell to either live or to die and main components of mammalian apoptotic machinery have been identified. These essential findings of basic science would certainly allow the development of novel therapeutic strategies and tools for treatment of major human diseases with an etiology of apoptosis dysfunction.

2.1.2 Apoptosis Pathways

As described above, apoptosis is an essential and evolutionary conserved process for normal embryogenesis, organ development, tissue homeostasis and elimination of deleterious cells from multicellular organisms. Any deregulation or aberrant activation of apoptosis can be involved in the pathogenesis of human diseases such as atherosclerosis, chronic heart failure, cancer, diabetes and neurodegenerative disorders [8,9]. Thus, the cellular and genetic integrity of a cell under stress or oncogenic stimuli have to be strictly controlled to maintain its functionality and viability. The cells are absolute targets for various extrinsic and intrinsic stimuli and they receive and process signals not only from the plasma membrane but also from different compartments within cytoplasm. This dynamic characteristic of cells enables them to sense signals and response quickly, a fundamental principal of cellular survival. Multiple death and survival signals are integrated to molecular apoptotic machinery via protein signaling networks, which are predominantly regulated by protein-protein interactions, subcellular localization and major protein modifications such as phosphorylation and cleavage. Mostly an array of protein kinase-mediated pathways targets "the players" of apoptotic machinery at transcriptional and post-translational level and regulates their level and/or function. The balance between pro- and anti-apoptotic signaling pathways determines the fate of a cell in response to an external or internal stimulus.

Apoptosis is a multi-component programmed cell death process, which is characterized by specific cellular morphological patterns such as chromatin condensation, nuclear fragmentation, cytoplasmic shrinkage, membrane blebbing, formation of apoptotic vesicles and consequent phagocytosis by immune cells [18]. The molecular changes that occur during apoptosis are redistribution of phosphotidylserine at outer and inner leaflets of plasma membrane and internucleosomal DNA cleavage. Apoptosis is usually induced by an initiation phase, which depends tightly on the cell type and the characteristic of the stimuli (origin, duration, amplitude and presence of co-stimuli). In a cell under an apoptotic insult either within or outside of the cell, multiple cellular signalling modules are activated synchronously. During this deterministic phase, molecular signaling modules serve as parts of central apoptotic machinery, which should be tightly controlled and finely tuned to maintain appropriate biochemical functioning of the cell.

Caspases are the main executioners of programmed cell death process and their mechanism of activation is first characterized in *Caenorhabditis elegans* [19]. In this model four gene products (CED-3, CED-4, CED-9 and EGL-1) are playing fundamental roles in the apoptosis process. The interaction between caspase protein, CED-3 and an adaptor-protein, CED-4 leads to formation of a complex defined as an "apoptosome", in which CED-3 zymogen is activated through close proximity and self-processing. CED-4 is sequestered and kept away from CED-3 via its interaction with a mitochondria-associated protein, CED-9 in unstimulated cells. In response to a death stimulus, a Bcl-2 homolog protein with a BH3 domain, Egl-1 is induced and interacts with CED-9, which frees CED-4 and formation of apoptosome as well as activation of CED-3 [19]. This simple and clear caspase activation model is evolutionary conserved. Caspases are also normally inactive or minimally active in unstimulated healthy mammalian cells and they are activated through a set of signaling events such as activation of a death receptor or a direct DNA damage by chemotherapeutics. However, there is one main difference; the apoptotic machinery becomes much more complex throughout evolution in parallel to increased complexity of the organisms at biochemical, molecular and physiological levels. Thereby, the molecular apoptotic signaling mechanisms in mammalian cells have been a subject of intensive studies for the past few decades and two main components with distinct and overlapping parts have been identified (Figure 2.1):

i. an extrinsic pathway which involves direct initiator cascades triggered by death receptors on cell surface

ii. an intrinsic pathway which involves mitochondria and intracellular death signals

These two pathways share a couple of adaptor proteins, proteases, protein kinases and protein phosphatases as a part of apoptotic signaling modules, but the potential intersections between these pathways which controls life and death decisions of a cell are not completely identified, yet.

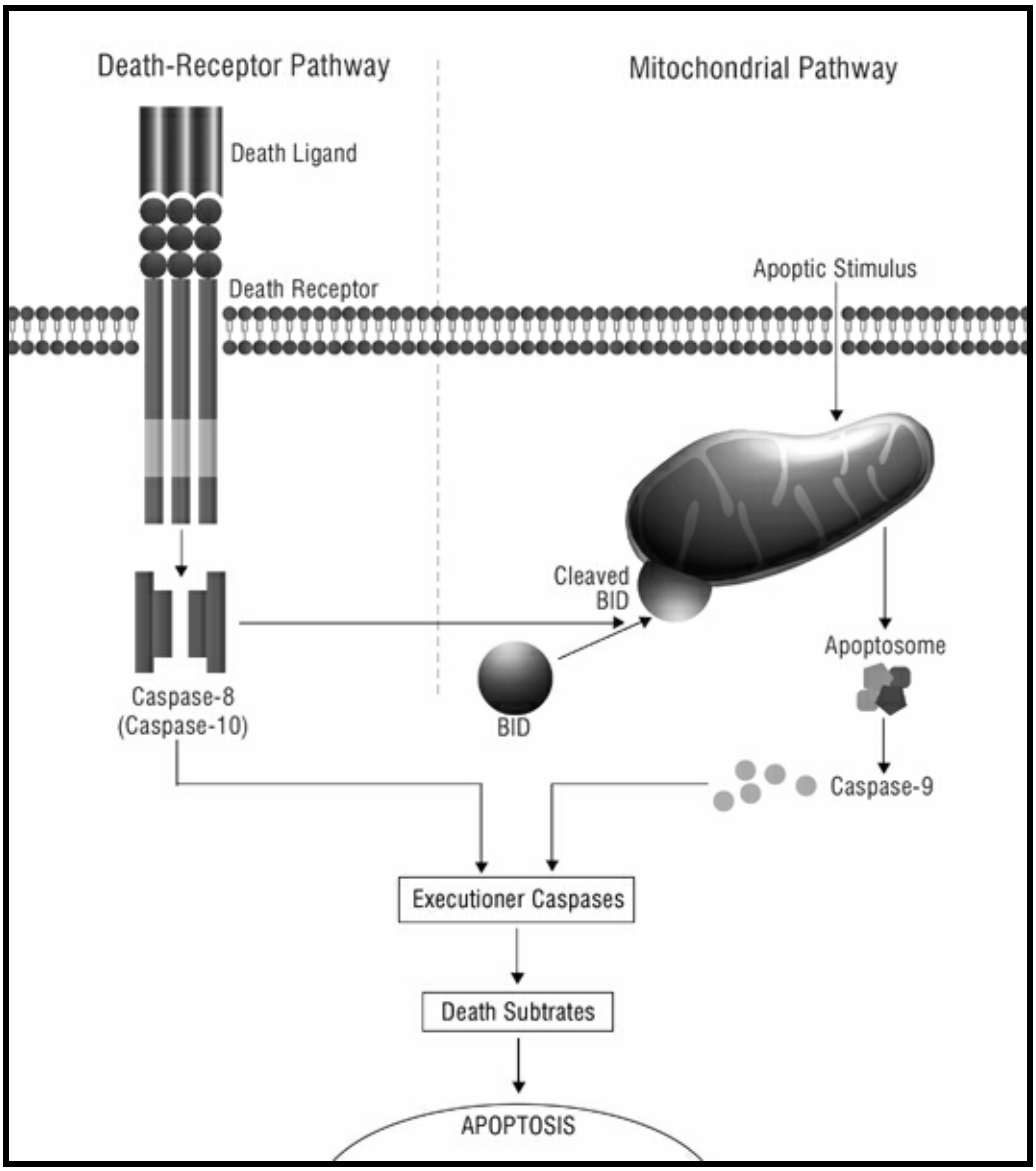


Figure 2.1 Basic overview of apoptosis pathways.

The extrinsic or death receptor-mediated pathway is activated in response to extracellular pro-apoptotic signals and integrated to the apoptotic machinery via specific death receptor adaptors (Figure 2.2). The death receptor family members are characterized by the presence of cysteine-rich repeats in their extracellular domains and protein-protein interaction modules known as the death domain (DD) in their cytoplasmic portions [20]. Binding of specific ligands induces receptor multimerization and formation of a signaling complex known as DISC (death inducing signaling complex), which is consist of various adaptor proteins including TRADD, FADD, Daxx, RIP, RAIDD and FLIP [20]. FADD acts as a bridge between DISC and caspase-8, which is critical for recruitment and oligomerization of caspase-8 in the DISC, as

well as autocatalytic activation caspase-8 and activation of death receptor-mediated programmed cell death [21-23].

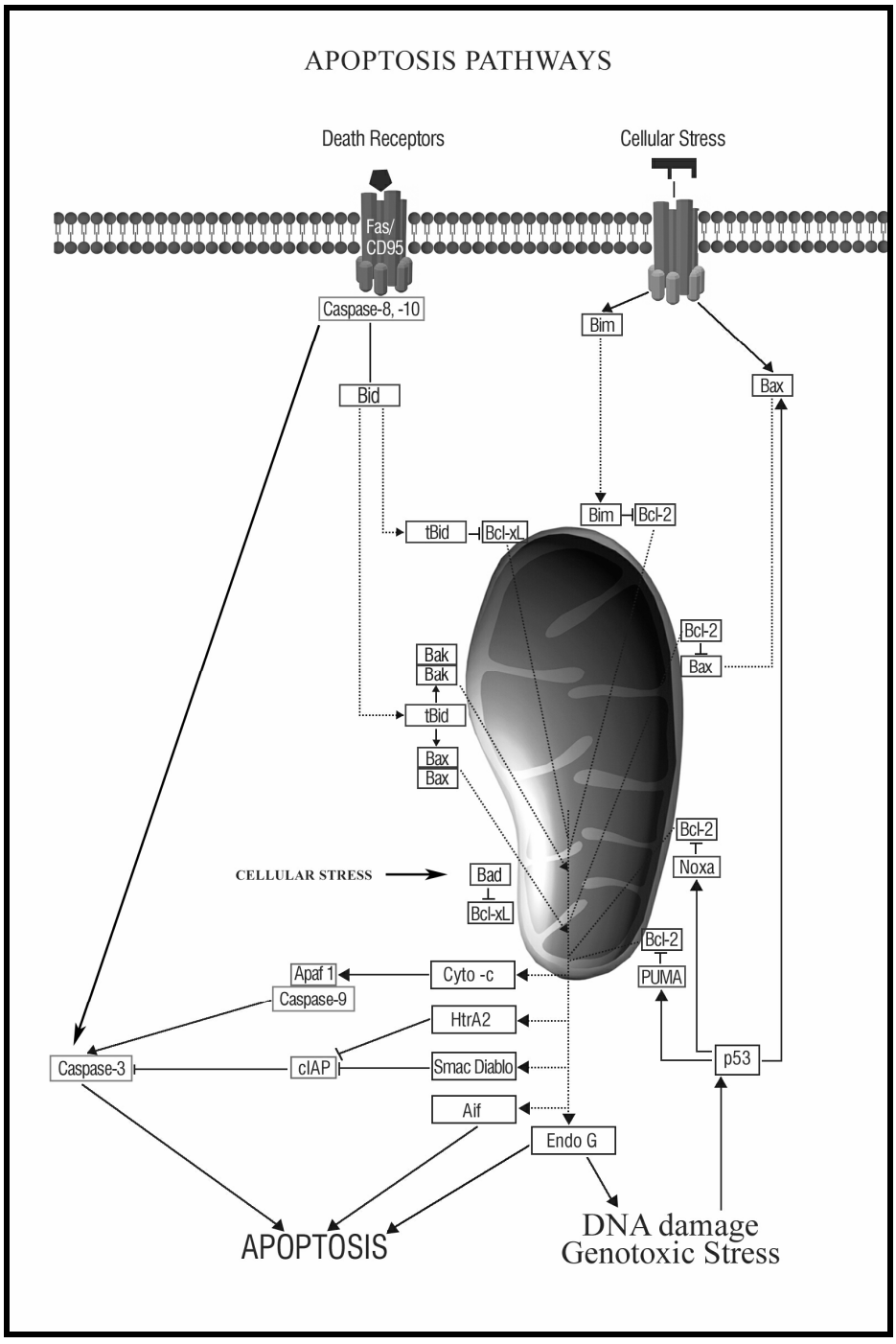


Figure 2.2 Detailed scheme of apoptosis pathways.

The direct activation of effector caspases-3 and -7 by caspase-8 may not necessarily involve mitochondrial events; however in some cell types death receptor-mediated apoptotic signaling requires a mitochondrial death amplification loop [21,24,25]. The reason for this discrimination has not been identified clearly, but insufficient amount of active caspases or abundance of downstream inhibitors of apoptotic machinery was suggested to be involved in this paradigm. The mitochondrial amplification loop involves the caspase-8-mediated cleavage of the cytosolic BH3-only pro-apoptotic Bcl-2 family member Bid; an integration of two apoptotic pathways on mitochondria. Upon processing by caspase-8, Bid translocates from cytosol to mitochondria where it oligomerizes with pro-apoptotic Bcl-2 family members Bax and Bak and mediates cytochrome *c* release [14,25,26]. The cytosolic cytochrome *c* induces the formation of the apoptosome complex, which is composed of seven Apaf-1 (Apoptotic protease activating factor-1) molecules, each bound to one molecule of cytochrome *c* and a dimer of caspase-9. Formation of apoptosome results in the activation of caspase-9, which thereby activates effector caspases 3 and 7 to initiate the execution of apoptosis [25,26]. The intracellular components that convey apoptotic stimuli to central apoptotic machinery are not identified completely, but there is one reality that has been shown clearly; mitochondria lie in the center of apoptotic machinery.

The intrinsic apoptosis pathway, which involves direct and active contribution of mitochondria, is initiated by receptor-independent apoptotic stimuli such as DNA-damaging agents, UV and γ-radiation, hypoxia and growth factor withdrawal [27-29]. These stimuli target intracellular signalling components, which transmit the apoptotic signal to main apoptotic machinery. In mammalian cells, Bcl-2 family proteins are one of the main "apoptotic sensors" mentioned above and they act primarily on the mitochondria, where they regulate the survival or death signals in a preventive or provocative fashion. Upon exposure to apoptotic insults many apoptosis regulator proteins such as cytochrome *c*, SMAC (second mitochondria-derived activator of caspases)/DIABLO (direct inhibitor of apoptosis-binding protein with low pI) and Omi/HtrA2 (high-temperature-requirement protein) are released from mitochondria (Figure 2.2) [30,31]. Some proteins responsible for caspase-independent DNA fragmentation and apoptosis-like nuclear morphology (apoptosis inducing factor (AIF)

and endonuclease G) are also released from mitochondria following apoptotic stimuli [32]. Thus, mitochondrial integrity is critical for maintaining cellular homeostasis and proper compartmentalization of apoptotic mediators. The mechanisms for the intrinsic apoptosis pathway and induction of mitochondrial permeabilization are not completely understood, but studies until today provide some clue how apoptotic stimuli induce permeabilization of mitochondrial membranes.

2.1.3 Bcl-2 protein family

2.1.3.1 Multidomain anti-apoptotic Bcl-2 proteins

The Bcl-2 family proteins can be classified into three groups based on their structural and functional properties (Figure 2.3). The first group involves the multidomain anti-apoptotic members Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1/Bfl-1, Boo/Diva and NR-13. They exhibit all four Bcl-2 homology domains (BH1-4) which are essential for their survival function through mediating protein-protein interactions and a transmembrane domain, which is formed by a stretch of hydrophobic amino acids near their C-terminal. The C-terminal domain is required for anchoring or insertion in cellular membranes of not only mitochondria but also nucleus and endoplasmic reticulum [33,34]. The α-helices of BH1, BH2 and BH3 domains form a hydrophobic pocket and the N-terminal BH4 domain further stabilizes this structure [35,36]. The protein structure of Bcl-xL complexed with the BH3 domain of Bak suggested a functional interaction of amphiphathic α-helix of Bak BH3 with the hydrophobic groove formed by BH1-3 domains of Bcl-xL [37]. Both BH3-only and multidomain pro-apoptotic Bcl-2 proteins appear to act through exposure of their BH3 domain following an apoptotic insult [38,39]. Therefore, this protein-protein interaction model proposed anti-apoptotic Bcl-2 members as functional traps of pro-apoptotic members, but is the cellular machinery always in a pro-apoptotic conditioning, which should be continuously blocked by anti-apoptotic Bcl-2 members? Or is it the pro-apoptotic members, which are present ubiquitously in the local cellular compartments, acting pro-actively to sequester the "silencers" of apoptotic machinery along with apoptotic process going by the way? Even though the exact biochemical mechanisms for the actions of anti-apoptotic Bcl-2 members remain to be elucidated, the efforts to clarify these mechanisms provided us many clues. Bcl-2 has been shown to modulate cellular

viability through regulating intracellular calcium homeostasis, cellular redox state, lipid peroxidation, as well as cytochrome *c* release from mitochondria [40-42]. Bcl-2 has been shown to attenuate apoptosis induced not only by ionizing radiation, chemotherapy, UV radiation but also by death receptors [28,43,44]. Overexpression of Bcl-2 protects SW480 cells from TRAIL-induced apoptosis via attenuation of caspase-8 activation and cleavage of Bid and caspase-3 [45]. In contrast, Fas/FasL-, TRAIL- and TNFα-mediated apoptosis pathways have been proposed to be insensitive to blockage by Bcl-2/Bcl-xL [46,47]; thereby the exact contribution of Bcl-2/Bcl-xL in receptor-mediated extrinsic apoptotic pathway remains undetermined. Mice deficient in Bcl-2 have been demonstrated to have gray hair, polycystic kidneys and decreased number of lymphocytes [48]. Mice deficient in Bcl-xL die at around day 13 of gestation due to massive neuronal and hematopoetic apoptosis [49]. Bcl-2 and Bcl-xL were also reported to abrogate mitochondrial translocation and oligomerization of Bax in the outer mitochondrial membrane [50,51].

The mechanistic insight, which is derived from cases characterized with Bcl-2 overexpression, remains insufficient to explain the consequences of Bcl-2 on resistance to apoptosis. In addition to level of expression, post-translational modifications such as such as phosphorylation and cleavage may regulate the activity of Bcl-2 and Bcl-xL. Phosphorylation of Bcl-2 at Ser-70 by PKC has been reported to be required for efficient anti-apoptotic function [52]. In contrast, microtubuli-targeting agents such as paclitaxel have been shown to induce hyperphosphorylation of Bcl-2 (Ser-70, Ser-87 and Thr-69) and abrogate its anti-apoptotic effect [53]. Phosphorylation of Bcl-xL at Ser-62 by JNK (c-Jun N-terminal kinase) in response to taxol or 2-methoxyestradiol treatment has been reported to oppose the anti-apoptotic function of Bcl-xL and sensitizes prostate cancer cells to apoptosis [54]. The caspase-dependent N-terminal cleavage of Bcl-2/Bcl-xL and exposure of their BH3 domains converts these anti-apoptotic proteins into pro-apoptotic ones.

2.1.3.2 The multidomain pro-apoptotic members of Bcl-2 protein family

This second group of Bcl-2 protein family mainly involves Bax, Bak and Bok/Mtd (Figure 2.3). Bax is mainly localized in the cytosol or loosely attached to the outer membrane of mitochondria or ER as a monomer. Following an apoptotic stimuli, Bax

undergoes a unique conformational change exposing its C-terminal hydrophobic domain, which is involved in its anchorage to mitochondrial membrane [55]. In the mitochondrial membranes, Bax forms dimers, oligomers or high-order multimers [56]. Another important multidomain proapoptotic Bcl-2 protein family member is Bak, which is an integral protein of outer mitochondrial membrane and ER. Similar to Bax, Bak also undergoes a conformational change -the open conformer- in response to apoptotic stimuli such as etoposide and cisplatin [57,58]. The inhibitory effect of Bcl-2 on Bak acts through selective interaction of Bcl-2 with open conformer (N-terminal exposed conformation) of Bak [59]. The principles of these conformational changes and oligomerization of Bak and Bax proteins remain to be explained at structural level. The involvement of Bak and Bax in apoptosis regulation is demonstrated by the insensitivity of Bak $^{-/-}$ Bax $^{-/-}$ MEFs to multiple apoptotic stimuli including chemotherapeutics and UV radiation [60]. Afterwards, the requirement of Bak and Bax in the apoptotic machinery has been confirmed many other studies. Bax-null cells have been shown to be resistant against TRAIL-induced apoptosis [61]. Bax deficiency did not effect the processing of caspase-8 or Bid cleavage by TRAIL, but the release of Smac/DIABLO, which is required for inhibition of IAP proteins and caspase-3 activation, was abrogated [61]. In TRAIL-resistant leukemic cells that are deficient in Bax and Bak, release of mitochondrial proteins appear to be abrogated and adenoviral transduction of the Bax, but not the Bak gene, to the Bax/Bak-deficient leukemic cells rendered them TRAIL-sensitive as assessed by enhanced apoptotic death and caspase-3 processing [62]. Recently activation of multiple caspases by DNA damage and ER stress has been shown to be directly regulated by Bax and Bak in double knock-out MEFs [63]. Post-translational modifications of Bax or Bak such as cleavage have been shown to regulate the functional impact of these proteins on apoptosis.

Calpain-mediated conversion of Bax into a truncated form (arises from cleavage of N-terminal 33 amino acids, p18 Bax) enhances its pro-apoptotic properties of the protein upon stimulation with chemotherapeutics [64]. After truncation into its p18 form, Bax behaves like a BH-3 only protein and the potentiation of apoptosis by p18 Bax has been proposed to be related to increased affinity for Bcl-xL. Furthermore, a cathepsin-like cysteine protease is involved in degradation of p18 Bax and stabilization of p18 Bax by cathepsin inhibitors enhances drug-induced apoptosis [65].

2.1.3.3 BH3-only members of Bcl-2 protein family

The third group of the family involves BH3-only proteins such as Bid, Bad, Bim, Bik, Blk, Hrk, BNIP3, Nix, BMF, Noxa and Puma. These proteins share only the amphipathic α-helical BH2 homology domain and mainly act through inhibition of Bcl-2/Bcl-xL and activation of Bak and Bax. They act as sentinels of cell death sensing machinery and in a point of view; they coordinate the fine-tuning of apoptotic response through their interactions with pro- and anti-apoptotic Bcl-2 members. This fine-tuning phenomenon has been attributed to the selective predisposition of certain BH3-only proteins for either anti-apoptotic or pro-apoptotic Bcl-2 proteins, but the definitive mechanisms that lie behind remain to be clarified [66]. There are two main pathways, which characterize the function of BH3-only proteins on mitochondria;

i) Direct activators: Some BH3-only members (Bid and Bim) interact with pro-apoptotic Bcl-2 proteins such as Bak and Bax and thereby induce their activation/oligomerization. This type of activity of BH3-only proteins can be attenuated by Bcl-2 through selective sequestration and functional silencing.

ii) Sensitizers: Other BH3-only members (Bad) interact with anti-apoptotic Bcl-2 proteins and prevent them binding and sequestering BH3-only members such as Bid and Bim, which can activate Bak and Bax.

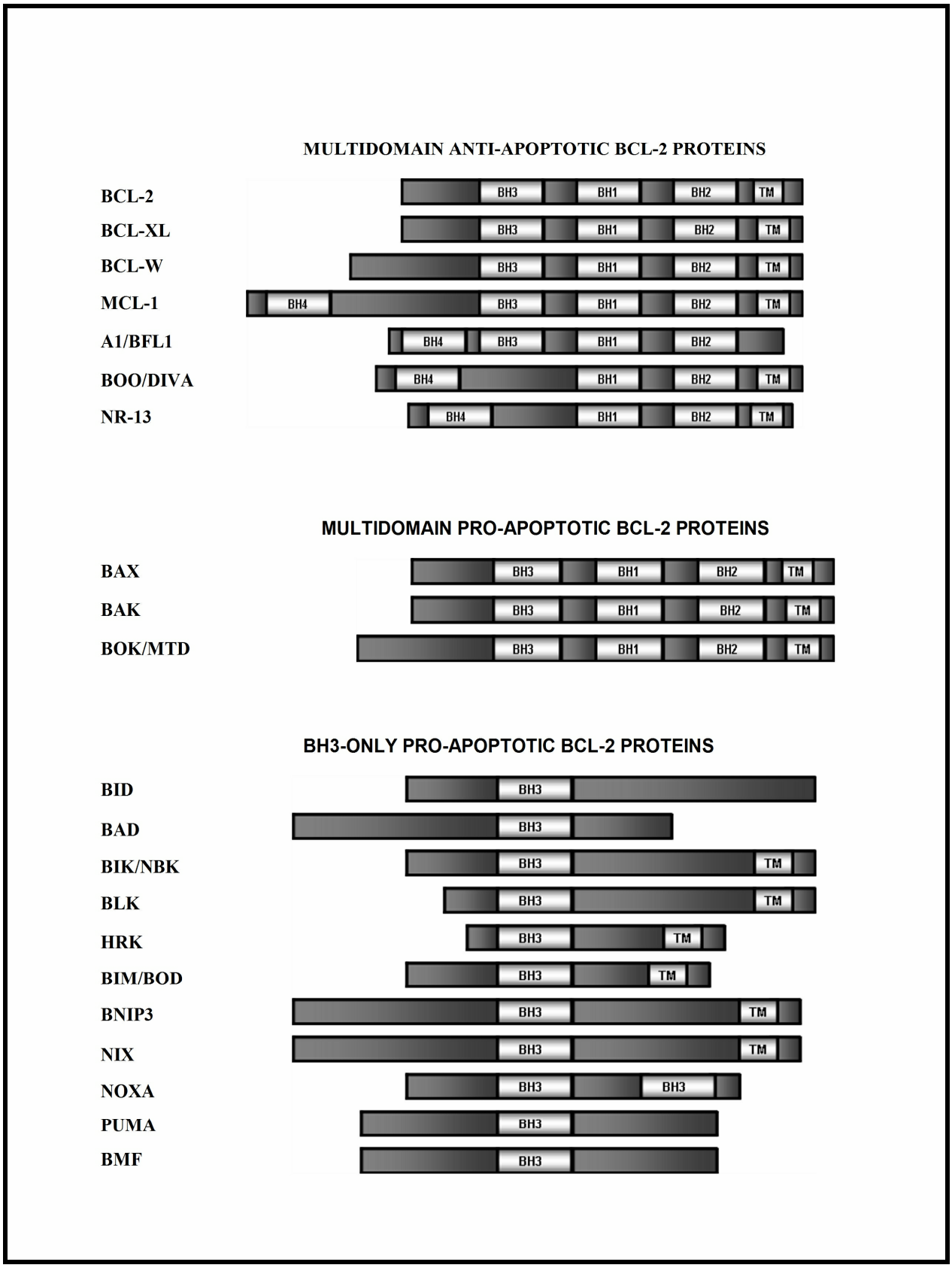


Figure 2.3 Bcl-2 protein family members are key regulators of apoptosis.

The functional regulation of BH3-only proteins at cellular level could be regulated by;

1)Phosphorylation

Selective phosphorylation of proteins at different residues may modulate different molecular and cellular responses. Considering apoptosis signalling and Bad phosphorylation, survival signals induce phosphorylation of Bad on Ser-112, Ser-136, and Ser-155, which leads to the sequestration and inactivation of Bad by 14-3-3 proteins [67,68]. Recently, a novel Cdc2- or JNK-mediated phosphorylation site of Bad has been mapped at Ser-128 and this modification has been demonstrated to inhibit sequestration of Bad by members of 14-3-3 family and potentiate its pro-apoptotic effect [69,70]. Cytokine-dependent phosphorylation of Ser-170 has been demonstrated to negatively regulate pro-apoptotic activity of Bad [71]. Furthermore, phosphorylation of Bim at Ser-65 by JNK has been shown to mediate trophic factor withdrawal-induced Bax-dependent apoptosis [72].

2)Transcriptional control

Puma (p53 up-regulated modulator of apoptosis) and Noxa are transcriptional targets for p53 [73,74]. PUMA is transcriptionally induced by the chemotherapeutics 5-FU and adriamycin in a p53-dependent fashion and it is localized to mitochondria where it interacts with Bcl-2 and Bcl-XL through its BH3 domain [75]. In contrast to Noxa, the pro-apoptotic effect of Puma has been shown to depend on conformational change and multimerization of Bax [76]. Induction of Noxa did not show any relevance to subcellular localization of Bax, but it selectively interacts with Bcl-2, Bcl-xL and Mcl-1 via its BH3-only domain [74].

3)Cleavage

Following death receptor signalling, the full-length 22 kDa Bid is cleaved within its unstructured loop and a 15 kDa truncated form of Bid is created, tBid [77,78]. Cleavage of Bid results in exposure of a new terminal glycine residue, which is N-

myristoylated [79]. Upon N-myristoylation, tBid is selectively routed to mitochondria and induces oligomerization of Bax and Bak.

2.1.4 Mitogen-activated protein kinases, AP-1 and apoptosis regulation

2.1.4.1 MAP kinase signaling pathways

Living cells can sense and respond to biological and chemical alterations, which may affect different cellular functions such as proliferation, migration, differentiation and cell death. The cellular response to stress depends on the type, strength and duration of the stimuli and involves a complex network of signal-transduction pathways.

Mitogen-activated protein kinases (MAP kinases) are among the best-characterized signaling pathways regulating cell survival and apoptosis. MAP kinase signaling cascade consists of a module of three cytoplasmic kinases: a mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAP kinase (MAPK) itself (Figure 2.4). MAPK cascades regulate proliferative and cellular stress signals into changes in protein interactions and/or gene expression. MAPKKKs are serine-threonine kinases that receive activating signals and then activate its substrate, a MAPKK, by phosphorylation. MAPKKs are dual-specificity kinases with ability to phosphorylate serine and threonine residues in their substrates, MAP kinases. MAP kinases are serine-threonine kinases, which phosphorylate both cytoplasmic and nuclear substrates. Transcription factors such as AP-1 are among direct targets of MAP kinases.

In mammals, three main MAP kinases are identified: extracellular signal regulated kinase (ERK), p38 and c-Jun NH2-terminal protein kinase/stress activated protein kinase (JNK/SAPK) groups of MAPKs [80].

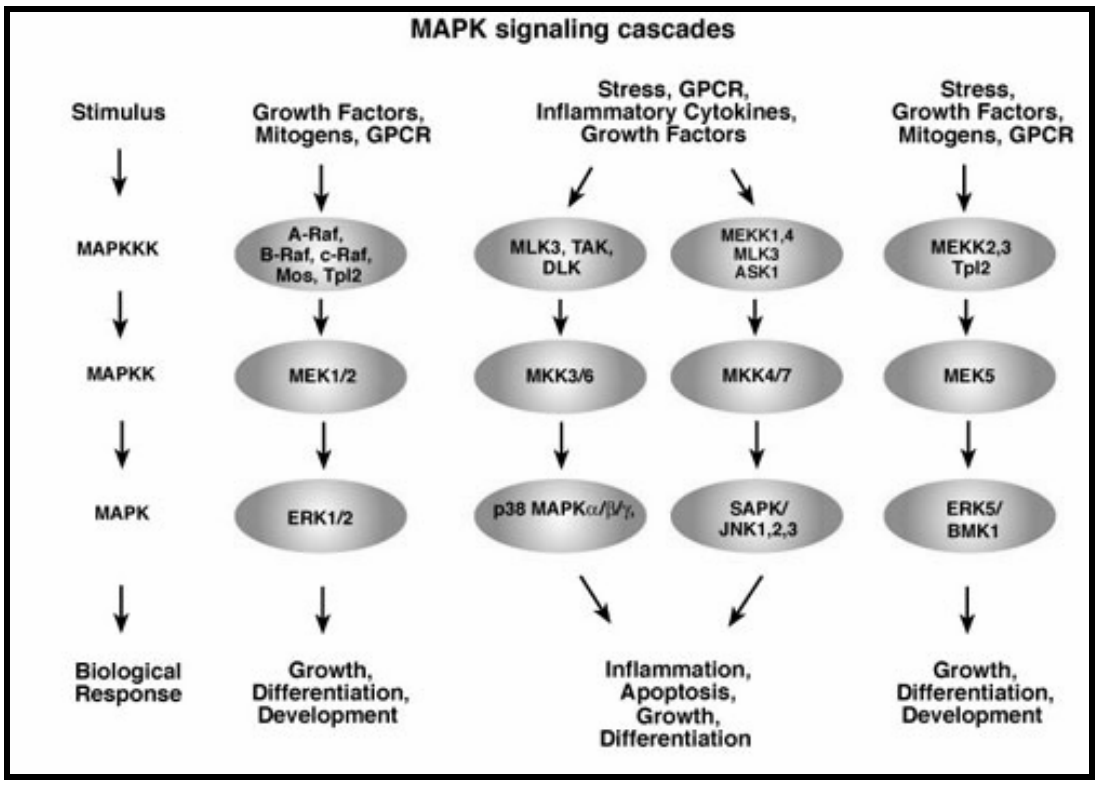


Figure 2.4 MAP kinase signaling pathways (adapted from [10]).

These different MAP kinases are regulated by distinct stimuli. JNK and p38 are mainly activated by cellular stress and by inflammatory cytokines. In contrast, the ERK signaling pathway is activated by mitogens and growth factors, and transduces survival, proliferation and differentiation signals. MAP kinases exert a selective substrates specifity, which are involved in the regulation of specific components of transcription factor AP-1. For example, c-Jun is phosphorylated by JNK, c-Fos is a substrate for ERK while ATF-2 can be phosphorylated by both JNK and p38 [80]. AP-1 is a major transcription factor complex composed mainly by homodimers of c-Jun and heterodimers of c-Jun/c-Fos [81]. AP-1 transcriptional activity may be related to either apoptosis or proliferation in different cellular systems. The outcome of AP-1-mediated transcriptional activity in response to mitogens or cellular stress has been proposed to be defined by the composition of dimers forming AP-1 transcription factor complex.

A major target of the JNK signaling pathway is activation of a transcription factor, AP-1, which is mediated by phosphorylation of c-Jun. JNK binds to the NH2-terminal

activation domain of c-Jun and phosphorylates it on Ser-63 and Ser-73, resulting in increased transcriptional activity of AP-1 [82,83]. The other major substrates are transcription factors: activating transcription factor (ATF)-2 and Elk-1 [84]. JNK appears to be essential for AP-1 activation caused by stress and some cytokines, but is not required for AP-1 activation in response to other stimuli [85]. Thus, the precise role of AP-1 in the response to JNK activation is not clear and is likely to be modified by the activity of other transcription factors that interact with AP-1 on the promoters of target genes.

The JNK protein kinases are encoded by three genes. The *Jnk1* and *Jnk2* genes are expressed ubiquitously whereas *Jnk3* expression is limited to brain, heart and testis. These genes are alternatively spliced resulting in at least ten JNK isoforms. Mice deficient in JNK1 or JNK2 are morphologically normal, but are immunodeficient due to severe defects in T cell function. In contrast, deletion of both *Jnk1* and *Jnk2* genes causes early embryonic death [86]. MEFs isolated from $Jnk1^{-/-}$ $Jnk2^{-/-}$ mice exhibit defects in AP-1 transcription activity, decreased proliferation and resistance to stress-induced apoptosis [86]. JNK is activated by two protein kinases, MKK4 (SEK1) and MKK7 (Figure 2.4). Although MKK4 and MKK7 are dual specificity kinases and can phosphorylate JNK on both Tyr and Thr. MKK4 and MKK7 appear to selectively phosphorylate JNK on Tyr and Thr, respectively. The difference in specificity suggests that MKK4 and MKK7 may act cooperatively to activate JNK [87]. In addition to MEKKs, several other MAPKKKs have been reported to activate the JNK signaling pathway such as *A*poptosis *S*timulating *K*inases (ASK1 and ASK2) and the mixed-lineage protein kinases (MLK1-3, DLK and LZK) [87]. Involvement of MEKK1 and MEKK3 have been implied to be involved in cytokine- and cellular stress-induced JNK activation [87].

ERK1 and ERK2 are widely expressed in human tissues and are involved in the regulation of cell cycle and proliferative functions in already differentiated cells. Many different stimuli, including growth factors, cytokines, viral infection, G protein-coupled receptor ligands, and carcinogens, activate the ERK1/2 pathway [88]. *Ras* proto-oncogene may activate the downstream components of kinase module (c-Raf1, B-Raf, or A-Raf). Mutations that convert Ras to an activated oncogene are common oncogenic mutations in many human tumors. Oncogenic Ras persistently activates the ERK 1/2

pathway, which contributes to the increased proliferative rate of tumor cells. For this reason, inhibitors of the ERK pathways are entering clinical trials as potential anticancer agents. In differentiated cells, ERKs have different roles and are involved in responses such as learning and memory in the central nervous system. The ERK 1/2 signal transduction pathway in mammalian cells has been extensively studied. Proliferative signals such as growth factors induce autophosphorylation and activation of receptor tyrosine kinases, such as Raf. Activation of Raf results in MEK and ERK 1/2 activation and regulation of proliferation and cell cycle progression [89,90]. Nevertheless, ERK pathway is generally regarded as survival-promoting, ERK activation may mediate the response to various stress-inducing stimuli including genotoxins and microtubule inhibitors. The balance between the activities of survival-promoting pathways such as ERK and pro-apoptotic pathways such as JNK and p38 has been proposed to determine the response of a cell under stress [91].

The p38 kinases were first defined in a screen for drugs inhibiting TNFα-mediated inflammatory responses. The p38 MAP kinases regulate the expression of many inflammatory mediators and exerts important role in activation of the immune response [80]. p38 MAP kinases are also activated by many other stimuli, such as hormones, G protein-coupled receptor ligands and cellular stress [80,92]. The p38 kinases are activated by TAB1, which is an adaptor protein with no known catalytic activity. This important finding indicates that other adaptor proteins should be investigated for potential roles in regulating MAPK activity.

The importance of MAPKs in controlling cellular responses to the environment and in regulating gene expression, cell growth, and apoptosis has made them a priority for research related to many human diseases. MAP kinase knockout phenotypes are summarized in Table 2.1. The ERK, JNK, and p38 pathways are all molecular targets for drug development, and inhibitors of MAPKs will surely be one of the new classes of drugs developed for the treatment of human disease.

Genes	Summary of phenotypes
	Decreased T cell responses in thymus
ERK 1/2	**Lack of mesoderm differentiation**
	Defects in the placenta
	Defects in T cell activation and apoptosis of thymocytes
	Less susceptibility to insulin resistance in diabetes models
JNK	**Defects in neural tube closure**
	Increased IL-2 production in T cells
	Resistance to UV-induced apoptosis in embryonic fibroblasts
	Defects in placental angiogenesis
p38	**Defects in Epo production**
	Angiogenic defects in the placenta and peripheral vessels

Table 2.1 Summary of MAP kinase knockout phenotypes.

2.1.4.2 The role of MAP kinases in apoptosis regulation

A finely controlled regulation of MAP kinases is required for physiological cell proliferation and differentiation; whereas an unregulated activation of these MAP kinases can result in oncogenesis or diseases related to disproportionate apoptosis. The role of JNK in pro-apoptotic, signaling has been investigated by identification of target genes induced by stress. The JNK/AP-1 pathway has been proposed to promote apoptosis by increasing the expression of pro-apoptotic genes such as Bak and TNFα and decreasing the expression of p53 and its target p21, which would prevent cell cycle arrest and promote apoptosis [93]. However, more recent studies demonstrate that JNK is not required for UV radiation-induced accumulation of p53 [86]. The potential role of p53 as a target of JNK signaling is therefore unclear. JNK has also been observed to increase expression of Fas-L [94]. However, murine embryo fibroblasts prepared from *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ embryos (*Jnk* null MEFs) exhibit no defects in Fas-induced apoptosis, indicating that JNK is not required for Fas- mediated apoptosis but it can contribute by increasing the expression of Fas-L. In contrast, *Jnk* null MEFs did exhibit a defective apoptotic response to stress- induced stimuli, including UV radiation, the DNA

alkylating agent methyl methansulfonate and the translational inhibitor anisomycin. The defect in apoptosis correlated with failure to induce mitochondrial depolarization, cytochrome c release and subsequent caspase activation [86]. Translocation of JNK to mitochondria has been reported in response to DNA damage supporting the involvement of mitochondria in JNK-mediated apoptosis [95]. The Bcl-2 proteins are potential targets of JNK involved in regulation of cytochrome c release [80]. Phosphorylation of Bcl-2 and Bcl-xL by JNK has been shown *in vitro* and is suggested to abrogate their anti-apoptotic functions [96,97]. JNK is also reported to phosphorylate the pro-apoptotic protein Bad resulting in abrogation of its pro-apoptotic function (Donovan et al., 2002). Although involvement of JNK in pro-apoptotic signaling is generally accepted, apoptosis does not represent the only possible outcome of JNK activation, since most forms of stress do not cause apoptosis under conditions that are sufficient for JNK activation [80]. This may be due to parallel activation of survival-mediating pathways such as ERK, Akt/PKB, NF-κB that can block pro-apoptotic signaling [91]. Increasing evidence in the literature suggests that the duration of JNK activation is important for the outcome, i.e., sustained JNK activation is associated with apoptosis, whereas transient activation primarily mediates pro-survival signaling [98].

Similar to JNK pathways, the involvement of p38 MAP kinase in apoptosis is also diverse. It has been shown that p38 signaling promotes cell death [99,100], whereas it has also been shown that p38-MAPK cascades enhance survival and cell growth [101,102]. Specific p38-MAPK inhibitor SB203580 blocks anti-CD3 mAb-induced T cell apoptosis [103]. MKK3 and MKK6 may therefore induce pro-apoptotic signals through the activation of p38 kinases and induce apoptosis. Inactivation of p38 results in embryonic lethality around embryonic day 11 [104]. In line with this finding, it has been shown that activation of p38 by exposure to UV leads to G2/M cell cycle arrest by suppressing CDC2 via the phosphatase CDC25 [105]. CDC2 can induce the phosphorylation of the BH3-only protein BAD and thus triggers neuronal apoptosis [69]. In conclusion, similar to the JNK pathways, p38 MAP kinase signaling plays multiple roles in cells such as differentiation, proliferation, cytokine secretion, as well as cell death.

ERK 1/2 are activated by various growth factors and induce transition from the quiescent state into the cell cycle. ERK signaling pathways are also involved in cell

proliferation, differentiation, actin cytoskeleton reorganization, and cell migration. Moreover, ERKs are also involved in the stress response and cell death [10,80,88]. It has been shown that irradiation stresses lead to phosphorylation of the epidermal growth factor receptor (EGFR) in cancer cell lines, which is mediated by radiation-induced free radicals and results in activation of ERKs [106]. Similarly, UV irradiation induces the activation of ERK in a number of cell types, and the UV-induced ERK activation involves the activation of EGFR [10,80].

The differential contribution of different MAP kinase family members to apoptosis has been examined after withdrawal of NGF from rat PC-12 pheochromocytoma cells [91]. NGF withdrawal resulted in sustained activation of the JNK and p38-MAP kinases and inhibition of ERK 1/2. The effects of dominant-negative or constitutively activate forms of JNK, p38, and ERK 1/2 signaling pathways demonstrated that activation of JNK and p38 and concurrent inhibition of ERK 1/2 are critical for induction of apoptosis in these cells. These results suggest that ERK is a survival factor, and JNK and p38 kinases exert opposing effects on apoptosis in this experimental cell system [91]. ERK $1^{-/-}$ mice are viable, fertile, and appear normal. However, mice that lack the upstream ERK activator MEK1 die at embryonic day 10.5 exhibiting defective placental vascularization [107]. The molecular frameworks by which ERK, JNK, and p38-MAPK signaling cascades cooperate, concert control of cell fates by other signaling, and networks in different cell types must be the focus of future studies.

2.1.4.3 AP-1 signaling and apoptosis regulation

AP-1 is one of the first mammalian transcription factors to be identified, but its physiological functions are still being unclarified. A wide range of physiological stimuli and environmental insults induces AP-1 activity. In turn, AP-1 regulates a wide range of cellular processes, including cell proliferation, death, survival and differentiation. The main checkpoints of AP-1 signaling have been summarized in Figure 2.5. However, despite increasing knowledge regarding the physiological functions of AP-1, the target-genes mediating cell proliferation and survival functions are not always obvious. AP-1 is composed of dimeric basic region-leucine zipper (bZIP) proteins that belong to the

Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1 and Fra2), Maf (c-Maf, MafB, MafA, MafG/F/K and Nrl) and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) sub-families, which recognize either TPA response elements (5′-TGAG/CTCA-3′) or cAMP response elements (CRE, 5′-TGACGTCA-3′) [81]. c-Jun is the most potent transcriptional activator in its group, whose transcriptional activity is attenuated and sometimes antagonized by JunB. The Fos proteins, which cannot homodimerize, form stable heterodimers with Jun proteins and thereby enhance their DNA binding activity. Regulation of AP-1 occurs through: first, changes in *jun* and *fos* gene transcription and mRNA level; second, c-Jun and c-Fos protein turnover; third, post-translational modifications of c-Jun and c-Fos proteins that modulate their transactivation potential; fourth, interactions with other transcription factors that can either synergize or interfere with AP-1 activity. Growth factors, cytokines, neurotransmitters, polypeptide hormones, cell–matrix interactions, bacterial and viral infections, and a variety of physical and chemical stresses induce AP-1 activity [81]. These stimuli activate mitogen activated protein kinase cascades that enhance AP-1 activity through the phosphorylation of distinct substrates [108]. Serum and growth factors that potently induce AP-1 do so by activating the extracellular-signal-regulated kinase subgroup of MAP kinases, whose members translocate to the nucleus to phosphorylate, and thereby potentiate, the transcriptional activity of ternary complex factors (TCFs) that bind to *fos* promoters [108]. Moreover, the ERKs directly phosphorylate Fra1 and Fra2 in response to serum stimulation, possibly enhancing their DNA binding in conjunction with c-Jun. JNK and p38 MAP kinase pathways mostly mediate the induction of AP-1 by proinflammatory cytokines and genotoxic stress [108,109]. Once activated, the JNKs translocate to the nucleus, where they phosphorylate c-Jun and thereby enhance its transcriptional activity. The JNKs also phosphorylate and potentiate the activity of ATF2**,** which heterodimerizes with c-Jun to bind divergent AP-1 sites in the *c-jun* promoter [110]. Importantly, the induction of c-Jun expression by certain genotoxic stresses, such as short-wavelength ultraviolet (UV) radiation, is much more robust and persistent than the induction seen after mitogenic stimulation [81]. Constitutive expression of activated oncogenes, such as *Ras*, also results in an elevation of AP-1 activity, mostly through persistent activation of ERK and JNK [111]. The contribution of p38 to AP-1 induction could be mediated by the direct phosphorylation and activation of ATF2 [112].

Inhibition of *fos* and *jun* expression in mouse fibroblasts using antisense-RNA demonstrated their requirement for proliferation and cell cycle progression [113]. Furthermore, microinjection of antibodies against c-Fos and c-Jun inhibits serum-stimulated quiescent mouse fibroblasts from re-entering the cell cycle [114]. Studies using fibroblasts derived from *fos* and *jun* knockout mice provide partial genetic support for these conclusions. Fibroblasts deficient in c-Fos or FosB alone proliferate normally, and only cells lacking both proteins have a reduced proliferative capacity [115]. Interestingly, c-$fos^{-/-}$ $fosB^{-/-}$ double-knockout mice, but not single knockouts, are smaller than their wild type counterparts [115]. Comparing the different AP-1 deficiency phenotypes, mouse embryo fibroblasts from *c-jun*$^{-/-}$ embryos exert the most severe proliferation defects [116]. These *c-jun*$^{-/-}$ cells could be passaged in cell culture only once or twice before entering a premature senescence. JNK-mediated phosphorylation of c-Jun specifically induces cell proliferation, as c-*jun* (Ala63/73) fibroblasts have a proliferation defect, which is less severe than that of *c-jun*$^{-/-}$ fibroblasts [117].

The first clues for apoptotic function for AP-1 raised from observations linking the induction of c-Fos and c-Jun to conditions that result in cell death. c-Fos is continuously induced in the brains of mice treated with kainic acid, which is a potent activator of glutamate receptors that induces apoptosis of hippocampal neurons [118]. The induction of apoptosis in neuronal and lymphoid cell cultures through withdrawal of growth factors is preceded by the induction of AP-1 proteins [119,120]. Nevertheless, these findings do not necessarily demonstrate whether c-Jun or c-Fos induction is functionally involved in triggering apoptosis. The research efforts demonstrating the pro-apoptotic functions of c-Jun and c-Fos was derived from transient overexpression experiments, in which c-Jun or c-Fos were found to induce apoptosis in various cell lines. The protein expression levels achieved in overexpression experiments are extremely high. Therefore, overexpression experiments could result in aberrant physiological function of the overexpressed protein. The anti-apoptotic activity of dominant negative c-Jun has been shown to depend, at least partially, on its ability to induce the expression of Bim, a proapoptotic Bcl-2 family member [121]. It was not clairifed that whether *Bim* transcription is directly regulated by c-Jun. Fas-ligand (FasL) is another important AP-1 target gene. c-Jun-dependent FasL induction was shown in several experimental models [81]. However, it should be underlined that the regulation

of FasL transcription is not promoted by c-Jun-dependent transcriptional activity. JNK-mediated phosphorylation has been shown to be involved in c-Jun-induced apoptosis in neuronal cells [122]. Expression of c-*Jun* mutated in the JNK phosphorylation sites has been shown to block apoptosis induced by NGF withdrawal. Furthermore, targeting JNK3, which is a JNK isoform specifically expressed in neuronal cells, has been demonstrated to reduce both AP-1 activation and kainate-induced apoptosis [123]. The exact function of AP-1 in cellular responses to genotoxic stress has not been entirely identified. The involvement of c-Jun in the induction of UV-induced apoptosis has been suggested by several studies [81]. The results have indicated that c-Jun deficient fibroblasts and $jnk1^{-/-}jnk2^{-/-}$ double knockout MEFs are less sensitive to UV-induced apoptosis [124]. In addition, dominant negative c-Jun reduces apoptosis in human monoblastic leukaemia cells exposed to genotoxic insults [125]. In contrast, there are other studies, which suggest that c-Jun protects cells against UV-induced cell death [126]. This protective activity of c-Jun has been proposed to be mediated through activation of STAT3 signaling pathway and inhibition of Fas expression.

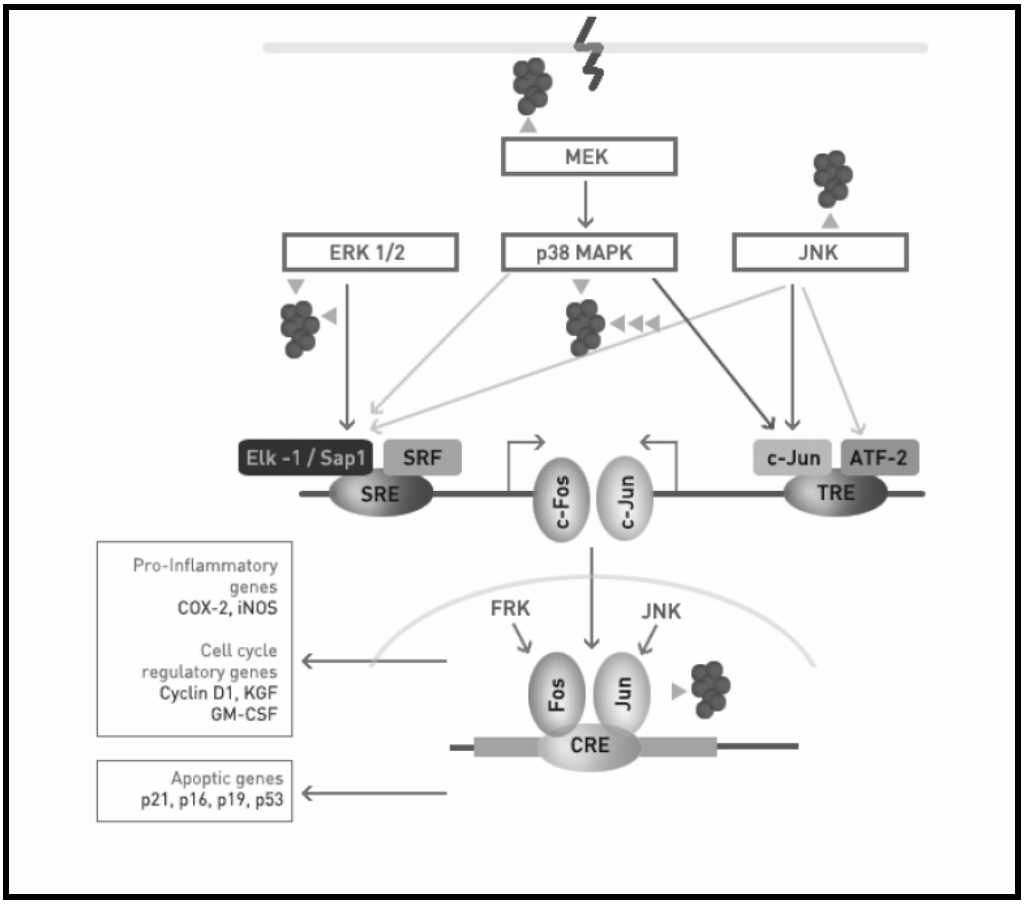


Figure 2.5 AP-1 signaling (adapted from [81]).

The balance between the pro-apoptotic and anti-apoptotic target genes determine whether the outcome will be cell survival or cell death. This balance may vary from one cell type to another, and may be dependent on the type and duration of stimulus used to activate AP-1, as well as on the activation of other transcription factors. AP-1 acts as a molecular switch that retains cells in a certain proliferative steady state or that activates apoptotic pathways in response to cellular damage.

2.1.5 4-Hydroxynonenal

2.1.5.1 Lipid peroxidation and cell signaling

Oxidative stress is recognized as a major upstream "key player" in the signaling cascades involved in many critical cellular functions, such as cell proliferation and apoptosis, inflammatory responses, cellular adhesion and chemotaxis. An increased amount of evidence suggests that many of the effects of cellular dysfunction under oxidative stress are mediated by products of non-enzymatic reactions, such as the peroxidative degradation of polyunsaturated fatty acids [1,2]. Lipid peroxidation is initiated and proceeds by a free radical chain reaction mechanism and lipid hydroperoxides are produced as reaction products. Moreover, the decomposition of lipid hydroperoxides leads to the formation of a number of breakdown products that display a wide variety of damaging actions. Aldehydic molecules generated during lipid peroxidation have been implicated as causative agents in cytotoxic and genotoxic processes initiated by the exposure of biological systems to oxidizing agents [2]. Compared to free radicals, the aldehydes are relatively stable and can diffuse within or even escape from the cell and attack targets far from the site of the original event.

Oxidative modifications of lipoproteins, which are either components of cellular membranes or circulating in blood, exert many considerable consequences in disease pathogenesis. These lipid peroxidation end-products, specifically ox-LDLs, play key roles in the evolutionary and progressive pathogenesis of atherosclerosis through activation of pro-inflammatory and pro-atherosclerotic signaling pathways [127]. The biological responses triggered by ox-LDLs have been shown to be associated with various signaling pathways including phospholipases, kinases, transcription factors,

calcium and proteases [2,127]. The ox-LDLs may act as ligands and trigger a chain of signaling events, which include calcium, cAMP and inositol phosphate pathway. Ox-LDLs have been shown to increase the cytosolic calcium levels of vascular endothelial and smooth muscle cells (SMCs) prior to cAMP activation [128]. It has been demonstrated that ox-LDL treatment of SMCs induces an increase in phospholipase C-mediated phosphoinositide turnover, which depends on receptor signaling and internalization of ox-LDLs [128]. In addition to membrane-related signaling events, cytosolic targets of ox-LDLS such as PKC family of kinases have been determined [129]. Recent studies have also shown that ox-LDLs directly induce tyrosine phosphorylation and activation of epithelial growth factor receptor (EGFR), which could be also initiated by HNE [130]. Furthermore, ox-LDLs have been shown to activate phosphatidylinositol-3-kinase (PI3-kinase) in a macrophage cell line and to induce a mitogenic effect via this signaling cascade [131]. Ox-LDLs activate STAT1, STAT3 (Signal transducer and activator of transcription) and MAP kinase pathways in SMCs and macrophages [132,133].

Gene expression regulation (such as vascular adhesion molecules ICAM and VCAM) and modulation of vascular signal transduction participate in development and progress of atherosclerotic lesion [127]. Ox-LDLs regulate the expression of genes through activation of nuclear transcription factors and their upstream activator/inhibitor kinases. Ox-LDLs have been shown to activate AP-1 and NF-κB DNA binding in endothelial cells, fibroblasts and SMCs [127,134]. PPARγ (peroxisome proliferator-activated receptor γ) , which is ligand-dependent nuclear transcription factor in macrophage cell lineage, has been shown to be modulated by ox-LDLs [135]. This event partly explains how lipid peroxidation induces a proinflammatory state in tissue microenvironment. All these data demonstrate how lipid peroxidation may influence pathophysiological progress not only by exerting direct cytotoxic effects but also through modulation of cellular signaling cascades.

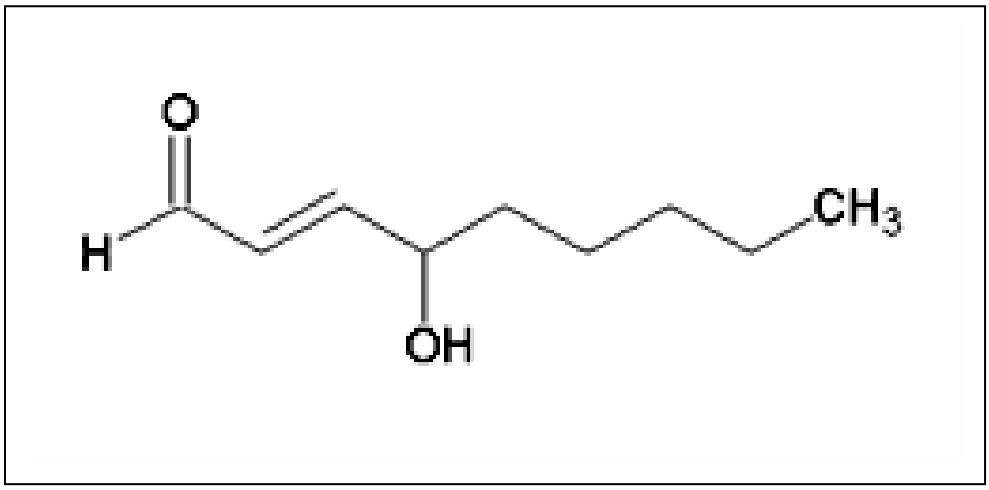


Figure 2.6 Structure of HNE.

2.1.5.2 Basic Chemistry of 4-Hydroxynonenal

HNE was initially identified and characterized by Hermann Esterbauer's group [136]. HNE is a degradation product of hydroperoxides of n-6 polyunsaturated fatty acids such as linoleic acid, linolenic acid and arachidonic acid (Figure 2.6). Physical and chemical properties of HNE are summarized in Table 2.2. Studies with hydroperoxides of linoleic acid have demonstrated that 9(*S*)-hydroperoxy-octadecadienoic acid (9(*S*)-HPODE) may decompose into HNE through a three-step reaction [137]. 9(*S*)-HPODE cleaves into nonenal and 9-oxo-nonaic acid and peroxidation of nonenal in the position 4 results in hydroperoxynonenal (HPNE). The hydroperoxy group of HPNE can then be reduced to form HNE.

- Molecular formula: $C_9H_{16}O_2$
- Molecular weight: 156.22
- FW: 156.2
- colorless liquid
- soluble in most organic solvents, e.g. alcohols, hexane, chloroform
- slightly soluble in water (6.6 g/L = 42 mM)
- UV maximum
  223nm, *e* 13750 (water)
  221nm, *e* 13100 (ethanol)
  215nm, *e* 14400 (hexane)

Table 2.2 Some physical and chemical properties of HNE

The basic chemical structure of HNE can be defined as a trifunctional molecule: a hydroxyl group, C=C double bond and a carbonyl group, all contribute to the high reactivity of HNE. HNE mainly reacts with thiol and amino groups, primarily with cysteine, histidine and lysine residues through formation of Michael adducts to the C=C bond (Figure 2.7).

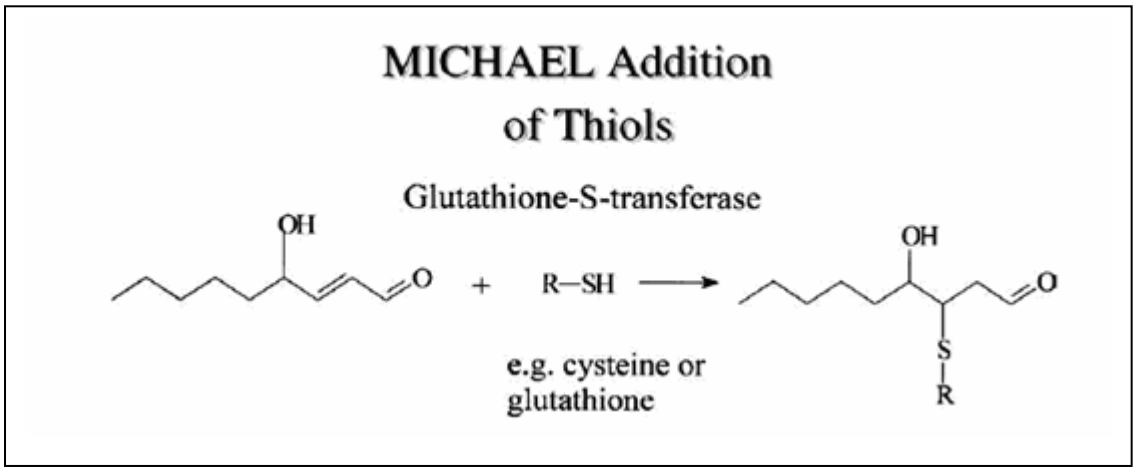


Figure 2.7 Michael addition of thiols.

C2-C3 bond becomes more flexible and further reactions involving the carbonyl and the hydroxyl group may occur. Primary amines may react with the carbonyl group and form Schiff bases (Figure 2.8).

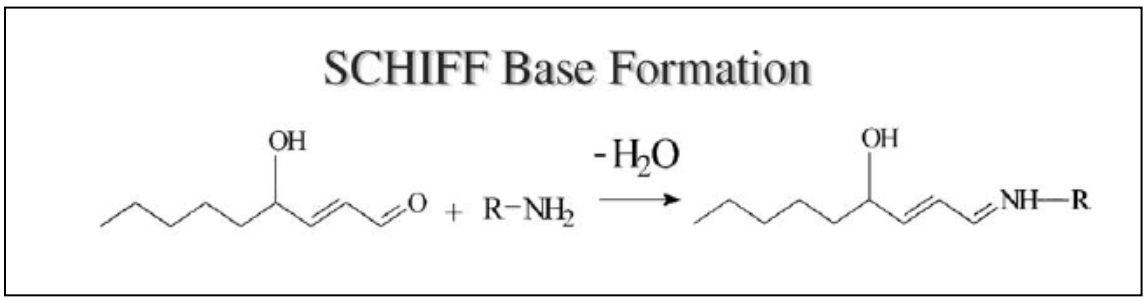


Figure 2.8 Schiff base formation by HNE.

There are also oxidation/reduction of carbonyl group and the epoxidation of the C=C double bond among the possible chemical reactions of HNE. When we consider the chemical structure of HNE, it is clear that the lipophilic properties are more

important than its hydrophilic properties and HNE have a tendency to concentrate in various biomembranes. HNE is also genotoxic at micromolar concentrations, which is exhibited by elevated chromosomal aberrations and formation of micronuclei [138]. The most common way for the formation of HNE-adduct is direct interaction with guanosine moieties of DNA, which are also observed in human and rodent genomes [138]. HNE treatment also results in the inhibition of DNA, mRNA and protein synthesis in various cell lines [139].

2.1.5.3 Cellular Metabolism of HNE

Lipid peroxidation is combined with the formation of reactive aldehydes in most of the cases; HNE is one of the most important of these aldehydes. HNE undergoes several reactions with cellular macromolecules (proteins, peptides, phospholipids, nucleic acids) and mammalian cells have many active pathways of HNE metabolism. The metabolic pathways of HNE have been investigated in various mammalian cells and organs, such as endothelial cells, fibroblasts, tumor cells and heart [140]. The physiological blood serum level of HNE was determined as 0.1-10 μM and HNE concentrations have been significantly elevated in tissues under oxidative stress [141,142]. Thereby, the organism utilizes highly effective mechanisms to prevent the accumulation of this highly reactive substance. HNE is mainly removed by its intracellular metabolism. In rat hepatocytes at pH 7.4 and 37 °C, nearly 95% of 100 μM HNE was shown to degrade within 3 min of incubation, presumably due to higher metabolism by glutathione-dependent enzymatic meachanisms (Figure 2.9) [140]. The degradation of HNE was also shown to be dependent on the cell concentration and initial HNE concentration. The main biochemical reactions of HNE has been demonstrated to lead to the formation of alcohol 1,4-dihydroxynonene, 4-hydroxynonenoic acid, and conjugated products such as aldehyde-glutathione conjugate. The corresponding HNE-metabolizing enzymes catalyze these reactions: alcohol dehydrogenases (NADPH-dependent reduction of the carbonyl group), aldehyde dehydrogenases (NAD^+-dependent oxidation of the carbonyl group) and glutathione S-transferases (Michael addition of thiols). Glutathione S-transferases (GST) and aldeyhde dehydrogenases are present in almost all mammalian cell types. GST activity is much faster than non-enzymatic Michael addition reaction.

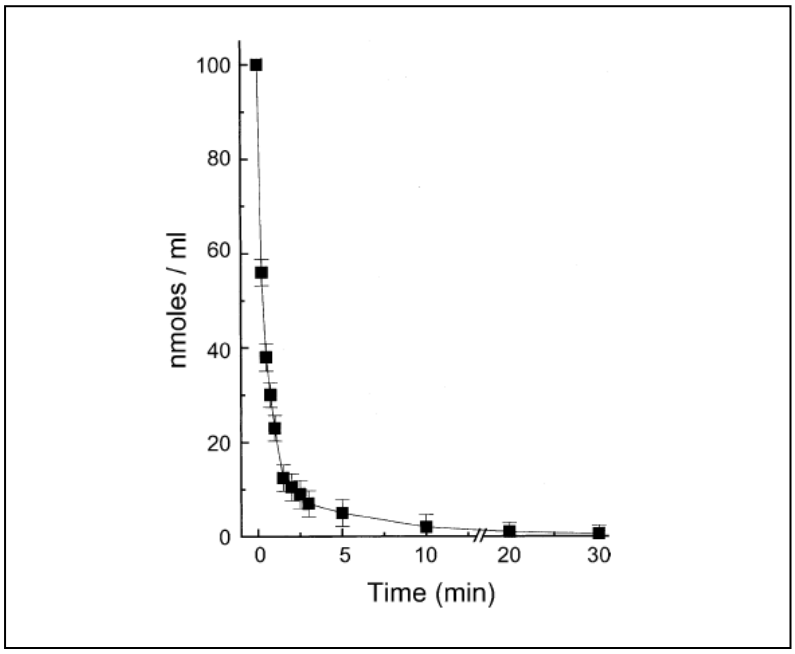


Figure 2.9 The typical degradation profile of HNE in mammalian cells [140].

Increased HNE levels or addition of HNE into the medium results in a rapid decrease of intracellular GSH levels and one of the main primary metabolites of HNE is the HNE-GSH adducts [143]. After this rapid and transient GSH decrease, synthesis of GSH is induced through activation of glutamate cysteine ligase (GCL), which catalyzes the initial step in de novo synthesis of GSH. Exposure of rat L2 cells and human HBE1 cells to HNE causes an increase in GSH biosynthesis, which may be responsible for the cellular protection against HNE-induced cytotoxicity [144,145]. An increase in the intracellular content of GSH is a result of an effort to balance between loss through use and *de novo* GSH synthesis. This induction could be related to either direct conjugation or depletion of GSH or activation of MAP kinases and AP-1. In rat L2 cells, activation of ERK 1/2 and JNK pathways has been shown to be involved in HNE-mediated signaling for GSH synthesis [144]. In contrast, there are also studies, which could not demonstrate the involvement of MAP kinases in GSH synthesis [145]. Thereby, the exact mechanism behind the induction of GSH by HNE remains to be identified. Furthermore, the total rate of HNE formation and degradation also depends on the ratio between oxidative and reductive pathways of the HNE metabolism in different cellular conditions and oxygenation/reperfusion. In spite of the rapid response against HNE accumulation via cellular metabolic/protective pathways, protein modification by HNE

could be detected in many experimental systems. HNE protein binding has been shown to be in the range between 1% and 8.5% of total cellular proteins [146]. Many different cellular proteins such as Na+-K+-ATPase, PARP, Complex I, MAP kinases and PKCs have been shown to be modified by HNE and the result was inhibitory in most of the cases [146]. The degradation of oxidized and oxidatively modified proteins is an essential component of cellular defense system. The fate of a HNE-modified protein is either degradation by the 20S proteosome pathway or cross-linking and accumulation [146]. Both of the pathways may lead to pathophysiological sequences of events, leading impairment of cellular and organ dysfunction. Nevertheless, intracellular HNE-metabolizing/degrading defense systems are critical for protection of proteins and other macromolecules from modification by aldehydic lipid peroxidation products.

2.1.5.4 HNE and cellular signaling

The manipulations of cellular signaling pathways by lipid peroxidation have been described above. HNE is one of the most abundant aldehydic components of ox-LDL and it exerts similar effects on intracellular and extracellular signaling cascades [1,2]. A brief synopsis of HNE-modulated signaling pathways is shown in Table 2.3. Here a short comprehensive summary of HNE-induced signaling pathways mainly focused on cell survival and cell death will be given. Protein kinases C are variously distributed protein kinases, which are differentially involved in signal transduction, differentiation and apoptosis [147]. Recent data has demonstrated that PKCs are among a group of cell-signaling molecules that are modulated by redox modification and functional alterations that mediated stress-mediated cellular responses [147]. HNE has been shown to activate phospholipase C, which results in an increased production of inositol triphosphate (IP3) and diacylglycerol (DAG) as well as protein kinase C activation [148]. Interestingly, subtypes of protein kinase C is differentially regulated by HNE. HNE treatment has been shown to induce a decrease in PKCα activity in a dose-dependent manner [149]. By contrast, its effect on PKCβ-I and PKCβ-II was biphasic and dependent on HNE concentration [150]. HNE was also shown to stimulate cathepsin D intracellular transport and secretion in isolated rat hepatocytes and enhanced production of amyloid-β protein in neuronal cells [151]. HNE at similar concentrations that activate JNK, also markedly activated novel PKC isoforms, in

particular δPKC in hepatocytes [152]. Through this effect, HNE has been shown to activate AP-1, which could be blocked by a specific novel PKC inhibitor, rottlerin.

Tyrosine kinase receptors (RTK) such as EGFR and PDGFR are also among the cellular targets of HNE. In intact living cells, both ox-LDL and HNE has been shown to trigger HNE-adduct formation and activation of PDGFR and EGFR [130]. HNE-PDGFR adducts have also been detected in atherosclerotic lesions, which may be involved in SMC migration and proliferation in vascular intimal area and synthesis of matrix molecules in the formation of atherosclerotic cap [153]. HNE at 1 μM concentration induced EGFR and PDGFR autophosphorylation, recruitment of SH2 domains to phosphorylated receptor and activation of downstream signaling cascades such as MAP kinases. HNE has also been reported to induced EGFR signaling and receptor clustering along with apoptosis, when used at higher concentrations [154]. Moreover, modulation of HNE-induced tyrosine kinase activities at the level of kinase level by phenolic compounds has been demonstrated [155]. Mitogen-activated protein kinase cascades are pivotal mediators in cell signaling and HNE-induced upstream pathways may lead to downstream activation/inhibition of both ERK 1/2 and other two stress-activated protein kinases (JNK and p38) [2,3]. HNE may also induce c-Fos or c-Jun gene expression and consequent activation of AP-1 downstream of MAP kinases. HNE has been shown to activate AP-1 in PC12 pheochromacytoma cells through activation of JNK without concurrent p38 activation [156]. In rat epithelial cells, HNE induced both JNK and p38 activation and in vascular smooth muscle cells HNE has been shown to activate all three kinases and c-Fos activation [157].

Regarding another important redox-sensitive transcripition factor, NF-κB, HNE did not seem to influence its activation and nuclear translocation by when utilized alone [158]. In contrast, HNE has also been shown to activate NF-κB and to induce apoptosis in vascular smooth muscle cells [159]. Interestingly, HNE has been shown to counteract the lipopolysaccharide-induced NF-κB activation in a macrophage cell lineage via inhibiting IKK signalsome pathway [160]. All these data confirm the complex and cell-dependent integration of signaling pathways activated by HNE.

HNE as a signal for biological functions

(I) Enzymatic activities/functions

- Stimulation of chemotaxis
- Activation of adenyl cyclase
- Activation of phospholipase C
- Activation of AP-1 binding
- Activation of JNK
- Activation of PKCβI and βII
- Stimulation of caspases
- Inhibition of PDGFβR tyrosine kinase

(II) Stimulation of gene expression

- Heat shock proteins (HSP 70)
- Procollagen A1
- Aldose reductase
- Heme oxygenase
- TGFβ1
- c-Glutamyl cysteine synthetase
- MCP-1
- β-secretase (BACE)
- TIMP-1

(III) Modulation of proto-oncogenes, cell cycle and proliferation

- Inhibition of c-Myc mRNA
- Inhibition of c-Myb mRNA
- Block of cell cycle in G0/G1
- Stimulation of c-Jun mRNA
- Stimulation of growth of vascular SMCs
- Stimulation of cyclins D1and D2
- Modulation of pRb/E2F pathway

Table 2.3 Cellular signaling pathways regulated by HNE**.**

2.1.6 Resveratrol

Phytochemicals are compounds found widespread in plants, which have beneficial effects on human health and have potential chemoprevention properties. The main families of phytochemicals involve flavonoids, phytoestrogens, isothiocyanates, monoterpenes, organosulfur compounds, saponins, capsaicin, and sterols. Recent molecular and cellular studies have focused on the beneficial effect of dietary phytoestrogens in reducing the risk of cardiovascular diseases, diabetes and cancer. Phytoestrogens are polyphenolic non-steroidal molecules acting as functional analogs of mammalian estrogen 17-β-estradiol (E2) and they have potential applications in hormone therapy to prevent menopausal syndromes and osteoporosis [161,162]. Resveratrol (trans-3,5,40-trihydroxystilbene) has been identified as the major active compound of the stilbene phytoestrogens (Figure 2.10) [163]. It exists as trans and cis isomers, however only the trans form of resveratrol has been reported to be estrogenic. The inverse relationship between risk of ischemic heart disease and French-pattern dietary intake, moderate consumption of red wine and consumption of foods high in saturated fats raised a wave of interest in monitoring the presence of resveratrol in red wine [164]. Resveratrol is found only in skin of grapes, not in grape flesh, making this stillbene the active ingredient of red wine but resulting in low levels in white wine [164]. The gastrointestinal absorption, distribution and metabolism of resveratrol have been evaluated. The bacterial microflora in the ileum and cecum plays an important role in the metabolism and absorption of resveratrol. Kinetics of trans- and cis-resveratrol (3,4',5-trihydroxystilbene) after oral administration of red wine in rats showed that a fraction of resveratrol (6.5 mg/l) was absorbed by rats and can be detected in considerable concentrations in plasma [165].

Resveratrol has been shown to protect against reactive oxygen species (ROS). Reactive oxygen species such as hydroxyl radical ($^{\cdot}OH$), superoxide radical ($O2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are generated as by-products of molecular cellular events and involved in initiation of oxidative stress. Antioxidants provide a redox balance and protect the cellular structures and macromolecules against ROS-induced damage. Nevertheless, when the defense system is deranged due to excess generation of ROS, cell injury or even cell death may occur due to oxidative damage to DNA, proteins and lipids.

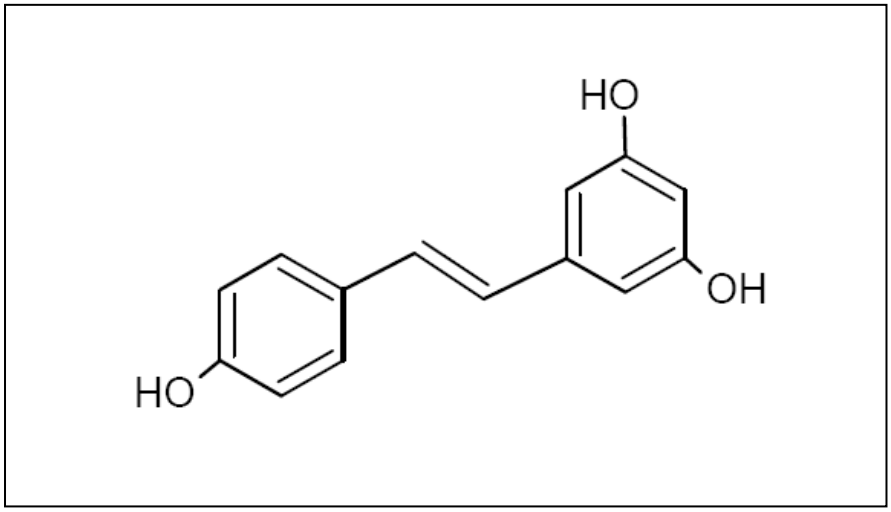


Figure 2.10 Structure of resveratrol.

Resveratrol has been shown to inhibit the pro-oxidant effect of tert-butyl hydroperoxide by scavenging of radicals in rat embryonic mesencephalic cells [166]. Furthermore, resveratrol has been demonstrated to exert an inhibitory effect on LPS-induced superoxide radical and hydrogen peroxide production by macrophages [167]. Regarding the proposed role of ox-LDL in the development of atherosclerosis, studies have been utilized to evaluate the role of resveratrol on inhibition of LDL oxidation and atherosclerosis. A 45% decrease in plasma LDL concentration, and a 25% reduction in plasma cholesterol levels was detected in hyperlipidermic hamsters fed with red wine [168]. Resveratrol has been shown to decrease VLDL/LDL and triglyceride levels in rats with hypercholesterolemia and hypertriglyceridemia, when orally administrated to these animals. Resveratrol has also been shown to inhibit the peroxidation of membrane lipids that destroys the phospholipid bilayer of the cellular membrane resulting in irreversible cell damage [169]. In human primary cell cultures and rat liver microsomes, it has been demonstrated that resveratrol inhibited the lipid peroxidation induced by free radical donors [170]. In addition to inhibition of LDL and cell membrane oxidation, resveratrol can protect cells against the cytotoxic effect of ROS by increasing their resistance [171].

Resveratrol was shown to exhibit protective effects against H_2O_2- and β-amyloid-induced oxidative stress and cell death in rat pheochromocytoma cells by increasing the

resistance of these cells to oxidative stress and cell death by attenuating intracellular ROS accumulation and restoring the levels of some marker proteins of apoptosis such as Bax, Bcl-x_L, JNK, and PARP [172,173]. Furthermore, resveratrol was also shown to inhibit TNFα- and LPS-induced activation of NF-κB binding and subsequent apoptosis in human myeloid cells [174]. The molecular mechanism of NF-κB inactivation by resveratrol is through inhibition of IKK signalsome and IκB-α phosphorylation and degradation.

Resveratrol is effective in blocking, reversing or retarding the process of carcinogenesis [175]. In this context, resveratrol functions as a cancer chemopreventive agent, which has been shown to inhibit the ribonucleotide reductase, which is involved in the cell proliferation and cell cycle progression [176]. In several human cancer cell lines, resveratrol has been demonstrated to induce cell cycle arrest and induced apoptosis [175]. The resveratrol-mediated cell death involved increased caspase-activity and Bax levels, and decreased Bcl-2 and Bcl-x_L levels [177]. The modulation of cellular signal transduction pathways by naturally occurring substances such as resveratrol has been recognized as a novel therapeutic approach against human diseases

2.1.7 Apoptosis in disease pathogenesis

Over the past decade, our understanding of apoptosis, or programmed cell death, has increased greatly, with the identification of some of the major components of the apoptotic program and the processes regulating their activation. Although apoptosis is an intrinsic process present in all cells, it can be regulated by extrinsic factors, including growth factors, cell surface receptors, cellular stress and hormones. In a simplified vision, the diseases in which apoptosis has been involved can be divided into two groups: those in which there is an increase in cell survival (or diseases associated to inhibition of apoptosis), and those in which there is an increase in cell death (diseases associated to increased apoptosis).

2.1.7.1 Diseases associated with inhibition of apoptosis

This group of diseases includes those diseases in which an excessive accumulation of cells occurs due to increased cell proliferation. Recently, it has been demonstrated that defective apoptosis is also a major part of excessive accumulation of cells. The role of apoptosis in some of these diseases will be described in this section (Table 2.4).

2.1.7.1.1 Cancer

In both solid and hematological tumors, it has been demonstrated that the malignant cells display an abnormal response to the induction of apoptosis. It has been demonstrated that in neoplasias, cell cycle regulator genes/oncogenes such as *p53, ras or c-myc* suffer mutations, inactivation or deregulations associated to malignant transformation; these genes also regulate apoptosis in those cells [178]. Further genes, such as *bcl-2*, also overexpressed in tumour cells, and the expression of *bcl-2* is currently considered a predictive factor for worse prognosis in prostate, breast and colon cancer and in neuroblastomas [179,180]. Further to the studies that reveal a correlation between apoptosis inhibition and malignant transformation, it has been also shown that resistance to apoptosis in tumor cells also contributes to chemotherapy resistance and tumor escape mechanisms [181].

Diseases associated to apoptosis	
Through excess apoptosis	**Inhibition of apoptosis**
AIDS	
T lymphocytes	Cancer
Neurodegenerative diseases	Colorectal
Alzheimer's disease	Glioma
Amyotrophic lateral sclerosis	Hepatic
Parkinson's disease	Neuroblastoma
Retinitis pigmentosa	Leukaemias and lymphomata
Epilepsy	Prostate
Haematologic diseases	Autoimmune diseases
Aplastic anaemia	Myastenia gravis
Myelodysplastic syndrome	Systemic lupus erythematosus
T CD4+ lymphocytopenia	Inflammatory diseases
G6PD deficiency	Bronchial asthma
Tissue damage	Inflammatory intestinal disease
Myocardial infarction	Pulmonary inflammation
Cerebrovascular accident	Viral infections
Ischaemic renal damage	Adenovirus
Polycystic kidney	Baculovirus

Table 2.4 Diseases related to apoptosis

2.1.7.1.2 Autoimmune diseases

The apoptotic death of clones of auto-reactive lymphocytes or of lymphocytes, which are not effective in the immune response, has been shown to be fundamental in the development of the immune system. Furthermore, it has been demonstrated that autoimmune diseases, such as systemic lupus erythematosus, are associated to mutations in genes rendering the lymphocytes resistant to apoptosis [182]. Normal thyrocytes could induce apoptosis of infiltrating activated T cells and protect against T cell-mediated apoptosis. In Hashimoto's thyroiditis (HT), Fas-mediated apoptosis of thyroid cells due to at least two distinct mechanisms, the first by infiltrating activated T cells,

and the other by FasL-positive thyroid cells in a suicidal fashion [183]. A common feature of autoimmune diseases such as systemic lupus erythematosus (SLE) is the breakdown of tolerance of self-antigens, a consequence of which is the production of autoantibodies reactive with multiple self-proteins [182]. Evidence is accumulating that modifications of autoantigens during apoptosis lead to the development of autoantibodies by bypassing the normal mechanisms of tolerance. Tissue homeostasis is maintained through a balance between cell proliferation and apoptotic cell death. Rheumatoid arthritis (RA) is characterized by pronounced hyperplasia of the synovial tissue, cell infiltration and periarticular osteoporosis. Enhanced Bcl-2 expression and NF-κB nuclear translocation of synovial cells are induced by inflammatory cytokines [184]. These synovial cells become resistant toward apoptosis triggered by various stimuli. The infiltrated cells, which are defective in activation-induced cell death, can cause autoimmunity by allowing the survival of autoreactive T and B cells [184]. These data suggest that apoptosis might be implicated with the pathogenesis of autoimmunity, whereas the mechanisms might be distinct in each autoimmune disease

2.1.7.2 Diseases associated with increased apoptosis

Increased cellular apoptosis has also been implicated in the pathogenesis of a number of diseases, which are characterized by excessive cellular damage and loss of physiological homeostasis.

2.1.7.2.1 Neurodegenerative diseases

Neurodegenerative diseases are characterized by extensive apoptosis of the neurons [185]. Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and retinitis pigmentosa are among the most common neurodegenerative disorders. Increased neurological susceptibility to apoptosis neuronal cells in Alzheimer’s disease has been shown to be related to oxidative stress, mitochondrial insults and neurotoxins [186].

2.1.7.2.2 Hematological disorders

Mature blood cells are derived from hematopoietic precursors located in the bone marrow. Haematopoiesis, and hence the presence of mature cell forms, is regulated by a number of hematotrophic factors (erythropoietin, colony stimulating factors, cytokines). Hematological turnover is a dynamic process in which the number of mature forms in peripheral blood regulates the generation of new cells. The presence of altered trophic factors, or of abnormal levels of normal hematotrophic factors, would favour the accumulation of immature cell forms, which would not be effectively regulated in the peripheral blood and excessive apoptosis [187].

2.1.7.2.3 Ischemia-reperfusion

In myocardial infarction, ischemic renal damage or cerebrovascular events, the cells in and around ischemic zone are lost through apoptosis. Alterations in the biochemical metabolism and pro-apoptotic signaling pathways accompany the ischemic process. However, in these diseases oxygenation/reperfusion also induces further apoptosis, as it is associated to a sudden increase in reactive oxygen radicals that could also induce apoptosis in these cells [188].

2.1.7.2.4 AIDS

The $CD4^+$ cells of the HIV (+) patients die through apoptosis when stimulated *in vitro*. Moreover, HIV infection of cells from healthy subjects induces apoptosis of CD4+ cells [189]. However, further to this, not only the infected cells but also non-infected cells undergo apoptosis. There is a large number of studies demonstrating that cell apoptosis plays a relevant role in the etiology of many diseases, and that a wide range of pharmacologic agents (cytotoxic agents, hormones, anti-inflammatory drugs) are effective through inducing apoptosis of target cells. Nevertheless, it appears to be evident that the future of such studies should be aimed both at documenting new associations between apoptosis and disease and at developing new therapies based on the modulation of apoptosis.

2.1.7.2.5 Atherosclerosis

Atherosclerosis and its complications such as acute myocardial infarction and stroke are the leading cause of mortality and morbidity in industrialized and developing countries. The pathophysiology of atherosclerosis is characterized by an initial fatty streak formation, which progresses by alteration of endothelial function, expression of adhesion molecules, inflammatory response as well as lipid retention/oxidation and engulfment by macrophages, which forms one of the most characteristic hallmarks of atherosclerosis: foam cells [127]. The cell proliferation, apoptosis and migration contribute to the progression of atherosclerotic lesion. The proliferation and intimal migration of vascular smooth muscle cells results in increased synthesis of collagen and formation of fibrous plaques around lipid cores, which are mainly formed by lipid-laden macrophages. In advanced lesions, the central parts of these lipid cores become highly instable because of the necrosis and apoptosis of cellular structures of the lipid core [127]. The rupture of the fibrous plaque occurs usually at the shoulder region of the lesion, which is followed mostly by formation of a thrombus and increased risk of acute coronary syndromes and stroke. The progression of an atherosclerotic lesion is evolutionary and chaotic, but we can describe the pattern of lesion development into two main phases with overlapping characteristics. The first phase is defined by endothelial dysfunction and inflammation with prominent lipid retention but minimal lipid peroxidation. This pro-inflammatory microenvironment predominantly provokes a proliferative response for vascular smooth muscle cells, which is followed by intimal migration and neointima formation. It has been also speculated that endothelial cell apoptosis has a role in development of endothelial cell dysfunction and atherosclerotic lesion areas show extensive endothelial cell turn-over with dysfunctional endothelial cells. [190]. Thereby, functional involvement of apoptosis in the first phase of lesion development targets two cell types: a preferential pro-apoptotic stimulation for endothelial cells and an anti-apoptotic and proliferative stimulation for smooth muscle cells. The second phase of lesion progression involves increased inflammatory response and lipid retention/peroxidation, which trigger the formation of oxidized low density lipoprotein (ox-LDL) particles in the vascular wall, alteration of redox balance and modification of cellular proteins, DNA and lipids [191]. This leads to plaque development and stabilization of the plaque by extracellular matrix and cellular support formed by vascular smooth muscle cells. In more advanced lesions extensive apoptosis

of cells, which form the plaque (lipid-laden macrophages and SMCs), may lead to thinning of fibrous support, plaque destabilization, rupture and thrombosis, which may result in clinical presentation of the lesion such as acute myocardial infarction [192,193].

In this study, our aim was to identify the HNE-induced apoptosis signaling pathways in 3T3 fibroblasts and the molecular signaling targets of resveratrol in these signaling cascades. The fact that that apoptosis is a major cellular event in disease pathogenesis underlines the importance of understanding signaling pathways in apoptotic machinery and development of small molecule approach targeting these signaling cascades.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Materials

All chemicals and growth mediums used in this study were purchased from SIGMA (Darmstadt, Germany) otherwise indicated.

3.1.1 Chemicals and antibodies

Chemicals and antibodies that are used are listed in Appendix A.

3.1.2 Molecular biology kits

Molecular biology kits that are used for DNA isolation, caspase activation, apoptosis screening, gene transfection, plasmid isolation and protein analysis are listed in Appendix B. Other specialty materials including DNA and protein markers are indicated in Appendix C.

3.1.3 Equipment

Equipment that is used for general laboratory procedures are listed in Appendix D.

3.1.4 Radioactivity

Radioactivity has been used under international radioprotection regulations. Adenosine 5'-[γ-^{32}P] triphosphate with a specific activity of 3000 Ci/mmol was obtained from Izotop (Hungary).

3.1.5 Buffers and solutions

Standard buffers and solutions used in cloning and molecular manipulations were prepared according to the protocols in *Cell Proliferation and Apoptosis*, Mehmet et al, 2004 and *Molecular Cloning: A Laboratory Manual*, Sambrook et al., 2001.

3.1.6 Buffer for agarose gel electrophoresis

1 X TAE (Tris-EDTA-Acetate) buffer was used for preparation of 1% and 1.5% agarose gels. Gels were run at 100mV for 45 minutes. DNA was visualized by including 0.005% ethidium bromide in the gel during its preparation.

3.1.7 Buffer for SDS polyacrylamide gel electrophoresis

1X Tris-Glycine-SDS (sodium dodecyl sulfate) buffer was used for polyacrylamide gel electrophoresis. Gels were run at constant voltage, 60 mV, for about 2 hours.

3.1.8 Oligos and plasmids

AP-1 TRE consensus oligonucleotides were obtained from Promega, Madison, WI, USA. The oligonucleotide sequences are as follows; 5´-CGC TTG ATG AGT CAG

CCG GAA-3′ and 3′-GCG AAC TAC TCA GTC GGC CTT-5′. The maps of pcDNA3 and pcDNA1 vectors are given in Appendix C.

3.1.9 Buffers for Western Blotting

Transfer buffer (Tris base, Glycine and methanol) was used for blotting the the proteins into PVDF membrane. The membranes were blocked with blocking solution, 5% milk powder in PBS-Tween 20 (0,25%) and washed with washing buffer, PBS-Tween 20 (0,25%). The antibodies were diluted in 10% milk diluent, 10% PBS-Tween 20 (0,25%) and 80% sterile distilled water.

3.2 Methods

All methods used in this study are explained below;

3.2.1 Cell Culture

Swiss 3T3 fibroblasts were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM Glutamine, 100 IU/ml penicillin and streptomycin. Cultures were maintained in 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded in 6-well culture plates (1 x 10^6 cells/well), 60 mm culture flasks (1x 10^7 cells/well) or 96-well plates (1 x 10^4 cells/well) and treated as indicated in the experimental protocols. Ethanol $\leq$ 0.05%, v/v) was added to all control wells in each experiment. For cryopreservation, cells were trypsinized and resuspended in complete medium containing 10% heat-inactivated FBS and 10% DMSO (freezing medium). The cell suspension in freezing medium transferred into cryovials, frozen at -70 º C for 24 hours, and then stored in liquid nitrogen to remain until thawing.

3.2.2 Protein isolation

3.2.2.1 Total protein isolation

Treated and control 3T3 fibroblasts were harvested, washed with ice-cold phosphate buffered saline and lysed on ice in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, Nonidet P-40 0.5%, (v/v), 1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, protease inhibitor cocktail (Complete from Roche, Mannheim, Germany) and phosphatase inhibitors (Phosphatase inhibitor cocktail 1 and 2, Sigma, Darmstadt, Germany). After cell lysis, cell debris was removed by centrifugation 15 min at 13000***g*** and protein concentrations were determined with Bradford protein assay.

3.2.2.2 Nuclear and cytoplasmic protein isolation

Nuclear and cytoplasmic proteins were isolated as described before with minor modifications [194]. Briefly, cells were treated as indicated and washed with ice-cold PBS, then scraped and harvested by centrifugation. They were resuspended in 1 ml of cold PBS and transferred to 1.5-ml Microfuge tubes. After centrifugation at 300***g*** for 30 s, cells were lysed by incubation for 10 min in 200 µl of cold hypotonic buffer [10 mM Hepes/KOH, (pH 7.9), 10 mM KCl, 2mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, protease inhibitors and Nonidet P-40 (0.2%)]. After centrifugation at 13000***g*** for 30 s, supernatants containing cytoplasmic proteins were removed and stored at -70°C. The nuclear pellet was washed and nuclear protein isolation was carried out by incubation for 20 min on ice in a cold saline buffer [20 mM Hepes/KOH (pH 7.9), 1.5 mM MgCl2, 0,2 mM EDTA, 650 mM NaCl, glycerol (25%, v/v), 1 mM DTT, 0.5 mM PMSF and protease inhibitors. After centrifugation at 13000***g*** for 20 min at +4°C, supernatants containing nuclear proteins were removed and stored at -70°C. Protein concentrations were determined by Bradford reagent (Bio-Rad, Munich, Germany).

3.2.2.3 Mitochondrial protein subfractionation

3T3 fibroblasts cells were seeded in 6-well plates (1x 10^6 cells/well) and after indicated treatments, cells were harvested, washed once with phosphate-buffered saline (PBS) and lysed for 30 s in 100 μl ice-cold lysis buffer [250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris, pH 6.8, 1 mM dithiothreitol (DTT), 0.1 mM PMSF and protease inhibitor cocktail (CompleteMini, Roche, Germany)]. Cell lysates were centrifuged at 13000***g*** at +4ºC for 5 min and supernatants (mitochondria-free cytosolic extracts) and the pellets (mitochondrial fraction) were separately obtained.

3.2.3 Electromobility Shift Assay (EMSA)

The gel shift method was performed as described before [194]. Briefly, the oligonucleotide probe was labeled with γ-^{32}P-dATP (3000 Ci/mmol) using T4 polynucleotide kinase and then labeled oligonucleotide was purified on a Sephadex G-25 column**.** Five μg of nuclear proteins were incubated for 20 min at room temperature with 0.2 ng of 32P-labelled oligonucleotide probe in gel shift binding buffer 1.25 μg of BSA and 1.25 μg of poly (dI-dC).poly (dI-dC) in 20 mM Hepes/KOH, 75 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM MgCl2, 1 mM DTT (pH 7.9) (final volume 10 μl). DNA-protein complexes then resolved on a non-denaturating 6% polyacrylamide gel run for 3 h at 180 V. The gel was then dried and autoradiographed on Kodak X-ray film.

The consensus sequences of the oligonucleotides used in this work were 5'-CGCTTGATGAGTCAGCCGGAA-3' and 3'-GCGAACTACTCAGTCGGCCTT-5'. For competition experiments unlabelled probe was added in excess (50x) in the binding buffer and for supershift experiment incubation with anti-c-Jun and anti-c-Fos antibodies was performed before addition of ^{32}P-labelled oligonucleotide probe into the binding buffer.

3.2.4 Immunoblots

Proteins (40 μg) were separated on a 10-15% SDS-PAGE and blotted onto PVDF membranes. The membranes were then blocked with 5% dried milk in PBS-Tween20

and incubated with appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech, Freiburg, Germany) in antibody buffer containing 10% (v/v) Milk Diluent/Blocking concentrate. After required washes with PBS-Tween 20, proteins were finally analyzed using an enhanced chemiluminescence detection system (ECL-Plus, Amersham Pharmacia Biotech, Freiburg, Germany) and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech, Freiburg, Germany).

3.2.5 Apoptosis and cell death

3.2.5.1 MTT assay

Cell death was determined using an MTT assay kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, Swiss 3T3 cells in 96-well plates were treated as indicated and 10 µl of MTT labeling reagent was added to each well, after which the plates were incubated for 4 h. The cells were then incubated in 100 µl of the solubilization solution for 12 h, and the absorbance was measured with a microtiter plate reader (Bio-Rad, CA, USA) at a test wavelength of 550 nm and a reference wavelength of 650 nm. Percent viability was calculated as (OD of drug-treated sample/control OD) X 100.

3.2.5.2 Crystal violet assay

Cell viability was measured by crystal violet assay, which is based on preservation of crystal violet dye by healthy cells. Briefly, after treatments as indicated in the legends, cells were washed with phosphate buffered saline, fixed with methanol for 5 min and stained for 10 min with 1.5% crystal violet solution. The dye was eluted with 0.1 mol/l trisodium citrate and in ethanol for 1 hour and optical density was measured at 630 nm. The data were expressed as mean ± SEM of untreated control.

3.2.5.3 Triple staining microscopy

For the determination of apoptosis using triple staining, cells were grown on glass bottom dishes in DMEM with 10% FBS. After confluency, cells were incubated with DMEM plus 0.2% FBS for 12 hours. After indicated treatments, cells were washed 3 times with PBS and then fixed with 2% paraformaldehyde and stained with Acridine Orange (AO), Propidium Iodide (PI) and Hoechst dye (HO) and cell death/apoptosis were assessed using fluorescence microscopy. At least 400 cells were counted in 5 high-power fields using Olympus BX51 microscope and data were expressed as expressed as mean ± SEM of control.

3.2.5.4 Cell Death Detection ELISA^{PLUS}

For evaluation of apoptosis, a Cell Death Detection ELISA^{PLUS} kit (Roche, Mannheim, Germany) was used according to the instructions of the manufacturer. This kit detects cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death. Briefly, cells were washed with PBS and incubated with 200 µl of lysis buffer for 30 min and centrifuged for 10 min at 300***g***, and 20 µl of the supernatant (cytoplasmic fraction) was assayed in the ELISA. The reaction was developed with a peroxidase system, and development of color was measured with a microtiter plate reader (Bio-Rad, Hercules, CA, USA) at a test wavelength of 405 nm and a reference wavelength of 490 nm. Results were determined as fold increases in absorbance over untreated control cells (enrichment factor).

3.2.5.5 Fluorometric caspase assay

The enzymatic activity of caspase-3 and caspase-9 was determined by using a caspase activation assay kit (Sigma, Darmstadt, Germany). 3T3 fibroblasts were treated as indicated, washed twice with ice-cold PBS and then resuspended in lysis buffer [250 mM HEPES, pH 7.4, 25 mM CHAPS, 25 mM DTT]. After 15 minutes of incubation on ice, samples were centrifuged for 10 minutes at 10000***g*** at 4ºC, supernatants were collected and protein concentrations were determined by Bradford protein assay. Ten µg of protein were assayed in 200 µl of reaction solution containing Ac-DEVD-AMC for

caspase-3-like DEVDase activity and Ac-LEHD-AMC for caspase-9 activity. The released fluorescent AMC was monitored at an excitation of 360 nm and emission of 460 nm using a Spectramax Gemini XS multiplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA). Results were calculated from a standard curve of AMC and specific caspase activities were derived as mean relative fluorescence units (RFU)/mg protein. The specificity of fluorometric caspase assays were always checked by inhibitor studies and internal positive controls. Data shown are mean ± SEM of three independent experiments performed in triplicate.

3.2.6 Transfections

Plasmid containing dominant form of c-Jun, pcDNAFLAG-D169 (c-JunD169; DN-c-Jun) was kindly provided by Dr. J. Ham (University College London, UK) and was described before [121]. The DN-JNK1 [pcDNA3-Flag-JNK1-APF] construct was kindly provided by Dr. R. Davis (Howard Hughes Medical Institute, Cambridge, MA, USA) and has the tyrosine 185 and threonine 183 amino acids, which require phosphorylation for activity replaced with alanine and phenylalanine, respectively. The empty pcDNA1 and pcDNA3 vectors were used as mock transfections and were purchased from Invitrogen (Gronmgen, the Netherlands). For transient transfections, Swiss 3T3 fibroblasts were plated in 60-mm dishes and transfected with plasmids (2–4 μg) using Lipofectamine Plus reagent (Gibco Europe, Breda, the Netherlands) according to the manufacturer's recommendations. The transfection medium was removed and replaced with fresh culture medium after 4 h, and the cells were incubated for another 16 h prior to treatments. The transfection efficiency was monitored via detection of FLAG-tag by immunofluorescence and immunoblot analysis.

3.2.7. Statistical Analysis

The results are expressed as mean ± SEM and the mean values were compared using Students *t*-tail test or one-way ANOVA analysis. Values of $P<0.05$ and $P<0.01$ were considered statistically significant.

CHAPTER 4

4 RESULTS

4.1 Determination of HNE-induced cytotoxicity

Although reactive oxygen species can cause cell death via apoptosis in many cell types and a number of antioxidants can block this effect, the molecular events. Molecular and genetic mechanisms activated in response to oxidative stress however are poorly understood. A number of reactive aldehydes originating from lipid peroxidation once have been formed induce severe cellular stress, including chromosome aberrations, sister chromatid exchanges, point mutations and cell killing.

Recently, considerable attention has been paid to HNE because of its potential as a second messenger and a direct mediator in the intracellular signaling network. The present results show that HNE at could trigger cell death in quiescent Swiss 3T3 fibroblasts in a dose-dependent manner (Figure 4.1). These cells are well characterized at the intracellular signaling level and can be reversibly arrested in the Go phase of the cell cycle thus removing the heterogeneity of response that may exist in an asynchronous population. In Figure 4.1A, cell viability has been determined by crystal violet assay and physiologically relevant doses of HNE can significantly affect viability of cells dose dependently. Concentrations above 50 μM of HNE induce gross non-specific cytotoxicity and are not involved in further experiments. Results are further confirmed by differential staining (Hoechst dye, AO, PI) of the cells treated with 1–50 μM HNE for 24 h, which indicates that cell death induced by HNE is mainly apoptotic (Figure 4.1B). HO has access to altered apoptotic nuclei, which allows labeling cells

from early to late apoptotic stage. AO exerts preference for normal nuclei AT-rich regions and PI can enter only late apoptotic and necrotic cells through intercalating nucleic acids without sequence preference. Thus, triple staining is generally a preferred method to discriminate healthy, early/late apoptotic and necrotic cells in a culture.

Apoptotic cell death is an active physiological process distinct from necrosis and is characterized by defined morphological and biochemical features including membrane blebbing, cytoplasmic shrinkage, chromatin condensation and DNA fragmentation. 3T3 fibroblasts incubated with HNE for 24 h were apoptotic and the nuclei showed characteristic diffuse granular staining with HO (Figure 4.2). Under these conditions HO stained nuclei were highly fluorescent and appeared pyknotic.

4.2 Protective effect of resveratrol against HNE-induced cytotoxicity

There is a growing interest in compounds present in human diet with antioxidant properties and with different biological activity, including cardioprotective and cancer chemopreventive action. Among them, polyphenols and vitamins are considered as pioneering compounds among these small molecules. Resveratrol is a natural molecule with antioxidant, anti-fungal, anti-inflammatory, anti-platelet and anti-cancer action. Here we investigated the possible protective effect of resveratrol on HNE-induced apoptosis in 3T3 fibroblasts. The results indicate that HNE-induced apoptosis could be prevented by 4 hours pretreatment of the cells with resveratrol in a dose dependent manner (Figure 4.3). The most effective concentration of resveratrol is between 20-50 μM. When we used resveratrol concentrations above 50 μM, it exerts further cytotoxic effects either alone or along with HNE treatment. Physiological level of resveratrol in plasma is very low and depends mainly on grapes and wine consumption. It has been shown that plasma level of resveratrol is about 5 μg/ml after 30-min drank of 100 ml wine containing 25 mg of resveratrol. The drug kinetic studies have shown that concentrations of resveratrol between 4.2-26 μM could be reached by nutrition. Thereby, we have used 20-50 μM of resveratrol in further experiments for the evaluation of molecular and cellular mechanisms of protective effects of resveratrol.

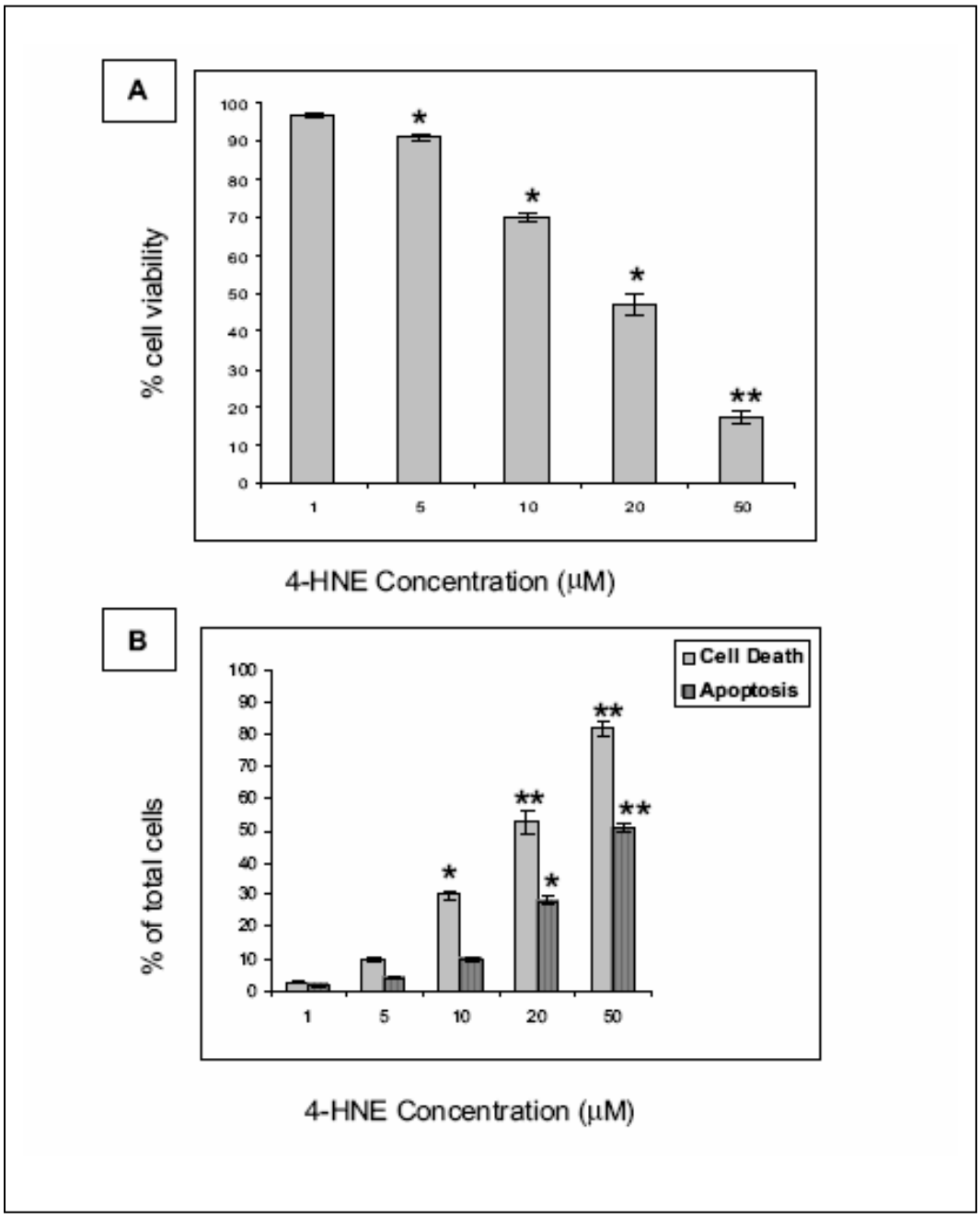


Figure 4.1 HNE induced apoptosis and cytotoxicity in fibroblasts. 3T3 cells were treated with indicated concentrations of 4-HNE for 24 hours. A: Cells were fixed and stained by 1.5% crystal violet for assessment of cell viability. The data are expressed as mean ± SEM % of untreated control and representative of six experiments. * $P < 0.05$, ** $P < 0.01$. B: Cells were fixed and stained with AO, HO and PI and healthy, necrotic and early/late apoptotic cells were scored as described in Materials and Methods. In each case, at least 400 cells were counted in 5 different microscopic fields. Data are expressed as mean ± SEM representative of six experiments. * $P < 0.05$, ** $P < 0.01$.

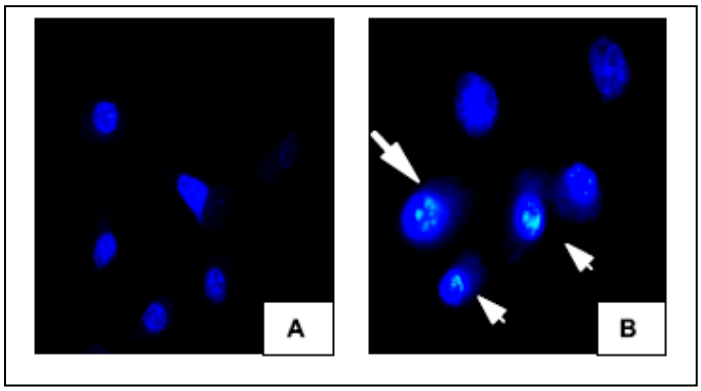


Figure 4.2 Analyses of apoptosis using Hoechst staining. HNE induces apoptosis. 3T3 cells grown on Glass Bottom Culture Dishes were treated with 50 μM of HNE for 24 hours, fixed and stained with Hoechst 33342. Cells were visualized by using Olympus Fluorescence Microscope at 40X magnification. A: Control, B: HNE-treated cells. Arrows indicate fragmented nuclei and condensed chromatin.

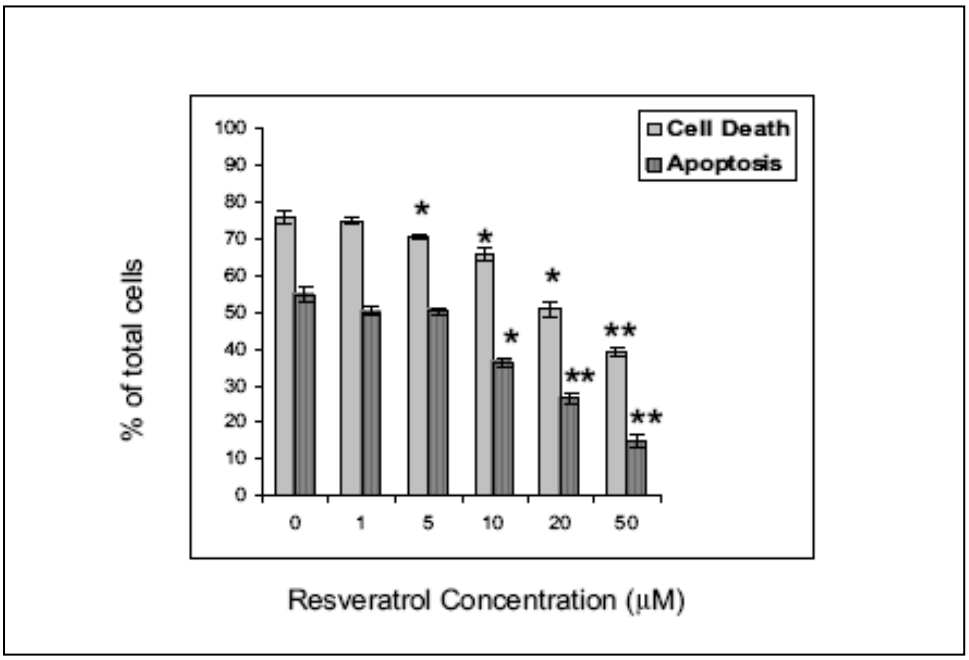


Figure 4.3 Protective role of resveratrol. Resveratrol prevents Swiss 3T3 fibroblast cell death and apoptosis. Cells were pretreated with indicated concentrations of resveratrol for 4 hours and then consequently treated with 50 μM HNE for 24 hours. Cells were fixed, stained with AO, HO and PI and healthy, necrotic, early and late apoptotic cells were scored as described in Materials and Methods. The data are expressed as mean ± SEM and representative of six experiments.* $P < 0.05$, ** $P < 0.01$.

One of the hallmarks of apoptosis is the endonuclease-mediated degradation of chromatin giving rise to DNA laddering. To investigate the effect of HNE and the role of resveratrol on DNA, laddering cells were incubated with HNE alone or pretreated with resveratrol. Cells treated with differing doses of HNE alone contained low molecular weight DNA species that migrated as a ladder with fragments differing by approximately 200 bp (Figure 4.4A). Pretreatment with resveratrol again prevented DNA laddering in a dose-dependent manner, where high molecular weight DNA was clearly observable (Figure 4.4B).

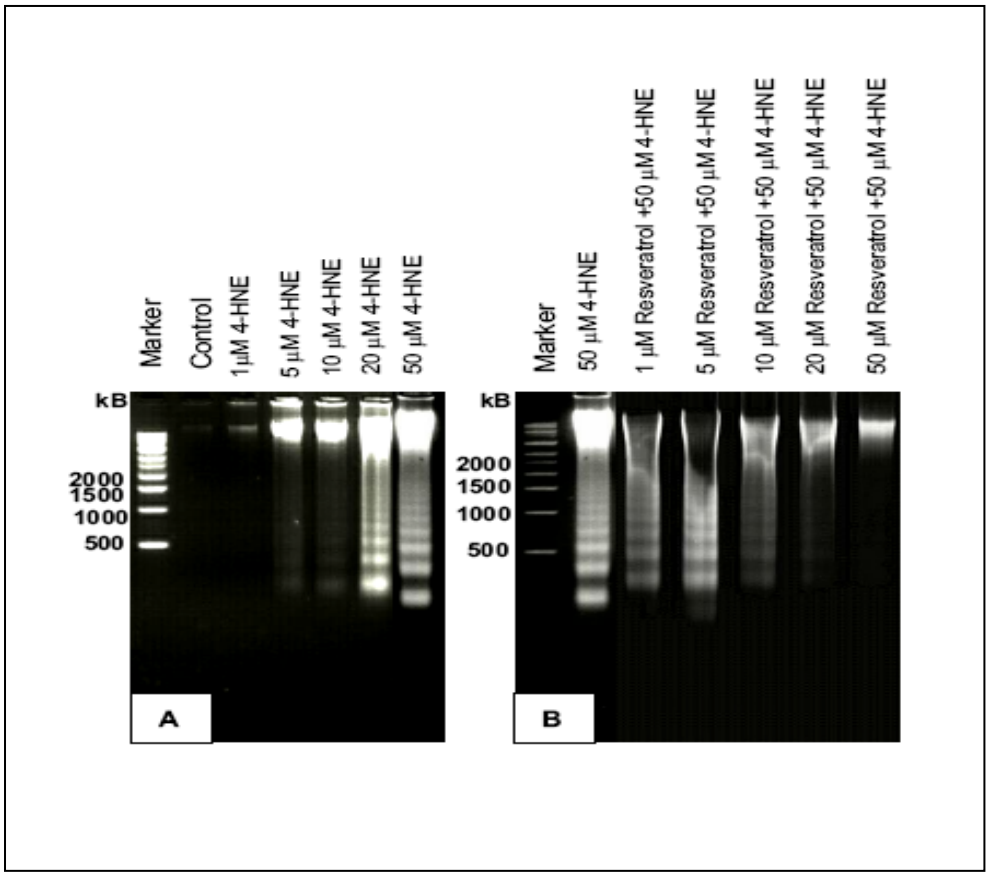


Figure 4.4 Resveratrol protects against HNE-induced apoptosis. Resveratrol prevents 4-HNE induced DNA fragmentation and apoptosis in a dose-dependent manner. A: 3T3 cells were treated with indicated concentrations of HNE for 24 h. DNA was isolated, resolved on 1.5% agarose gel and visualized by staining with ethidium bromide. B: 3T3 cells were pretreated with indicated concentrations of resveratrol for 4 h and then treated by 50 μM of HNE for 24 h. DNA was isolated, resolved on 1.5% agarose gel and visualized by staining with ethidium bromide.

4.3 Determination of HNE-induced ROS production

The effect of HNE on the redox state of cells was determined with the fluorescent probe DCF-DA as shown in Figure 4.5. The results indicate that 2 hours incubation with 50 μM HNE induced an increase in ROS intracellular level. At subtoxic concentrations and as low as 5–10 μM HNE is able to increase the production of ROS which again could be overcome by resveratrol (data not shown). In parallel, the antioxidant resveratrol prevented the HNE-induced increase in ROS again dose dependently (Figure 4.5). Resveratrol acts as an antioxidant via free radical scavenging or direct interference with enzymatic systems involved in hydrogen peroxide production; which remains to be elucidated. Our data concerning the ROS level are consistent with previous works indicating that HNE increased the production of superoxide and hydroxyl radicals in neutrophils. Detection of ROS as early as 2 hours of incubation with HNE suggests an early response or a primary event leading to apoptosis. Although there are the HNE-detoxifying enzymes; such as the GSH transferase, one can suggest that HNE through its reactions with cellular thiols can induce alterations in cellular oxidative status. It may well be suggested that HNE accumulated in mitochondria can alter mitochondrial functions and trigger free radical generation. In fact, cytochrome *c* release has been detected in cells undergoing apoptosis.

4.4 HNE-induced modulation of c-Jun Expression/Phosphorylation, c-Fos expression and AP-1 Binding

To investigate the effect of HNE on AP-1 proteins in 3T3 fibroblasts, we evaluated the levels of c-Jun, phospho-c-Jun, and c-Fos proteins. This event could be the first signal that might be highly relevant to the onset of the stress response represented by the induction of immediate early genes. As shown in Figure 4.6A, HNE (20 μM) dramatically stimulated the expression of c-Jun protein in a time-dependent manner with a maximal response by 1 h; in addition, increased phospho-c-Jun levels were detected at 1 h following HNE treatment. The increased expression of c-Jun/phospho-c-Jun was sustained until 4 h after treatment. In contrast, time-course

experiments up to 4 h have shown that downregulation of c-Fos by HNE (20 μM) was evident at 30 min and remained downregulated until 4 h. Furthermore, the effect of HNE on AP-1 c-Jun, phospho-c-Jun, and c-Fos was concentration-dependent, in which HNE at 20 lM concentration induced increased c-Jun and phospho-Jun, and decreased c-Fos levels efficiently after 1 h of treatment (Figure 4.6A). Our data indicate that induction of AP-1 proteins by HNE is highly specific to c-Jun. Because c-Jun is the major constituent of the transcription factor AP-1, and because HNE increased the expression of this proto-oncogene, we tested the effect of HNE on AP-1 activation. AP-1 activity was measured by its ability to bind to the palindromic TPA response element (TRE). The nuclear extracts of HNE-treated cells displayed greater binding activity than extracts of untreated cells, as determined by gel shift assay. The maximum increase in AP-1 binding activity in response to HNE (20 μM) treatment was observed by 1 h, and these responses persisted for at least 2 h (Figure 4.6B). HNE also induced a dose-dependent activity in AP-1 binding, and an increased AP-1 binding could be observed with as little as 5 μM HNE treatment for 1 h. A clearly significant degree of binding was detected when cells were treated with 20 μM HNE for 1 h as compared to control untreated cells (Figure 4.6B). These results suggest that HNE induces activation of AP-1 transcription factor complexes along with increased expression and phosphorylation of c-Jun and decreased expression of c-Fos.

4.5 HNE-induced modulation of MAP kinases

A number of transcription factors such as c-Jun/AP-1 and c-Fos/AP-1 have been shown to be phosphorylated by distinct members of MAP kinase proteins triggered by a large variety of extracellular stresses [81]. Based on the finding that HNE is a potential inducer of c-Jun expression and phosphorylation, a possible involvement of MAP kinases in HNE-induced c-Jun expression/phosphorylation was examined. To determine the possibility that HNE treatment of the cells results in the activation/phosphorylation of MAP kinases, whole cell lysates were probed with the antibodies specific for JNK, phospho-JNK, p38, phospho-p38, ERK 1/2, phospho-ERK 1/2 by means of immunoblot analysis.

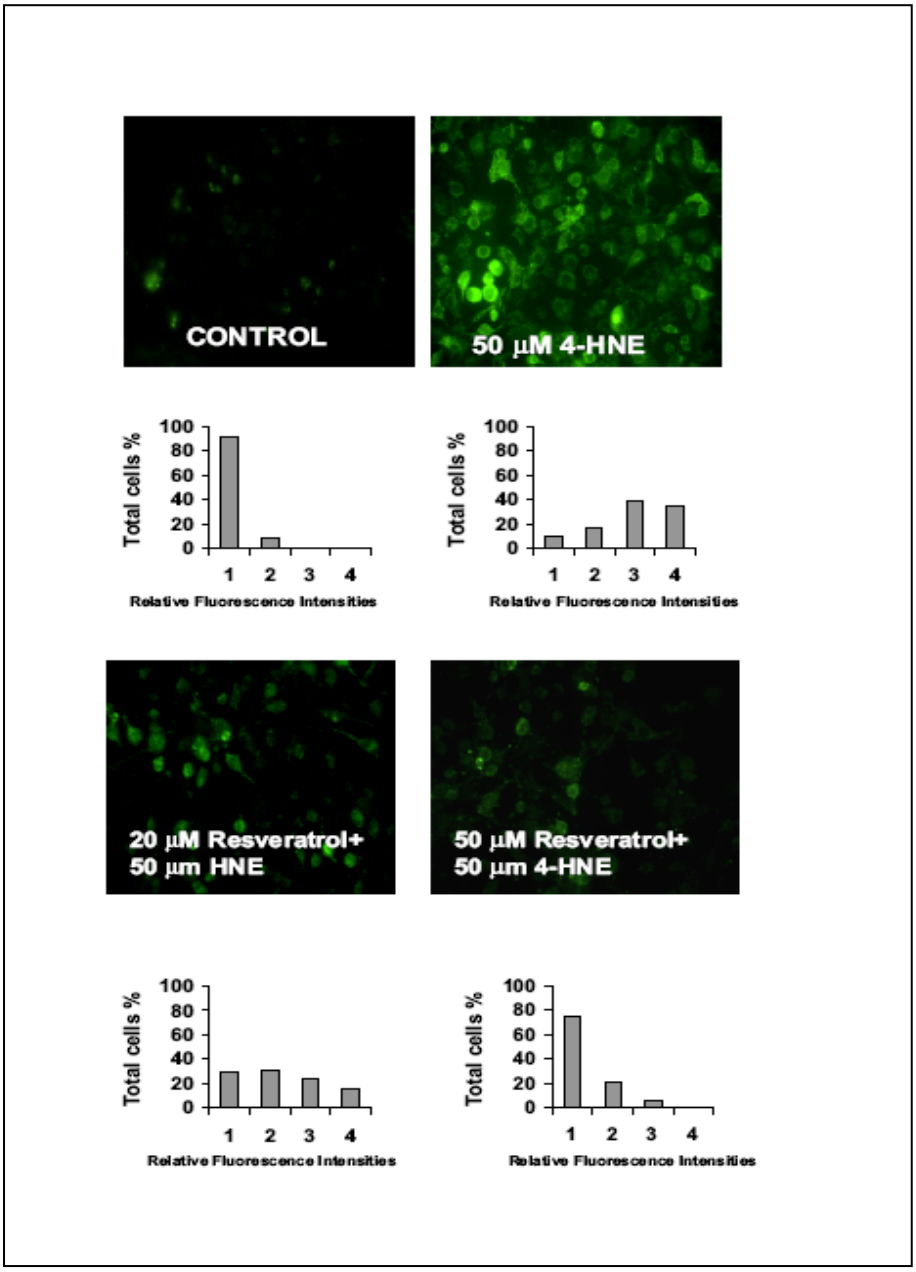


Figure 4.5 Protective role of resveratrol against HNE-induced ROS formation. Fibroblasts grown on Glass Bottom Dishes treated with 50 µM 4-HNE for 2 h with or without pretreatment with indicated concentrations of resveratrol. Cells were subsequently stained by ROS sensitive fluorescence probe DCHF-DA and visualized by Olympus fluorescence microscope at 40x magnification. Relative fluorescence intensities for each condition were determined as pixel intensity for relative fluorescence quantification and classified into four brightness categories as percentage of total number of cells.

As shown in Figure 4.7, HNE (20 μM) induced an abrupt increase in phosphorylation of JNK, which peaked at 15 min after stimulation without any significant change in total JNK protein levels. The activity of JNK began to diminish at 1 h of HNE treatment and became undetectable at 4 h. Interestingly, ERK 1 phosphorylation became undetectable after 30 min of HNE treatment, but we were able to observe phosphorylated ERK 2 until 2 h after HNE stimulation. HNE induced a complete inhibition of ERK 1/2 phosphorylation after 2 h of treatment without any significant change in total ERK 1/2 protein levels. Immunoblot analysis of phospho-p38 proteins revealed an early and transiently increased level of phospho-p38, which could be exclusively detected at 15–30 min after HNE treatment. HNE did not influence the total p38 protein level, as shown in Figure 4.7. In addition, loading of proteins for all immunoblots were checked by reprobing of membranes by β-actin antibody; a representative β-actin blot is shown in lower panel of Figure 4.7. These findings demonstrate that HNE alternates the activity of all three MAP kinases in 3T3 fibroblasts with different time kinetics and characteristics.

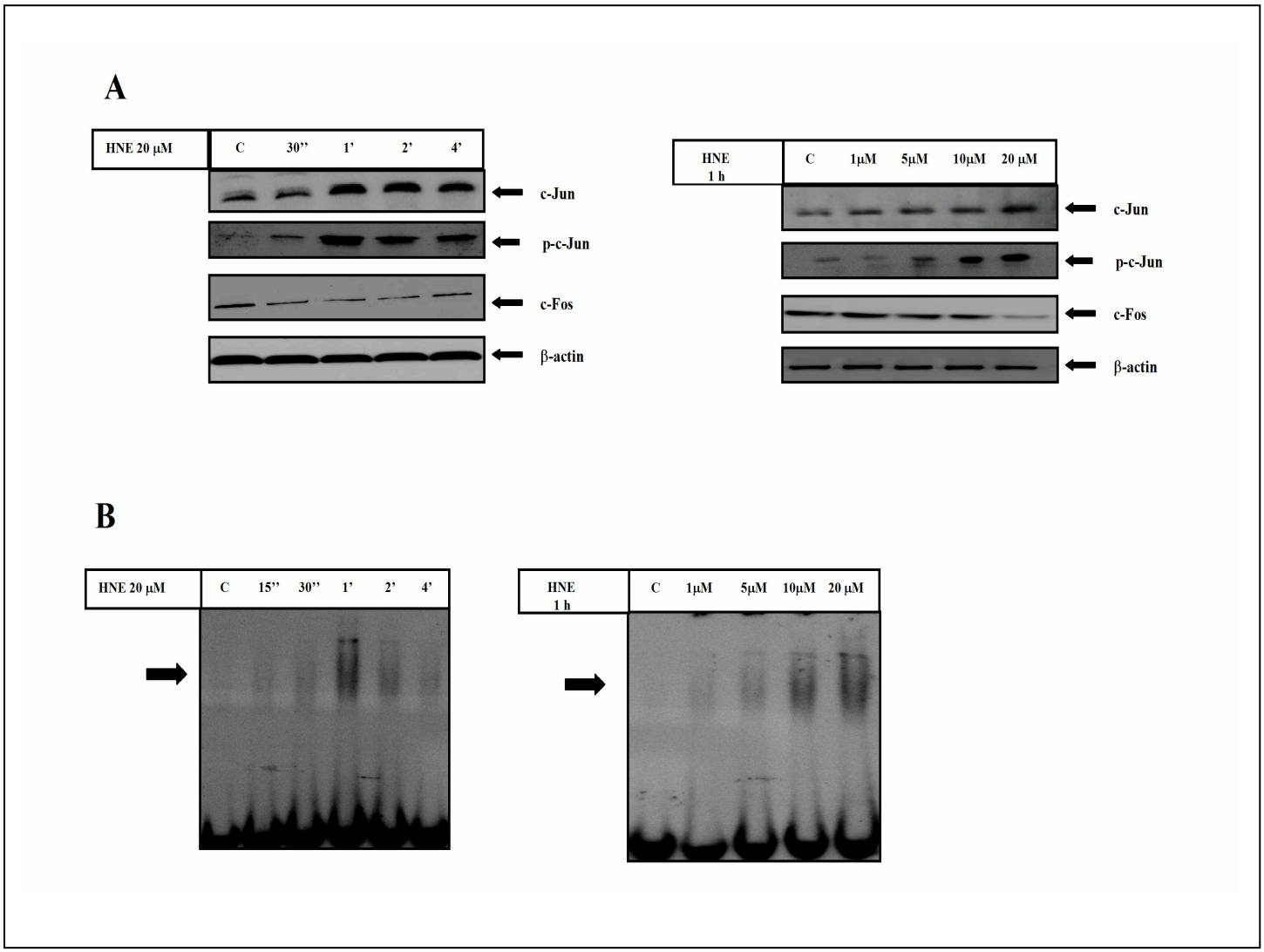


Figure 4.6 HNE-induced modification of c-Jun, c-Fos and AP-1 DNA binding. HNE modulates c-Jun and c-Fos proteins and AP-1 activation in Swiss 3T3 cells. 3T3 cells were grown on 60 mm culture flasks (1x 10^7 cells/well) and treated with 20 μM HNE for 0-4 h or treated with 0, 1, 5, 10 and 20 μM HNE for 1 h. A: Total protein lysates were isolated and c-Jun, phospho-c-Jun and c-Fos levels were detected by immunoblot analysis using specific antibodies. β-actin was probed as a loading control for immunoblots and results are representative of three independent experiments. B: 3T3 cells were grown on 6-well culture plates (1 x 10^6 cells/well) and treated with 20 LM HNE for 0-4 h or treated with 0, 1, 5, 10 and 20 μM HNE for 1 h. Nuclear proteins were isolated and gel shift assays were performed using ^{32}P-labelled AP-1 oligonucleotide probe. Black arrows indicate the specific AP-1 complex.

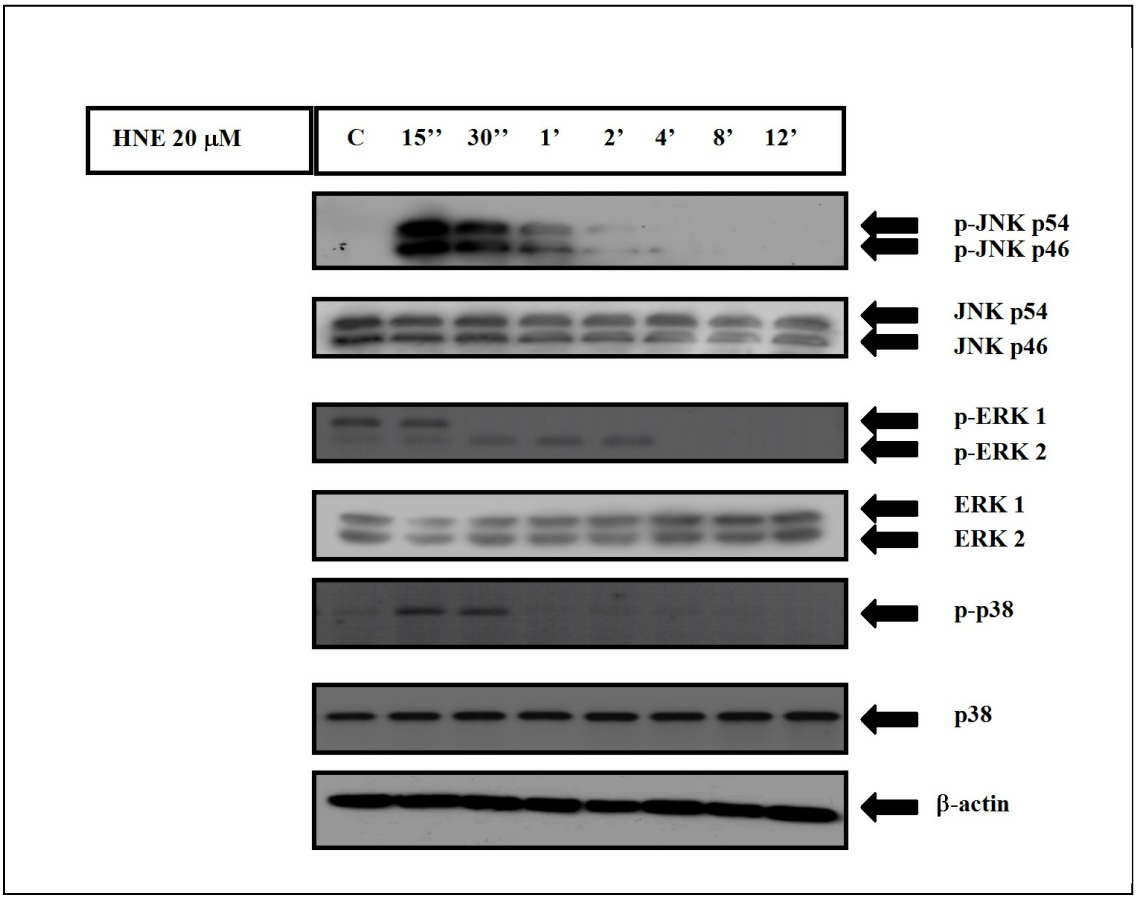


Figure 4.7 HNE-induced regulation of MAP kinases. 3T3 cells were grown on 60 mm culture flasks (1x 10^7 cells/well) and treated with 20 μM HNE for 0-12 h. Activities of JNK, p38 and ERK 1/2 MAP kinases were detected by immunoblot analysis. Specific antibodies against total and phospho-JNK, total and phospho-p38 and total and phospho-ERK 1/2 were used for immunoblot analysis. β-actin was probed as a loading control for immunoblots and results are representative of three independent experiments.

4.6 Effect of HNE on caspase activation and cytochrome *c* release from mitochondria

To identify the possible role of caspases in this apoptotic process, we investigated the activation of caspase-9 and caspase-3 in response to HNE treatment by using immunoblot analysis for active caspase fragments and fluorometric caspase assays. HNE (20 μM) induced the appearance of an active caspase-9 fragment (35 kDa) at 1 h,

which was followed by a more evident and strong band in immunoblot analysis at 4–8 h after HNE treatment (Figure 4.8). The parallel analysis of caspase-9 activation through its ability to cleave its specific substrate (Ac-LEHD-AMC) and formation of the fluorogenic AMC compound displayed a quite different caspase-9 activation pattern compared to immunoblot analysis (Figure 4.8). Although we could detect a pronounced active caspase-9 band at 4 h of HNE treatment, a complete activation of caspase-9 activation could only be detected at 8 h of HNE treatment in fluorometric caspase assays. In the mitochondrial apoptosis pathway, caspase-3 operates downstream of cytoplasmic cytochrome *c* translocation and caspase-9 activation. As shown in Figure 2B, HNE (20 μM) induced the appearance of cleaved active caspase-3 fragments within 4 h, and complete active bands were detected at 8 h posttreatment. The fluorometric caspase assays of caspase-3 activation through its ability to cleave its specific substrate (Ac-DEVD-AMC) and formation of the fluorogenic AMC compound presented a time-course similar to immunoblot analysis for caspase-3 activation (Figure 4.8).

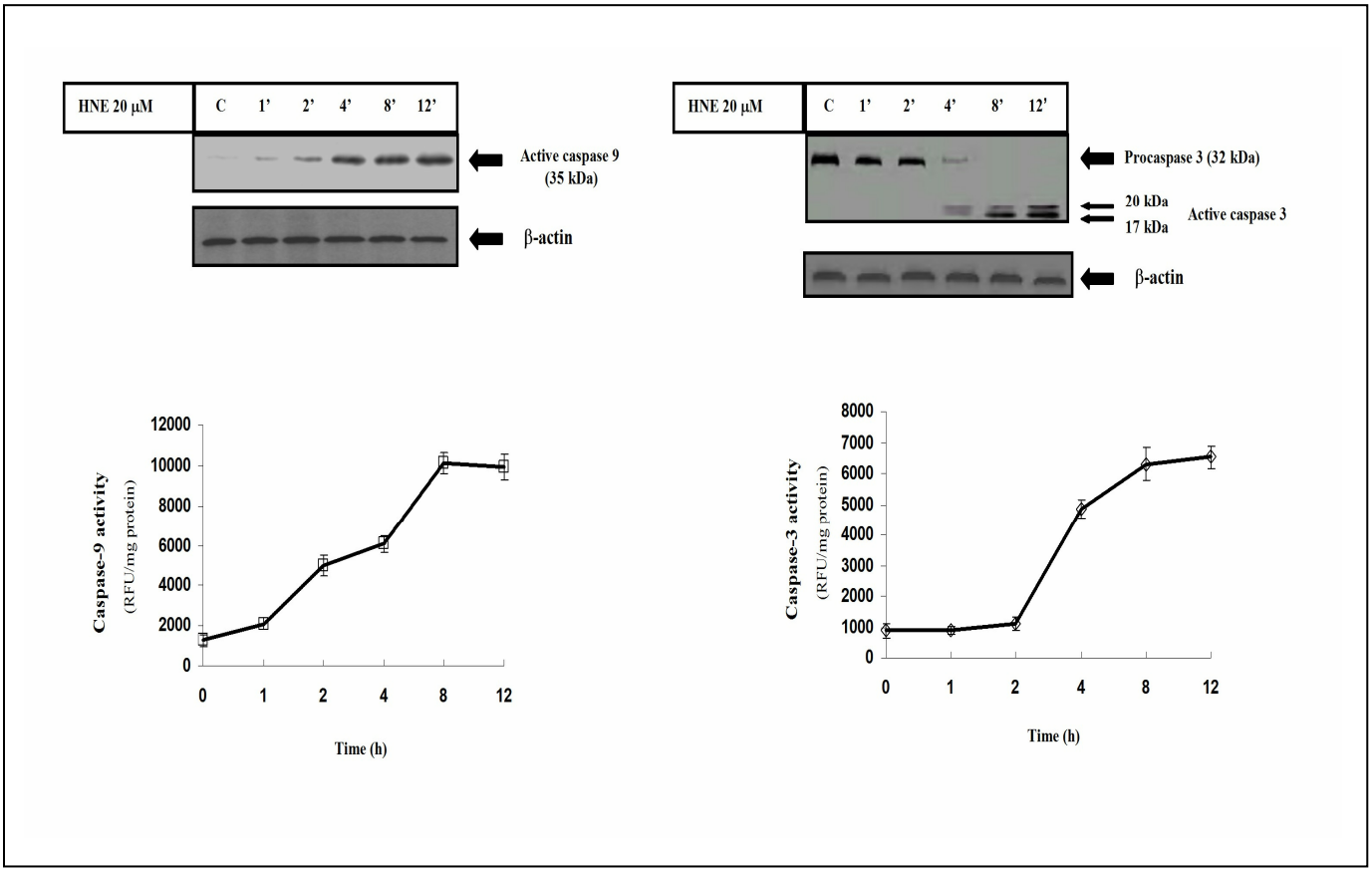


Figure 4.8 HNE-induced activation of caspases 3 and 9. 3T3 cells were grown on 6-well culture plates (1x 10^6 cells/well) and treated with 20 µM HNE for 0-12 h. The activation of caspase-9 and caspase-3 were evaluated by immunoblot analysis and fluorometric caspase assays. For immunoblot analysis antibodies against active caspase-9 and pro/active-caspase-3 were used and β-actin was probed as a loading control. In caspase assays, results were expressed as mean ± SEM from three independent experiments performed in triplicate.

The release of cytochrome *c* from mitochondria in response to apoptosis inducers (such as chemotherapeutics, UV radiation, growth factor withdrawal) and its binding to Apaf-1 leads to formation of apoptosome complex and caspase-9 activation [18]. As shown in Figure 4.9, HNE treatment resulted in an early and complete release of cytochrome *c* from mitochondria and its appearance in cytoplasm 1 h after HNE treatment, which is compatible with caspase-9 and caspase-3 activation patterns. Taken together, these results present evidence for intrinsic mitochondrial apoptotic machinery involved in HNE-induced apoptosis.

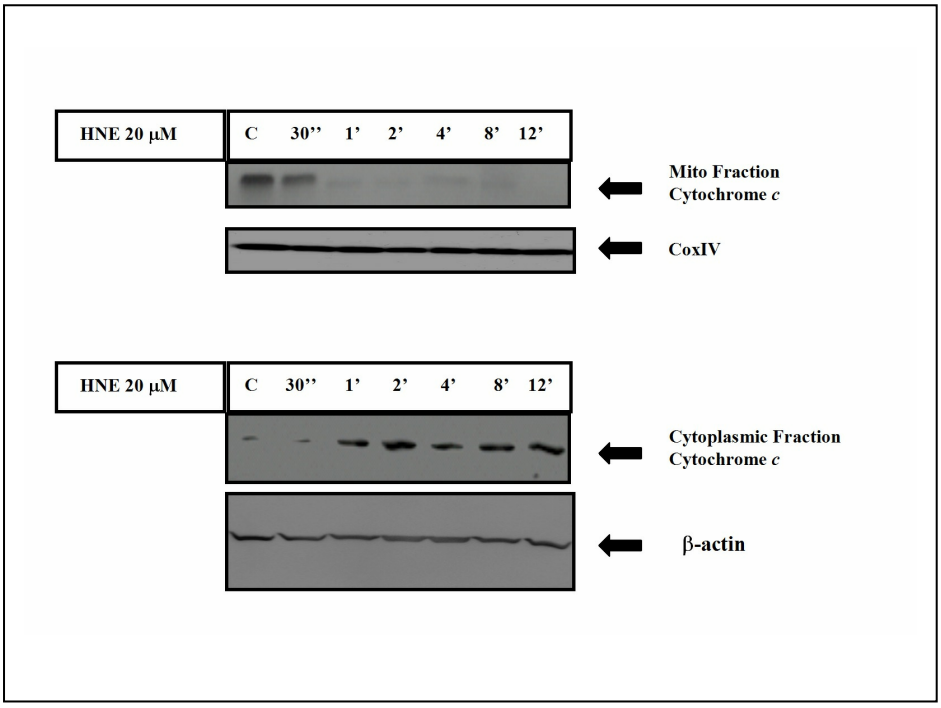


Figure 4.9 HNE-induced cytochrome *c* release. 3T3 cells were grown on 6-well culture plates (1x 10^6 cells/well) and treated with 20 μM HNE for 0-12h. The mitochondrial and cytoplasmic fractions of cytochrome *c* were detected by immunoblot analysis. CoxIV and β-actin were probed as a loading control for mitochondrial and cytoplasmic fractions respectively. Results are representative of three independent experiments.

4.7 JNK and caspases are functionally involved in HNE-induced apoptosis; protective effect of resveratrol

Activation of MAP kinases either upstream or downstream of caspase has been postulated in many *in vitro* and *in vivo* experimental apoptosis models [80,87]. Regarding HNE-induced modulation of MAP kinases and caspases, we investigated the functional involvement of MAP kinases in HNE-induced apoptosis. It has been previously reported that HNE-induced cell death is predominantly apoptotic rather than necrotic Therefore, we utilized MTT assay for determination of cell viability in response to specific MAP kinase or caspase inhibitors and HNE treatment. Treatment of cells with HNE (20 μM) reduced cell viability to 37.39 ± 1.68% (Figure 4.10, lane 1),

and pretreatment of cells with 10 μM SP600125 (JNK inhibitor) for 1 h protected 3T3 fibroblasts against HNE-induced apoptosis (Figure 4.10, lane 2, ** $P < 0.01$, compared to HNE-treated cells), but 10 μM SB203580 (p38 inhibitor) or 10 μM PD98059 (ERK 1/2 inhibitor) did not exhibit any significant protective effect (Figure 4.10, lanes 3 and 4, respectively). Pretreatment of cells with 20 μM pancaspase inhibitor Z-VAD-FMK (Figure 4.10, lane 5); 20 μM caspase-9 inhibitor, Z-LEHD-FMK (Figure 4.10, lane 6); or 20 μM caspase-3 inhibitor, Z-DEVD-FMK (Figure 4.10, lane 7) for 30 min significantly prevented HNE-induced apoptosis (**$P < 0.01$ for Z-VAD-FMK and Z-LEHD-FMK; *$P < 0.05$ for Z-DEVD-FMK, compared to HNE-treated cells). The protective effect of resveratrol (20 μM), a polyphenol with anti-carcinogenic and anti-inflammatory properties, is also comparable to protective effects of SP600125 and caspase inhibitors (Figure 4.10, lane 8, ** $P < 0.01$, compared to HNE treated cells).

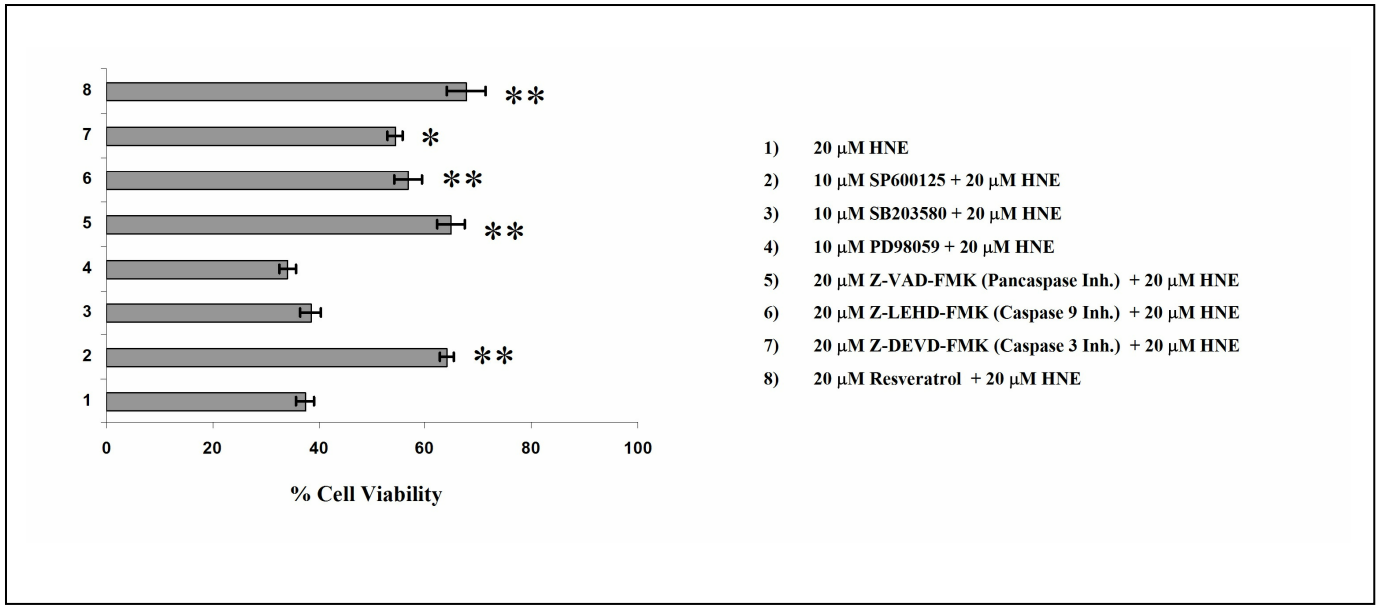


Figure 4.10 JNK and caspases are involved in HNE-induced apoptosis.3T3 cells were grown on 96-well plates (1 x 10^4 cells/well) and pretreated with specific MAP kinase and caspase inhibitors; (10 μM ERK 1/2 inhibitor (PD98059), 10 μM p38 inhibitor (SB203580) and 10 μM JNK inhibitor (SP600125) for 1 h; 20 μM pancaspase inhibitor (z-VAD-FMK), 20 μM caspase-9 inhibitor (z-LEHD-FMK), 20 μM caspase-3 inhibitor (z-DEVD-FMK) for 30 min and 20 μM resveratrol for 4 h, which is followed by 20 μM HNE treatment for 24 h. Cells treated with 20 μM HNE without inhibitor pre-treatment were also involved in experimental panels. The lanes for specific treatments are indicated in the figure. After incubation, the effects of specific kinase and caspase inhibitors on HNE-induced apoptosis were evaluated by MTT cell viability assay. MTT results are expressed as means ± SEM from three independent experiments performed in duplicate. (* P<0.05; ** P<0.01 compared with 20 μM HNE-treated sample)

To determine the interdependency of MAP kinases and caspases, as well as to identify the critical steps involved in resveratrol-mediated protection against HNE-induced apoptosis, we followed HNE-induced activation of caspases after pretreatment with specific MAP kinase inhibitors and resveratrol. As demonstrated in Figure 4.11, pretreatment of cells with 10 μM SP600125 or 20 μM resveratrol significantly inhibited

both caspase-3 and caspase-9 activation with similar efficiencies. In contrast, 10 μM SB203580 or 10 μM PD98059 did not confer any effect on caspase activation induced by HNE consistent with MTT results. Pretreatment of cells with caspase inhibitors did not show any effect on modulation of MAP kinases with HNE treatment (data not shown).

We next explored the involvement of cytochrome *c* release in inhibitory effect of JNK and resveratrol on caspase activation. Figure 4.12 shows that pretreatment of cells with 10 μM SP600125 or 20 μM resveratrol led to blockage of cytochrome *c* release from mitochondria and thereby disrupted formation of the apoptosome complex and caspase-9 activation. Our results suggest a JNK-dependent caspase-activation module in HNE-induced apoptosis in which JNK acts upstream of mitochondrial apoptotic machinery and regulate cytochrome *c* release in response to HNE treatment. Resveratrol also acts upstream of mitochondrial apoptotic machinery and exerts its protective effect through prevention of cytochrome *c* release and consequent caspase activation.

4.8 c-Jun/AP-1 transcriptional activity is involved in HNE-induced apoptosis

The protective effect of a pharmacological inhibitor of JNK (SP600125) against HNE- induced apoptosis has put forth functional involvement of c-Jun/AP-1 activation for consideration in this process. To examine whether c-Jun/AP-1 activation has a similar role in HNE-induced apoptosis, we transfected 3T3 cells with the expression vector for DN-c- Jun mutant. DN-c-Jun mutant lacking the N-terminal c-Jun transactivation domain (amino acids 1–168) can efficiently dimerize, but it cannot activate transcription and will prevent any member of the Jun and Fos family from activating transcription of AP-1-dependent target genes and may do so by occupying AP-1 binding sites in the place of functional Jun/ Jun or Jun/Fos dimers. We also transfected 3T3 fibroblasts with the expression vector for DN-JNK1 to verify the protective effect of the pharmacological JNK inhibitor. The transfected cells were treated with HNE (20 lM) for 24 h and assayed for apoptosis using the Cell Death Detection ELISA^{PLUS} kit. As shown in Figure 4.13, lane 3, overexpression of DN c-Jun significantly attenuated apoptosis when compared to HNE-treated untransfected cells (Fig. 4, lane 1), but the protective effect of DN-JNK1 overexpression against HNE-induced apoptosis was more pronounced (Fig. 4.13, lane 5). The expression of mock

vectors did not exhibit any effect on HNE-induced apoptosis (Figure 4.13, lanes 2 and 4). These results demonstrate that HNE-induced apoptosis in 3T3 fibroblasts involve c-Jun/AP-1 activation downstream of JNK, but JNK also has downstream targets other than c-Jun/AP-1.

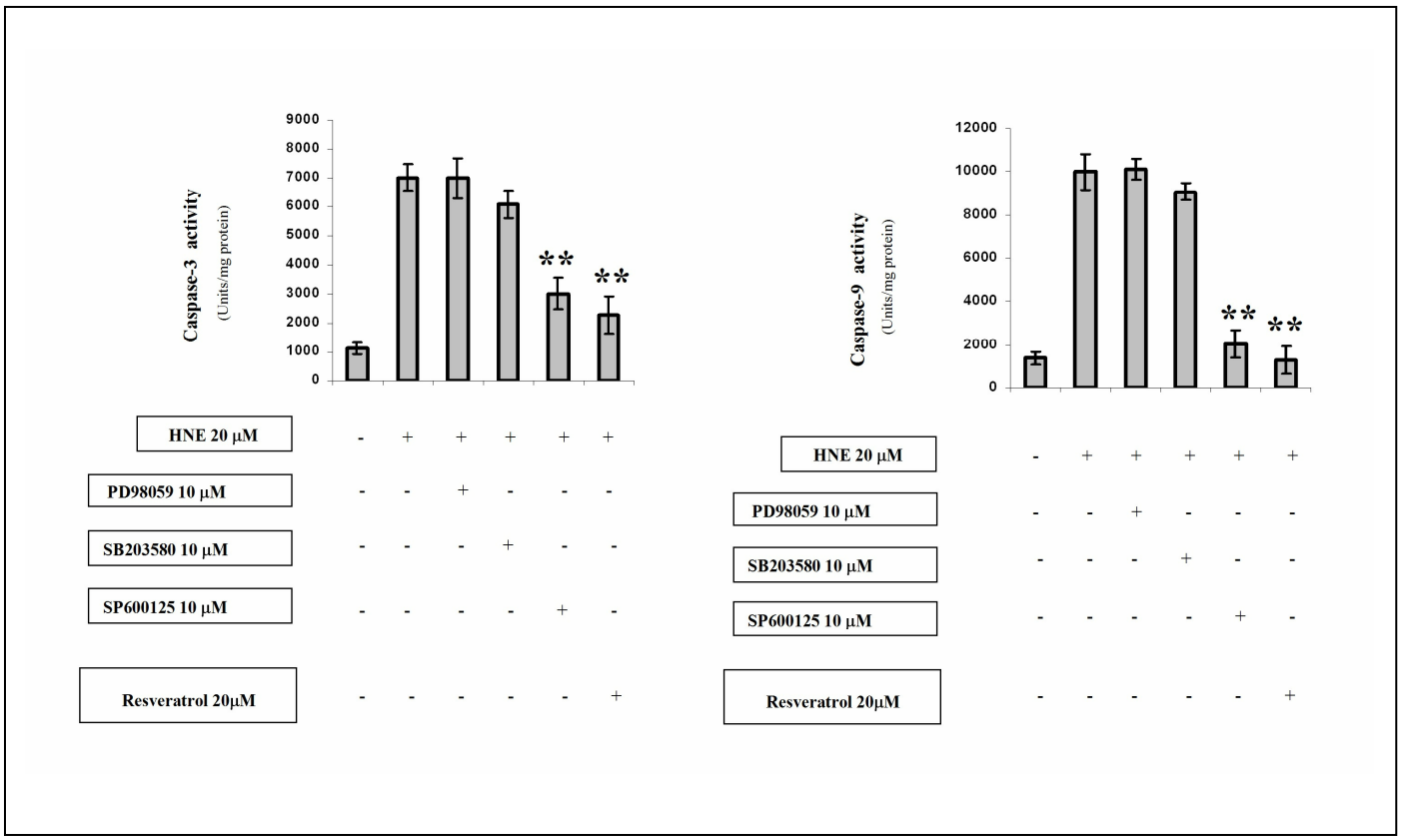


Figure 4.11 Modulation of HNE-induced caspase activation by MAP kinase inhibitors and resveratrol. 3T3 cells were grown on 6-well culture plates (1x 10^6 cells/well) and pretreated with specific MAP kinase and caspase inhibitors 10 μM ERK inhibitor (PD98059), 10 μM p38 inhibitor (SB203580), 10 μM JNK inhibitor (SP600125) for 1 h and 20 μM resveratrol for 4 h, which is followed by 20 μM HNE treatment for 8 h. The activation of caspase-9 and caspase-3 were evaluated fluorometric caspase assays. Results are expressed as means ± SEM from three independent experiments performed in duplicate. (** P<0.01 compared with 20 μM HNE-treated sample.)

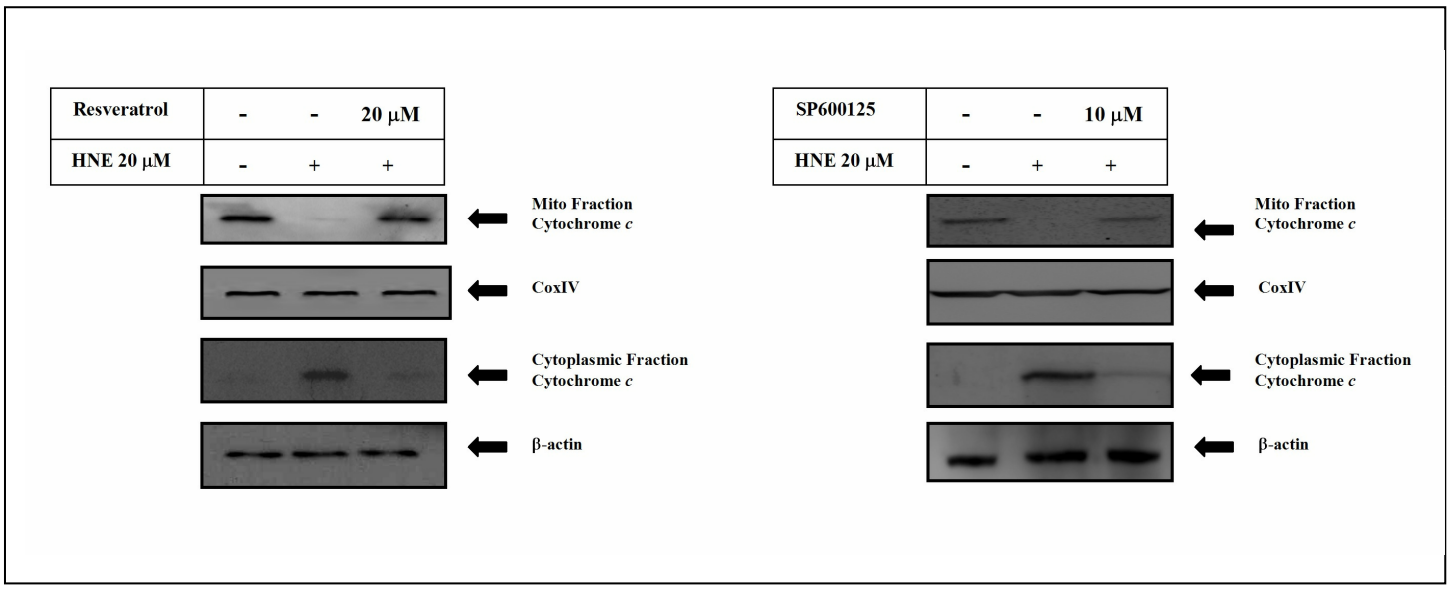


Figure 4.12 Modulation of HNE-induced cytochrome *c* release by resveratrol and JNK inhibitor SP600125. 3T3 cells were grown on 6-well culture plates ($1x10^6$ cells/well) and pretreated with 10 μM JNK inhibitor (SP600125) for 1 h and 20 μM resveratrol for 4 h, which was followed by 20 μM HNE treatment for 8 h. The mitochondrial and cytoplasmic fractions of cytochrome *c* were detected by immunoblot analysis. CoxIV and β-actin were probed as a loading control for mitochondrial and cytoplasmic fractions respectively. Results are representative of three independent experiments.

4.9 Resveratrol inhibits HNE-induced JNK and p38 Activation, c-Jun expression, and phosphorylation

To address the mechanisms of protection by resveratrol against HNE-induced apoptosis, 3T3 cells were pretreated with or without resveratrol (1, 10, or 20 μM) for 4 h, followed by treatment with HNE (20 μM). Total protein lysates were analyzed for phosphorylation and expression of MAP kinases and c-Jun and expression of c-Fos by means of immunoblot analysis. Resveratrol attenuated HNE-induced JNK activation/ phosphorylation in a dose-dependent manner, and it completely blocked HNE-induced p38 activation/phosphorylation at 20 μM concentration without any effect on total JNK and p38 levels (Figure 4.14). Resveratrol also restored basal ERK 1/2 activation/phosphorylation downregulated by HNE without any effect on total ERK1/2 protein level (Figure 4.14). Immunoblot analysis also revealed that resveratrol pretreatment efficiently inhibited HNE-induced increase in c-Jun expression, even at the

10 μM concentration, but HNE-induced phospho-c-Jun levels could only be attenuated when cells were pretreated with 20 μM resveratrol (Figure 4.14). Resveratrol pretreatment also restored c-Fos protein level, which was downregulated by HNE treatment. Although a moderate effect of resveratrol was observed even at 1 μM concentration, a complete restoration of c-Fos protein levels similar to untreated cells could be achieved when cells were pretreated with 20 μM resveratrol (Figure 4.14). These results suggest that resveratrol could protect against HNE-induced apoptosis through alteration of cellular signaling modules formed by MAP kinases and their downstream targets.

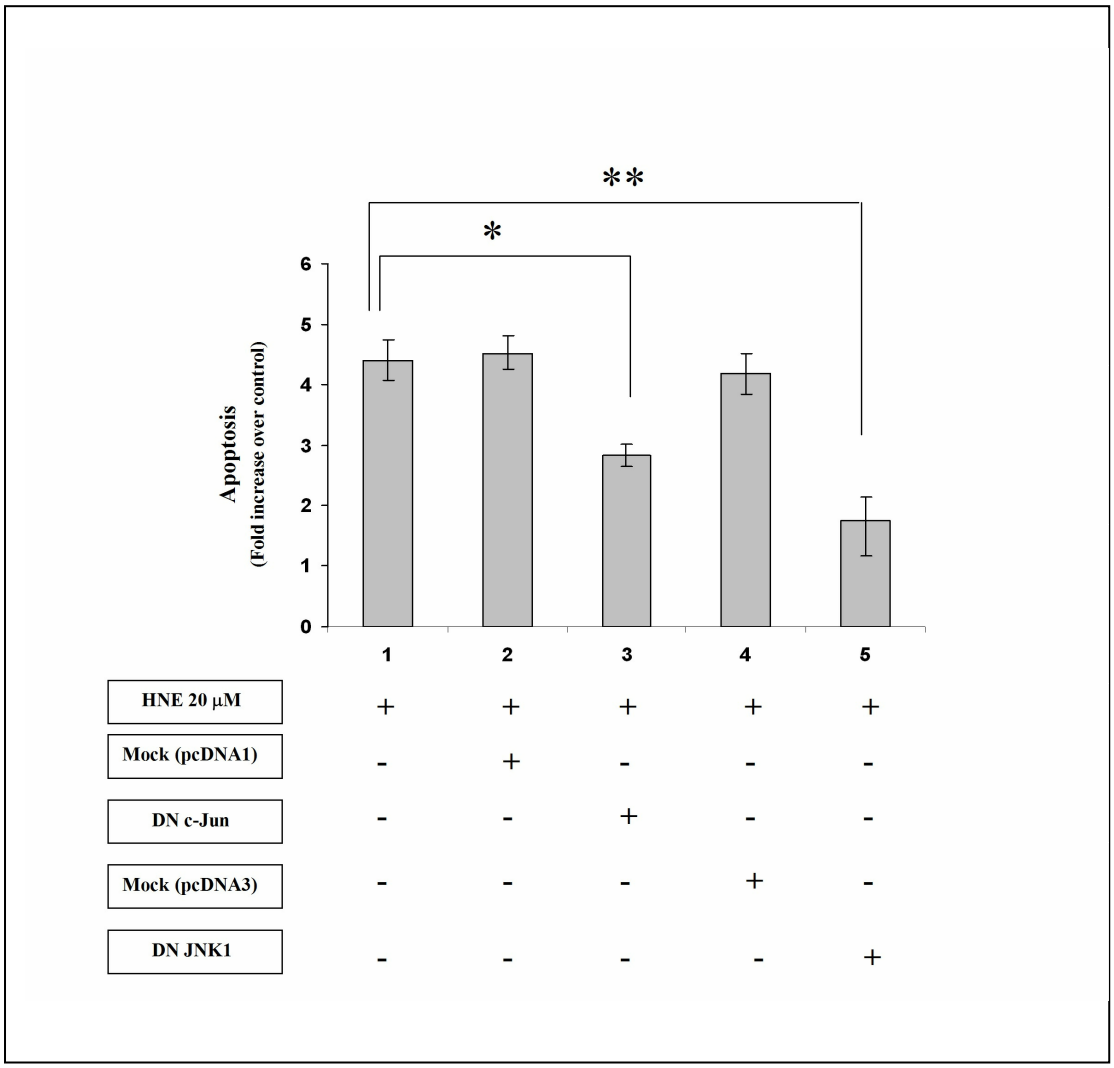


Figure 4.13 Effect of dominant negative JNK1 and c-Jun expression on HNE-induced apoptosis. Modulation of HNE-induced apoptosis in 3T3 cells by transfection of DN-c-Jun and DN-JNK1. 3T3 fibroblasts were plated in 60 mm dishes and transfected with DN-c-Jun and DN-JNK1 plasmids. In order to evaluate the effect of DN-c-Jun and DN JNK1 overexpression on HNE-induced apoptosis, Cell Death Detection ELISA^{PLUS} kit was used. The empty pcDNA1 and pcDNA3 vectors were used as mock transfections. Results are expressed as fold increases in absorbance over untreated control cells (enrichment factor) and means ± SEM from four independent experiments performed in duplicate. (* P<0.05; ** P<0.01 compared with 20 μM HNE-treated sample).

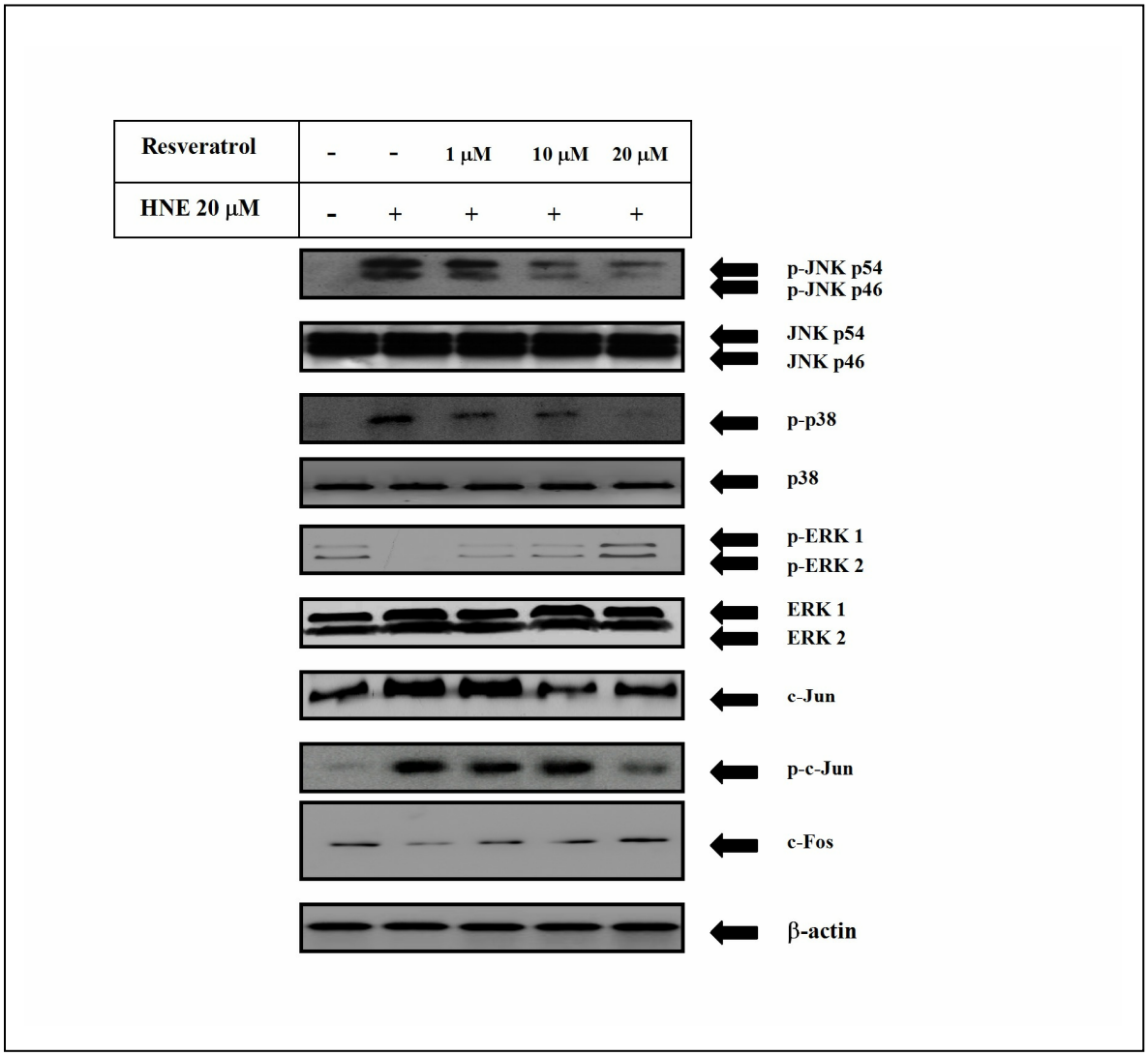


Figure 4.14 HNE-induced modification of MAP kinases, c-Jun, phospho-c-Jun and c-Fos is altered by resveratrol pretreatment. 3T3 cells were grown on 6-well culture plates (1x 10^6 cells/well) and pretreated with 20 μM resveratrol for 4 h, which is followed by 20 μM HNE treatment for 15 min to detect JNK and p38 activation, 4 h for ERK 1/2 activation and 1 h for c-Jun, phospho-c-Jun and c-Fos levels. Total proteins were isolated and analyzed by means of immunoblot. β-actin was probed as a loading control for immunoblots and results are representative of three independent experiments.

4.10 Modulation of HNE-induced AP-1 activation by SP600125 and resveratrol

To examine whether AP-1 activation is a target for resveratrol, we evaluated the effect of resveratrol on HNE-induced AP-1 activation. 3T3 cells were pretreated with resveratrol (1, 5, 10, 15, or 20 μM) for 4h, followed by HNE (20 μM) for 1 h, and nuclear proteins were analyzed by means of AP-1 gel shift assay. Resveratrol dose-dependently inhibited HNE-induced AP-1 activation with a maximum efficiency at 20 μM concentration, and resveratrol (20 μM) alone did not trigger any significant effect on AP-1 activation (Figure 4.15). Because MAP kinases reside upstream of AP-1 activation, we next investigated the inhibitory potential of specific MAP kinase inhibitors on AP-1 activation compared to resveratrol. Pretreatment of cells with SP600125 (10 μM) prior to treatment with HNE (20 μM) attenuated AP-1 activation at a comparable efficiency to resveratrol (20 μM) (Figure 4.15, lanes 4 and 3, respectively). In contrast, SB203580 (10 μM) and PD98059 (10 μM) did not exhibit any prominent effect on HNE-induced AP-1 activation (Figure 4.15, lanes 5 and 6, respectively). The specificity of AP-1 band was confirmed by competition experiments with an excess of unlabeled AP-1 oligonucleotide probe (Figure 4.15, lane 7), and by the ability of highly specific antibodies directed against individual AP-1 proteins to deplete nuclear extracts of DNA-binding activities, which confirmed that AP-1 complexes are mainly formed by c-Jun/c-Jun dimers (Figure 4.15, lanes 8, 9, and 10).

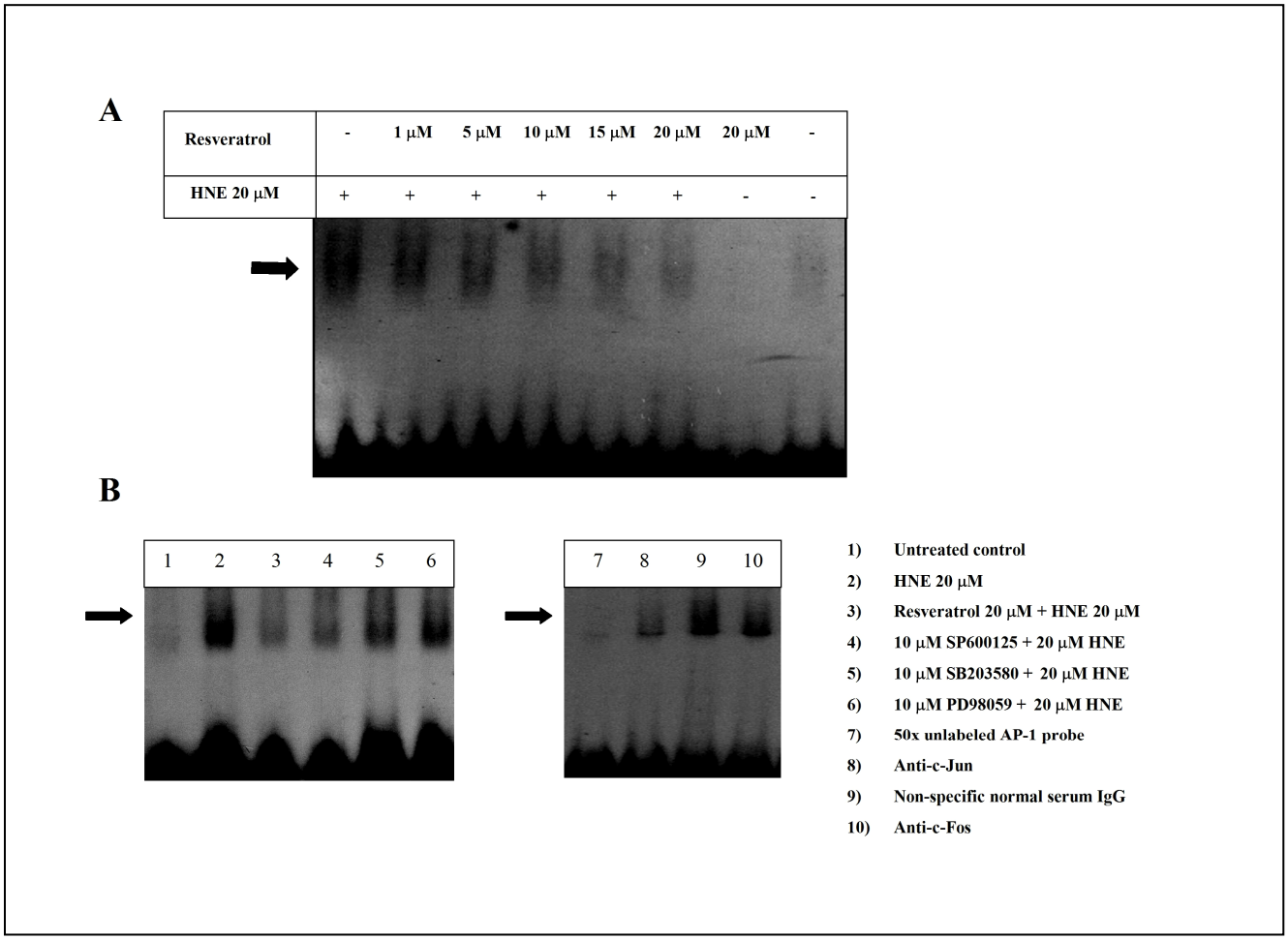


Figure 4.15 Effect of resveratrol and MAP kinase inhibitors on HNE-induced AP-1 activation. **(A)** 3T3 cells were grown on 6-well culture plates (1 x 10^6 cells/well) and pretreated with 1, 5, 10, 15 and 20 μM resveratrol for 4 h and consequently treated with 20 μM HNE for 1 h. Cells treated with 20 μM HNE for 1 h without resveratrol pretreatment or treated 20 μM resveratrol alone for 4 h were also involved in experimental panels. Nuclear proteins were isolated and gel shift assays were performed using ^{32}P-labelled AP-1 oligonucleotide probe. Black arrows indicate the specific AP-1 complex. **(B)** 3T3 cells were grown on 6-well culture plates (1 x 10^6 cells/well) and pretreated with 10 μM ERK inhibitor (PD98059), 10 μM p38 inhibitor (SB203580), 10 μM JNK inhibitor (SP600125) for 1 h and 20 μM resveratrol for 4 h, which is followed by 20 μM HNE treatment for 1 h . The lanes for specific treatments are indicated in the figure. The specificity of AP-1 binding was examined by competition with the 50x excess unlabelled probe (Lane 7) and the composition of AP-1 bands were defined by supershift experiments using c-Jun antibody (Lane 8), non-specific IgG (Lane 9) and c-Fos antibody (Lane 10).

4.11 Modulation of Bcl-2 proteins by HNE treatment

Bcl-2 proteins are key regulatory proteins of apoptosis signaling, mainly acting on mitochondrial membrane regulating protein trafficking and permeability [66]. The actual mechanism by which the Bcl-2 proteins maintain or ablate the integrity of the outer mitochondrial membrane is still unclear. Based on the finding that HNE is a potential inducer of cytochrome *c* release and caspase activation, HNE-induced modulation of Bcl-2 protein family members was examined. Total protein lysates were analyzed for expression of anti-apoptotic proteins Bcl-2, Bcl-xL and pro-apoptotic proteins Bax, Bak and Bim by means of immunoblot analysis. As demonstrated in Figure 4.16A, HNE induced decreased Bcl-2 protein levels at 12 h of treatment, but it did not induce any alteration of Bcl-x_L. In contrast, HNE induced increased Bax and Bak levels with a maximum effect at 4-8 h of treatment, which was first evident within 2 h for Bax. HNE also induced moderately increased Bim levels at 8 h of treatment, which returned to basal levels at 12 h. The results suggest that both anti-apoptotic and pro-apoptotic Bcl-2 protein family members are involved in HNE-induced apoptotic signaling. To determine whether resveratrol could exert any effect on HNE-mediated alterations in Bcl-2, Bax, Bak and Bim levels, a detailed immunoblot analysis panel was obtained. The results indicated that resveratrol could prevent HNE-induced decrease of Bcl-2 protein level and HNE-induced upregulation of Bax, Bak and Bim proteins (Figure 4.16B).

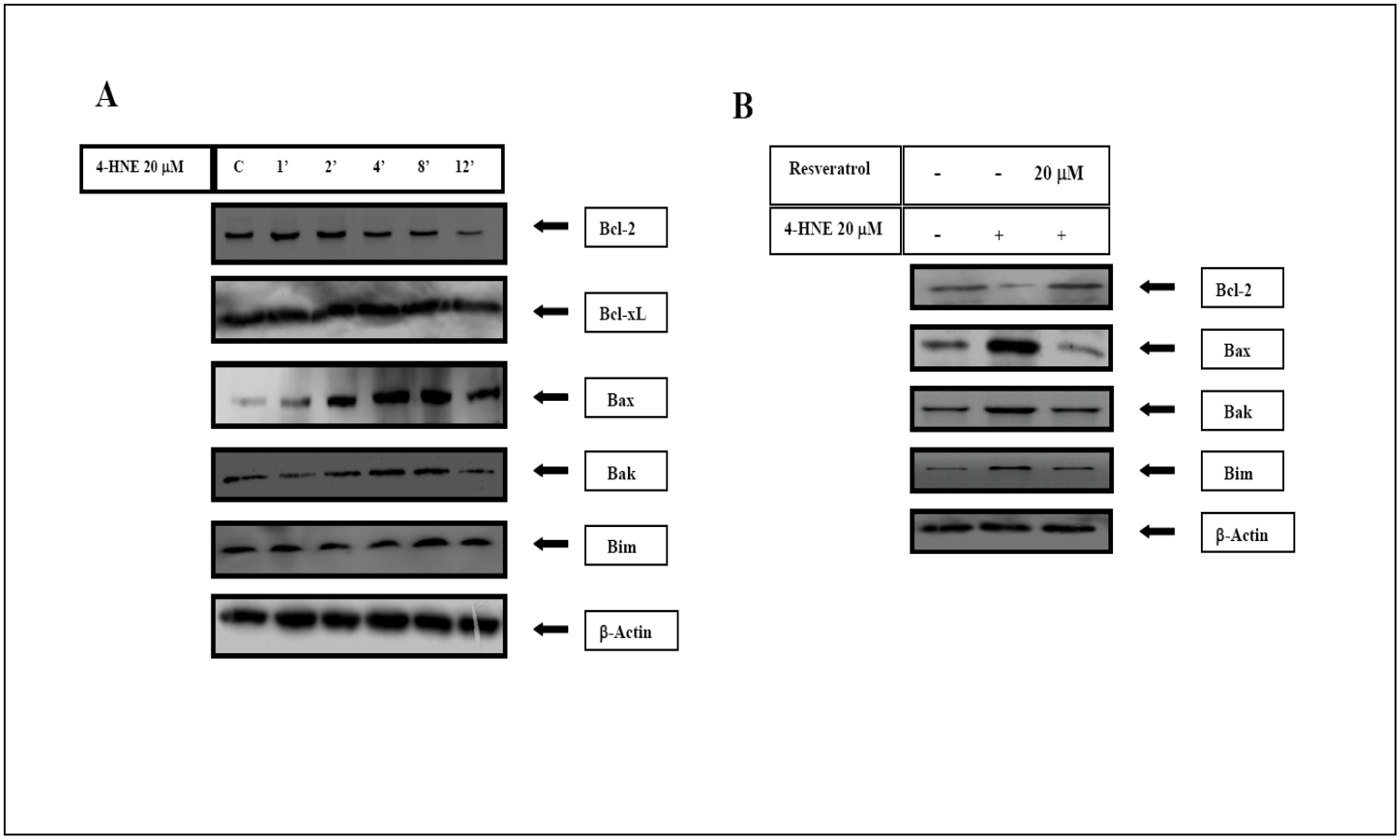


Figure 4.16 Modulation of Bcl-2 proteins by HNE: effect of resveratrol. **(A)** 3T3 cells were grown on 6-well culture plates (1 x 10^6 cells/well) and with 20 μM HNE for 0-12h. Total protein lysates were isolated and Bcl-2, Bcl-$x_{L,}$ Bax, Bak and Bim protein levels were detected by immunoblot analysis using specific antibodies. β-actin was probed as a loading control for immunoblots and results are representative of three independent experiments. **(B)** 3T3 cells were grown on 6-well culture plates (1x 10^6 cells/well) and pretreated with 20 μM resveratrol for 4 h, which is followed by 20 μM HNE treatment for 12 h for Bcl-2, 4 h for Bax and Bak, and 8 h for Bim protein levels. Total proteins were isolated and analyzed by means of immunoblot. β-actin was probed as a loading control for immunoblots and results are representative of three independent experiments.

CHAPTER 5

5 DISCUSSION

5.1 HNE induces apoptosis and ROS production in 3T3 fibroblasts: protection by resveratrol

HNE is a lipid peroxidation end product of arachidonic and linoleic acids. Because of its relatively high lipophilicity, HNE tends to concentrate within cell membranes where reasonably reaches steady-state amounts over 10^{-5} M [195]. Further, HNE may readily pass across the cellular membranes to modify critical molecules such as proteins and DNA [146,196]. As mentioned before, HNE with its highly electrophilic carbon could directly form covalent adducts with histidine, lysine, cysteine, serine and tyrosine residues of proteins and thereby alternate their functions [146,195]. Increased levels of HNE adducts have been detected in various human pathologies, such as neurodegenerative disorders, atherosclerosis and chronic obstructive pulmonary disease [195].

In the present study, we show that incubation of 3T3 cells with increasing concentrations of HNE results in decreased cell viability in a dose-dependent manner at 24 h of treatment. This reduced viability of 3T3 cells either could be due to acute necrosis of fibroblasts or programmed cell death. Thereby, we further characterized the type of cell death using Triple staining or Hoechst dye staining under fluorescence microscopy. The experiments have shown that HNE-induced cell death is mainly apoptotic when concentrations between 1 and 50 μM have been used. The fibroblasts have undergone acute necrotic and non-specific cytotoxicity above 50 μM HNE concentrations. At cellular level, HNE has been shown to reduce intracellular glutathione levels and modulate intracellular signaling pathways [2,197]. Glutathione is one of the major cellular defense systems against oxidative stress and the reduction of glutathione by HNE could lead to production of reactive oxygen species. Consequently,

we have investigated HNE-induced ROS production in response to HNE treatment via utilization of a ROS-sensitive fluorescence probe. The fluorescence microscopic analyses have shown that HNE induced increased ROS production, which could be effectively repressed by resveratrol. These findings are concordant with the pro-oxidative effect of HNE.

Resveratrol is a phytoalexin, which is one of most widely distributed flavones in nature and highly found in grape skin and seed, mulberries and peanuts. Resveratrol has been shown to exert anti-inflammatory and anti-proliferative effects in various cellular systems [198]. The cardioprotective effect of resveratrol as an important constituent of red wine has been proposed to be responsible for the "French paradox", which reflects a low incidence of cardiovascular diseases along with a diet rich in fat [199]. Apoptosis and inflammation has been shown to be involved in the pathogenesis of atherosclerosis and this anti-apoptotic effect of resveratrol may be involved in its protective function against atherosclerosis [200]. Recent reports have mainly demonstrated that resveratrol acts as a cancer chemopreventive molecule mainly acting through triggering of apoptosis. Resveratrol was shown to induce pro-apoptotic p53 pathway through activation of ERK 1/2 and p38 MAP kinases in a mouse epidermal cell line [201]. In contrast, resveratrol has been shown to suppress TNF-induced NF-κB and AP-1 activation and apoptosis [174]. Ischemia-reperfusion- and β-amyloid-mediated cellular injuries were shown to be prevented by resveratrol pretreatment [173,202]. Our results here demonstrate that resveratrol could effectively prevent HNE-induced cytotoxicity and apoptosis at physiologically achievable concentrations. Interestingly, when we loaded 3T3 cells at concentrations higher than 50 μM, resveratrol could induce an additive, but not a synergistic pro-apoptotic effect with HNE. This important finding underlines the activation or inhibition of distinct cellular pathways in response to high or low dose resveratrol treatment including HNE-induced ROS production.

An extrinsic pathway, which involves direct initiator cascades triggered by death receptors on cell surface and an intrinsic pathway, which involves mitochondria and intracellular death signals, characterize mammalian apoptosis cascades. HNE has been shown to induce either apoptosis or proliferation in a concentration-dependent manner. HNE, at concentrations of 1-2.5 μM stimulated proliferation in vascular smooth muscle

cells [203]. In contrast, HNE has been demonstrated to induce apoptosis in various cell types, which involves either intrinsic or extrinsic apoptosis pathways [204,205]. In spite of intense research conducted, the exact mechanisms, which are involved in HNE-induced apoptosis, remained to be elucidated. In our hands, HNE at 20 μM concentration efficiently induced apoptosis in 3T3 cells and after characterization of kinetics in HNE-induced apoptosis, we then tried to identify the apoptotic mechanisms involved in this apoptotic process. Here we show that HNE leads to the release of cytochrome *c* from mitochondria and caspase-9 and caspase-3 activation with kinetics similar to that for induction of apoptosis. Contrarily, we could not detect any activation of caspase-8, which indicates that HNE-induced apoptosis does not involve an extrinsic apoptosis pathway. Similarly, we could not detect Smac/DIABLO release or caspase-2 activation in response to HNE, which eliminates the possibility of an alternative mitochondrial apoptotic process or ER stress pathway.

5.2 HNE-induced modulation of AP-1 activation

AP-1 transcriptional activity has been demonstrated to be related to either apoptosis or proliferation in different cellular systems (Shaulian and Karin, 2002). Being the major component of transcription factor AP-1, c-Jun once activated can subsequently activate the transcription of several genes. The fact that c-Jun gene itself also contained an AP-1 element in its promoter indicates that phosphorylation of c-Jun allows signals in this pathway to be amplified. Activation of AP-1 is regulated by complex mechanisms, which consist of distinct effects on the preexisting AP-1 complex or on *de novo* synthesis of AP-1 subunits [81]. Another major member of the AP-1 transcription factor protein complexes, c-Fos, has been proposed to be necessary for cell proliferation and cell cycle progression in response to growth factor stimulation [206,207]. In contrast, both *c-jun* and *c-fos* mRNa levels have been reported to increase in lymphocyte apoptosis [208,209]. In HL60 leukemia cells HNE-induced apoptosis has been shown to take place following JNK activation, c-Jun phosphorylation and AP-1 activation, which could be attenuated by transfection of HL60 cells with glutathione S-transferase isozymes [210]. Activation of AP-1 in response to HNE treatment has also been reported to occur through caspase activation in cultured rat cortical neurons [211].

HNE has been shown to upregulate both c-Jun and c-Fos protein levels in aortic smooth muscle cells [203]. It was assumed that induction of protein kinases by HNE might cause activation of signaling pathways, which phosphorylated and activated c-Jun [157, 212]. Additionally, inhibition of proliferation of HeLa cells by HNE has been demonstrated to be mediated by upregulation of *c-fos* gene expression [213]. The experimental evidence presented here indicated the involvement of HNE in AP-1 activation, as shown by the phosphorylation and potent expression of c-Jun. Interestingly, in our experimental conditions HNE at 20 μM induced a moderate decrease in c-Fos protein levels. HNE has been shown to upregulate the transcription factor AP-1 in all cell types studied so far, including hepatocytes, neuronal cells and vascular smooth muscle cells [157,203,211]. In brief, our results have presented that HNE induced the activation of an AP-1 complex mainly formed by c-Jun/c-Jun dimers, which compatible with increased c-Jun and phospho-c-Jun levels and decreased c-Fos level. Thereby, HNE may concurrently activate a pro-apoptotic c-Jun-mediated pathway and de-activate an anti-apoptotic c-Fos-mediated pathway, which aggravates the apoptotic response of fibroblasts in response to HNE treatment.

5.3 Mining the signaling pathways involved in HNE-induced apoptosis

5.3.1 c-Jun and MAP kinases

Amino-terminal phosphorylation of c-Jun at serines 63 and 73 has been reported to regulate both proliferation and stress-induced apoptosis [116]. Furthermore, overexpression of a dominant-negative c-Jun (DN-c-Jun) mutant with a cleaved N-terminal fragment has been shown to attenuate neuronal apoptosis induced by survival factor withdrawal [121,214]. In contrast, DN-c-Jun gene transfer has also been demonstrated to inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia [215]. Additionally, overexpression of wild type c-Jun has been shown to trigger an apoptotic response in 3T3 fibroblasts and human vascular endothelial cells [216,217]. Our results indicated a pro-apoptotic role for c-Jun/AP-1 activation by HNE

because transfection of 3T3 fibroblasts with a DN-c-Jun mutant significantly attenuated HNE-induced apoptosis in 3T3 fibroblasts.

Since we had demonstrated the requirement of c-Jun in HNE-induced apoptosis, we investigated the activation of upstream MAP kinases, which are proline-directed protein kinases involved in regulation of critical cellular processes such as proliferation, cell cycle progression, gene expression and apoptosis. It has been implicated that ERK 1/2, which are primarily activated in response to growth factor stimulation, are involved in process of cellular proliferation and differentiation. Whereas, JNK and p38 MAP kinases are characterized by their strong response to cellular stresses such as UV light, serum deprivation, DNA damaging agents and pro-inflammatory cytokines [218,219]. In contrast, activated MAP kinases could remain unrelated to apoptotic process. It should be noted that multiple cellular and molecular parameters should be considered when defining a signal transduction pathway either pro-apoptotic or anti-apoptotic, such as magnitude and duration of the signal and origin of stimuli. MAP kinases were reported to be activated by reactive oxygen intermediates (ROI) and oxLDL, but the mechanisms by which ROI and lipid peroxidation end products activate MAP kinases are unclear and the precise molecular targets are yet to be identified [220]. Previous reports have shown differential regulation or activation of MAP kinase pathways by HNE. HNE-induced activation of JNK has been initially presented on hepatic stellate cells and direct modification of p46 and p54 isoforms of JNK has been demonstrated [221]. In addition, in hepatic stellate cells no alteration of ERK pathway or c-Fos protein level has been observed. In vascular endothelial cells, stimulation of cells with HNE resulted in activation of ERK 1/2, p38 and JNK MAP kinases [222]. Besides, HNE has been shown to induce significant phosphorylation of JNK and p38, but not ERK 1/2 in rat hepatic epithelial cells [157]. Interestingly, it has been demonstrated that HNE induced a strong activation of JNK in PC12 cells during HNE-induced apoptosis, while ERK 1/2 and p38 MAP kinases remained unaffected [156]. JNK3 has also been reported to mediate a caspase-dependent HNE-induced apoptotic pathway in sympathetic neurons [223]. Further, treatment of IMR-90 human lung fibroblasts with 25 μM HNE markedly activated ERK 1/2 and p38 without any effect on JNK MAP kinases [224]. The present findings suggest that HNE leads to activation of JNK and p38 MAP kinases, but with different time kinetics and amplitudes. HNE (20 μM) led to a transient activation of p38 and an immediate increase of p-JNK levels, which reached

a maximum at 30 min. In contrast, phospho-ERK 1/2 down-regulation was induced at 30 min following HNE treatment and no phospho-ERK 1/2 could be detected at 4 h following HNE treatment. An interesting finding, which was derived from dose-dependent activation patterns of MAP kinases by HNE, was that activation of JNK and p38 kinases was induced maximally at 20 μM HNE concentration. The involvement of MAP kinases in HNE-induced apoptosis was evaluated using their specific inhibitors. Pretreatment with JNK inhibitor (SP600125) effectively prevented the apoptosis induced by 20 μM HNE. ERK inhibitor (PD98059) or p38 inhibitor (SB203580) pretreatment did not have any significant effect on the apoptosis induced by 20 μM HNE.

In order to establish the molecular mechanisms of this differential MAP kinase involvement in HNE-induced apoptosis, we investigated the release of cytochrome *c* and caspase-9 and caspase-3 activation by utilizing pretreatments with specific MAP kinase inhibitors before HNE stimulation. Pretreatment with JNK inhibitor prevented cytochrome *c* release and caspase activation by 20 μM HNE, which indicates that JNK acts upstream of mitochondria in the apoptotic signaling machinery. Furthermore, pretreatment of cells with JNK inhibitor prior to HNE stimulation attenuated AP-1 DNA binding activity, but we could not able to observe any significant effect with p38 or ERK 1/2 inhibitors. Additionally, overexpression of a dominant-negative JNK1 mutant efficiently inhibited HNE-induced apoptosis, which confirmed the involvement of JNK in HNE-mediated apoptosis in 3T3 fibroblasts. The protective effect of DN-JNK1 overexpression was more pronounced than DN-c-Jun overexpression, which underlines JNK targets other than c-Jun in HNE-induced apoptotic pathway, such as Bcl-2 protein members. Interestingly, a recent report has proposed the involvement of the Bcl-2 family member, Bim in c-Jun-mediated apoptosis and cytochrome *c* release in neuronal cells, which indicates c- Jun target genes that are involved in HNE-induced apoptosis should be identified [121,214].

5.3.2 Protective mechanisms of resveratrol through JNK/AP-1 pathway

To investigate the mechanisms of this protection by resveratrol, we further evaluated its effects on HNE-induced c-Jun/AP-1 activation. Resveratrol prevents increased c-Jun expression and phosphorylation, as well as AP-1 DNA binding activity

at a comparable efficiency with JNK inhibitor. It is noteworthy that HNE-induced p38 and JNK activation could be inhibited efficiently by resveratrol. Resveratrol has also restored the decreased c-Fos and phospho-ERK 1/2 levels. The exact mechanism responsible for the inhibitory effect of resveratrol on HNE-induced JNK activation remained to be elucidated, but it is possible that resveratrol exerts its activity either by inhibiting upstream kinases of JNK such as protein kinase C (PKC) or by activating JNK-specific protein phosphatases. Additionally, caspase activation and cytochrome *c* release from mitochondria have also been attenuated by resveratrol pretreatment. Considering the involvement of c-Jun/AP-1 and JNK pathway in HNE-induced apoptosis, resveratrol blocks the apoptotic machinery through inhibition of these pathways upstream of mitochondria. Moreover, inhibition of HNE-induced ROS production may also be interpreted as a secondary mechanism regarding the protective capacity of resveratrol.

5.4 HNE-induced apoptosis and Bcl-2 proteins

The release of cytochrome *c* from mitochondria is regulated, in turn, through the activation of pro-apoptotic Bcl-2 protein family members, along with possible inactivation of anti-apoptotic Bcl-2 protein family members [12,33,60,66]. HNE-induced apoptosis involves a mitochondria-dependent caspase activation cascade. Interestingly, there is little available data on HNE-induced modulation of Bcl-2 protein family members. Bcl-2 protein expression has been shown to protect against HNE-induced apoptosis of PC12 cells induced by oxidative stress and HNE [225]. PC12 cells expressing Bcl-2 has been demonstrated to exhibit higher levels of glutathione and lower levels of HNE after oxidative stress [225]. Furthermore, Bcl-2 and 4-hydroxynonenal modified proteins have been shown to colocalize in microglial cells and neurons of rat brain following transient focal ischemia [226]. Moreover, overexpression of anti-apoptotic protein Bcl-2 in stably transfected RAW 364.7 murine macrophages has been demonstrated to prevent HNE-induced internucleosomal DNA fragmentation and apoptosis [227]. Furthermore, constitutive expression of high levels of Bcl-2 protected cells from HNE-mediated apoptosis and inhibited cytochrome *c* release from mitochondria and subsequent caspase-2, -3, and -9 activation [228]. In our experimental system, HNE induced a prominent decrease in Bcl-2 levels, but we could not detect any alteration of Bcl-x_L protein level. The decrease in Bcl-2 protein at a

delayed time point in HNE-induced apoptosis implied a late response role for Bcl-2 against activated apoptotic machinery. The expression of Bcl-2 is mainly controlled by the transcription factor NF-κB and HNE has been shown to downregulate NF-κB activation in various studies. Indeed, we were expecting an early upregulation of Bcl-2 to prevent HNE-induced apoptosis. Thereby, this late response of Bcl-2 could be because of inhibition of NF-κB and failure of Bcl-2-mediated anti-apoptotic response. HNE-induced Bax expression has been reported in SK-N-BE neuroblastoma cell line [229]. Our results have shown that HNE also induced increased pro-apoptotic Bax, Bak and Bim proteins. Bak $^{-/-}$ Bax $^{-/-}$ MEFs have been shown to be insensitive to multiple apoptotic stimuli including chemotherapeutics and UV radiation [60]. Furthermore, Bax-null cells have been shown to be resistant against receptor-mediated apoptosis [61]. Activation of caspase cascades by DNA damage and ER stress has been shown to be directly regulated by Bax and Bak in double knock-out MEFs [63]. The involvement of Bax and Bak in HNE-induced apoptosis is evident as the time kinetics of cytochrome c release and caspase activation is compatible with increased Bax and Bak proteins. Bim is a direct activator BH3-only protein, which interact with pro-apoptotic Bcl-2 proteins such as Bak and Bax and thereby induce their activation/oligomerization [66]. HNE-induced upregulation/activation of Bim could induce Bax or Bak activation, which could overcome the protective effect of Bcl-2 through selective sequestration and functional silencing.

We further investigated the effect of resveratrol treatment on HNE-induced modulation of Bcl-2 proteins. The results have shown that resveratrol prevented HNE-induced downregulation of Bcl-2 and upregulation of Bax, Bak and Bim. These results indicate that Bcl-2 protein members are among the targets of resveratrol in addition to JNK/AP-1 pathway. The modulation of upstream signaling pathways regulating the expression of Bcl-2 protein by resveratrol is a possible explanation for this phenomena, but the exact upstream molecular targets remain to be identified.

The formation of Michael adducts with free thiol groups has been described as the main reaction of HNE. In addition to thiols, HNE could also presumably form Michael adducts with the imidazole moiety of histidine residues. By way of this reaction, the aromatic nitrogen of histidine could attack the olefinic moiety of HNE at

its β position just as with any Michael-addition to an α,β-unsaturated carbonyl compound (Figure 5.1A).

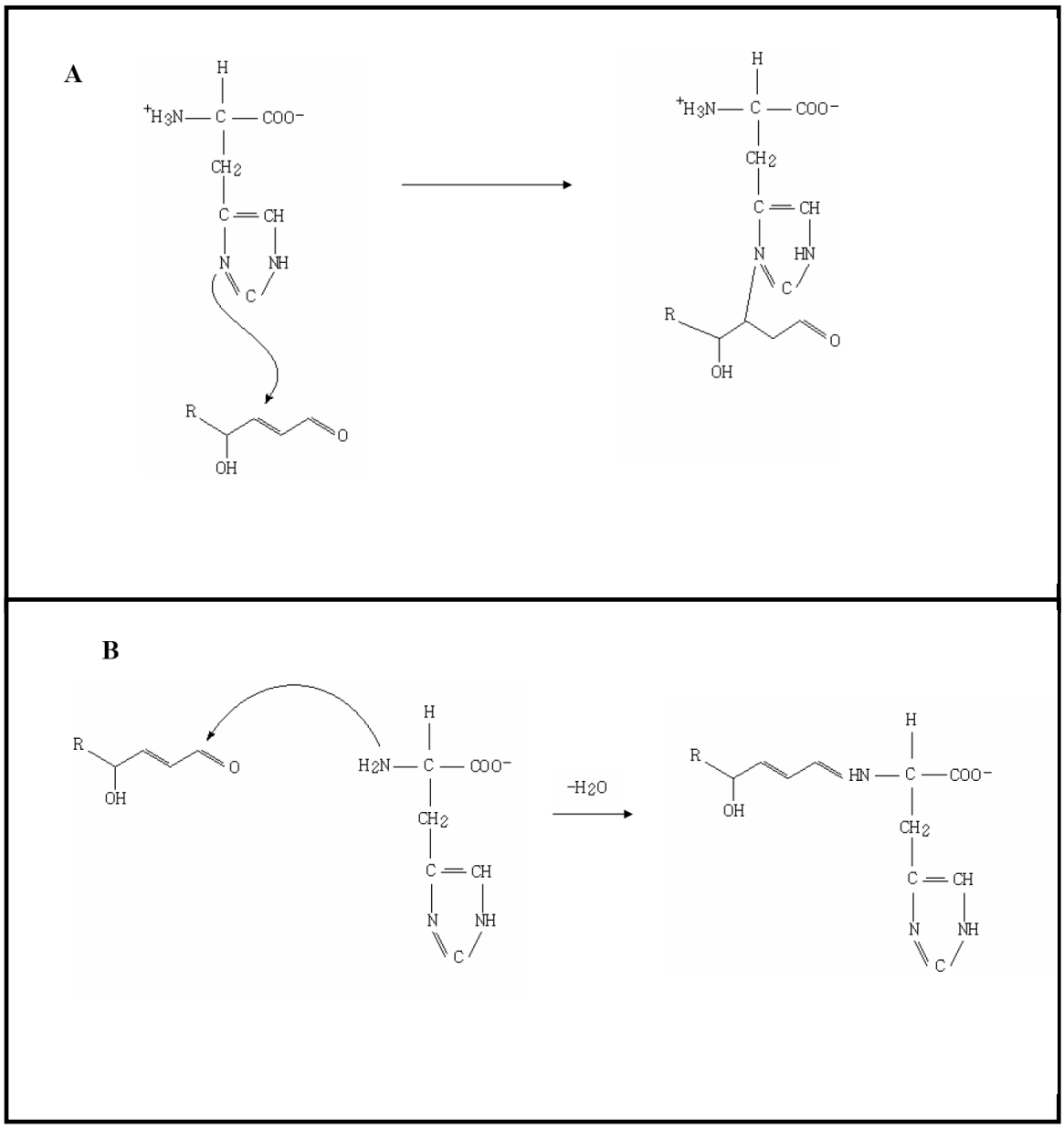


Figure 5.1 Proposed mechanisms of HNE-induced modification of histidine residues. (A) HNE could participate in a Michael addition reaction with the imidazole moiety of histidine residues. (B) HNE could react with the primary amine group histidine to form a Schiff base conjugate.

HNE may also react with the primary amine group of histidine to form a Schiff base conjugate (Figure 5.1B). In principle, other products could also be formed, given appropriate conditions. For instance, it is possible to have a

Michael addition to an α,β-unconjugated Schiff base or a double Michael adduct to histidine residues, affording a charged aromatic ring in the process. These direct modifications potentially have the ability of modulating cellular and molecular functionality of critical signaling proteins, which may be responsible for the cellular toxicity of HNE.

While much work has been conducted on the consequences of HNE, surprisingly little effort has been directed at identifying the direct products of HNE reaction with proteins and other biological components. Perhaps the technical difficulties of such an undertaking have been one factor that has discouraged such investigations. At any rate, in light of the implications related to the work herein, it would follow to reason that the time has come to readdress the question as to which direct chemical derivatives are responsible for the cellular response. A radiolabeling approach might be one way to address this issue. For instance, tritiated or 14-C radiolabeled HNE could be incubated under the same conditions as non-radioactive HNE was used in the work conducted herein. The direct reaction products could then be isolated, chromatographically separated, located by autoradiography, and identified using appropriate standards

6 CONCLUSION

In conclusion, we report here on the activation of apoptotic signaling pathways by HNE, which involves the activation of JNK-c-Jun/AP-1 signaling cascade, mitochondrial cytochrome *c* release and caspase-9/3 activation (Figure 6.1). Furthermore, involvement of Bcl-2 proteins in HNE-induced apoptosis is evident. As summarized in Figure 6.1, resveratrol prevents JNK, c-Jun/AP-1, caspase activation and apoptosis induced by HNE. Resveratrol also prevents HNE-induced downregulation of anti-apoptotic Bcl-2 protein and upregulation of pro-apoptotic Bax, Bak and Bim proteins. Thus, our studies provided a new insight into the molecular mechanisms of HNE-induced apoptosis and the cytoprotective properties of resveratrol.

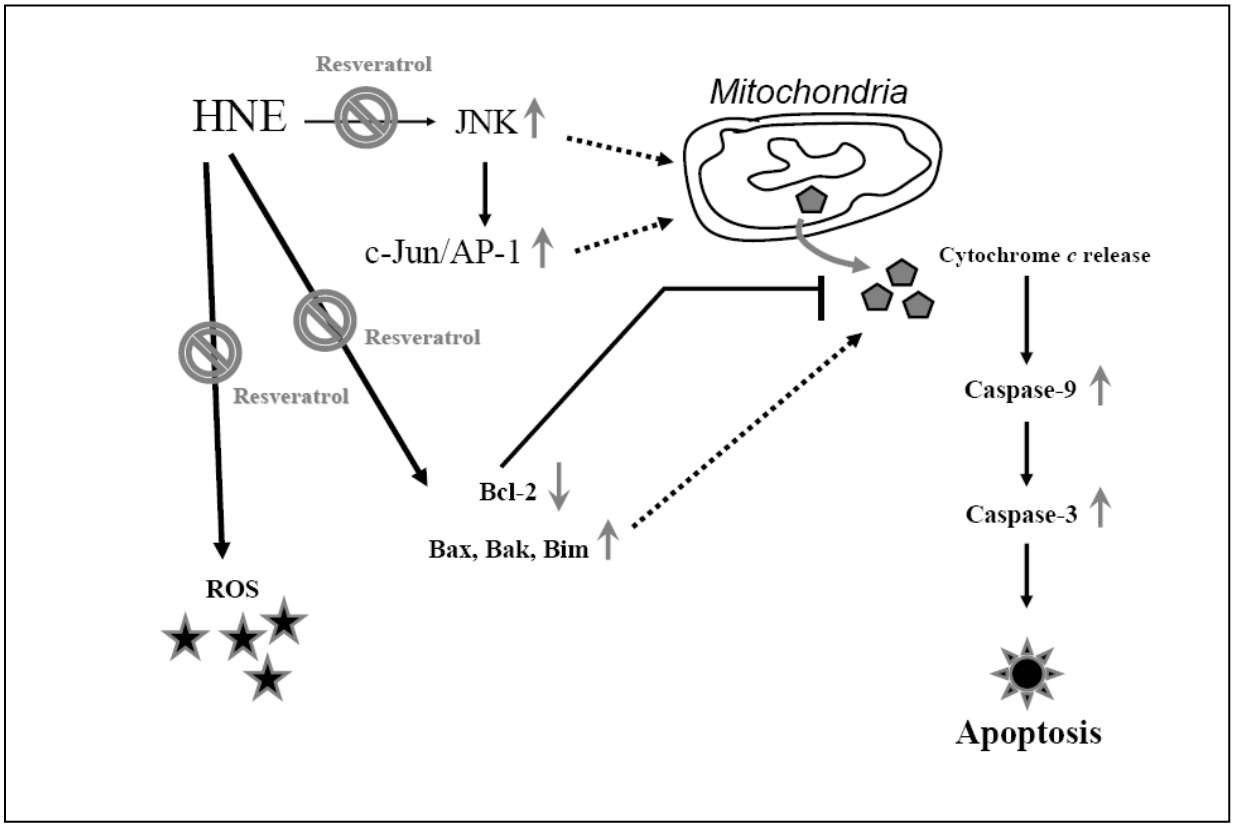


Figure 6.1 Proposed mechanisms of HNE-induced apoptosis signaling pathways and molecular targets of resveratrol in HNE-induced apoptotic cascades.

The present results argue strongly that c-Jun-mediated genes as well as direct JNK target proteins other than c-Jun/AP-1 pathway, which are involved in HNE-induced apoptosis, remained to be identified. The pro-apoptotic Bim has been proposed as a direct mediator of c-Jun-mediated apoptosis and it is also a potential mediator of HNE-induced transcription-dependent apoptotic signaling. Moreover, identification of these mechanisms along with utilization small-molecule inhibitors such as resveratrol may enable the development of specific and rationale therapies against human diseases caused by deregulation of apoptosis.

7 REFERENCES

1. Leonarduzzi G, Arkan MC, Basaga H, Chiarpotto E, Sevanian A, Poli G. Lipid oxidation products in cell signaling. (2000) *Free Radic Biol Med.* **28:**1370-1378

2. Leonarduzzi G, Robbesyn F, Poli G. Signaling kinases modulated by 4-hydroxynonenal. (2004) *Free Radic Biol Med.* **37:**1694-1702.

3. Awasthi YC, Sharma R, Cheng JZ, Yang Y, Sharma A, Singhal SS, Awasthi S. Role of 4-hydroxynonenal in stress-mediated apoptosis signaling. (2003) *Mol Aspects Med.* **24:**219-230.

4. Yang Y, Sharma R, Sharma A, Awasthi S, Awasthi YC. Lipid peroxidation and cell cycle signaling: 4-hydroxynonenal, a key molecule in stress mediated signaling. (2003) *Acta Biochim Pol.* **50:**319-336.

5. Minekura H, Kumagai T, Kawamoto Y, Nara F, Uchida K. 4-Hydroxy-2-nonenal is a powerful endogenous inhibitor of endothelial response. (2001) *Biochem Biophys Res Commun.* **282:**557-561.

6. Schaeferhenrich A, Beyer-Sehlmeyer G, Festag G, Kuechler A, Haag N, Weise A, Liehr T, Claussen U, Marian B, Sendt W, Scheele J, Pool-Zobel BL. Human adenoma cells are highly susceptible to the genotoxic action of 4-hydroxy-2-nonenal. (2003) *Mutat Res.* **526:**19-32.

7. Hyun DH, Lee MH, Halliwell B, Jenner P.Proteasomal dysfunction induced by 4-hydroxy-2,3-trans-nonenal, an end-product of lipid peroxidation: a mechanism contributing to neurodegeneration? (2002) *J Neurochem.* **83:**360-370.

8. Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. (2005) *J Intern Med.* **258:**479-517.

9. Krantic S, Mechawar N, Reix S, Quirion R. Molecular basis of programmed cell death involved in neurodegeneration. (2005) *Trends Neurosci.* **28:**670-676.

10. Chang L, Karin M. Mammalian MAP kinase signalling cascades. (2001) *Nature.* **410:**37-40.

11. Daniel PT. Dissecting the pathways to death. (2000) *Leukemia.* **14:**2035-2044.

12. Chao DT, Korsmeyer SJ. BCL-2 family: regulators of cell death. (1998) *Annu Rev Immunol.* **16:**395-419.

13. Glucksmann A. Cell deaths in normal vertebrate ontogeny. (1951) *Biol. Rev.* **26**:58–86.

14. Lockshin RA, Zakeri Z. Programmed cell death and apoptosis: origins of the theory. Nat Rev Mol Cell Biol. 2001 Jul;2(7):545-50.

15. Vogt C. Untersuchungen über die Entwicklungsgeschichte der Geburtshelferkroete (Alytes obstetricans) (1842) *Solothurn*. **:**130.

16. Lockshin RA, Williams CM. Programmed cell death. II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmoths. (1964) *J Insect Physiol.* **10:** 643-649.

17. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. (1972) *Br J Cancer.* **26:**239-57.

18. Strasser A, O'Connor L, Dixit VM. Apoptosis signaling. (2000) *Annu Rev Biochem.* **69:**217-245.

19. del Peso L, Gonzalez VM, Inohara N, Ellis RE, Nunez G. Disruption of the CED-9.CED-4 complex by EGL-1 is a critical step for programmed cell death in Caenorhabditis elegans. (2000) *J Biol Chem.* **275:**27205-27211.

20. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. (1998) *Science.* **281:**1305-1308.

21. Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. (1999) *J Biol Chem.* **274:**5053-5060.

22. Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, Blenis J, Krammer PH, Walczak H. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. (2000) Immunity. **12:**599-609.

23. Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P, Blenis J, Tschopp J. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. (2000) *Nat Cell Biol.* **2:**241-243.

24. Sartorius U, Schmitz I, Krammer PH. Molecular mechanisms of death-receptor-mediated apoptosis. (2001) *Chembiochem.* **2:**20-29.

25. Soderstrom TS, Poukkula M, Holmstrom TH, Heiskanen KM, Eriksson JE. Mitogen-activated protein kinase/extracellular signal-regulated kinase signaling in activated T cells abrogates TRAILinduced apoptosis upstream of the mitochondrial amplification loop and caspase-8. (2002) *J Immunol.* **169:**2851-2860.

26. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signaling pathways. (1998) *EMBO J.* **17:**1675-1787.

27. Ochs K, Kaina B. Apoptosis induced by DNA damage O6-methylguanine is Bcl-2 and caspase-9/3 regulated and Fas/caspase-8 independent. (2000) *Cancer Res.* **60:**5815-5824.

28. Slee EA, Keogh SA, Martin SJ. Cleavage of BID during cytotoxic drug and UV radiation-induced apoptosis occurs downstream of the point of Bcl-2 action and is catalysed by caspase-3: a potential feedback loop for amplification of apoptosis-associated mitochondrial cytochrome c release. (2000) *Cell Death Differ.* **7:**556-565.

29. de Moissac D, Gurevich RM, Zheng H, Singal PK, Kirshenbaum LA. Caspase activation and mitochondrial cytochrome c release during hypoxia-mediated apoptosis of adult ventricular myocytes. (2000) J Mol Cell Cardiol. **32:**53-63.

30. Adrain C, Creagh EM, Martin SJ. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. (2001) *EMBO J.* **20:**6627-6636.

31. van Loo G, van Gurp M, Depuydt B, Srinivasula SM, Rodriguez I, Alnemri ES, Gevaert K, Vandekerckhove J, Declercq W, Vandenabeele P. The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. (2002) *Cell Death Differ.* **9:**20-26.

32. van Gurp M, Festjens N, van Loo G, Saelens X, Vandenabeele P. Mitochondrial intermembrane proteins in cell death. (2003) *Biochem Biophys Res Commun.* **304:**487-497.

33. Nguyen M, Millar DG, Yong VW, Korsmeyer SJ, Shore GC. Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. (1993) *J Biol Chem.* **268:**25265-25268.

34. del Mar Martinez-Senac M, Corbalan-Garcia S, Gomez-Fernandez JC. Study of the secondary structure of the C-terminal domain of the antiapoptotic protein bcl-2 and its interaction with model membranes. (2000) *Biochemistry.* **39:**7744-7752.

35. Aritomi M, Kunishima N, Inohara N, Ishibashi Y, Ohta S, Morikawa K. Crystal structure of rat Bcl-xL. (1997) Implications for the function of the Bcl-2 protein family. *J Biol Chem.* **272:**27886-27892.

36. Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong SL, Ng SL, Fesik SW. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. (1996) *Nature.* **381:**335-341.

37. Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW. Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. (1997) *Science.* **275:**983-986.

38. Korsmeyer SJ, Gross A, Harada H, Zha J, Wang K, Yin XM, Wei M, Zinkel S. Death and survival signals determine active/inactive conformations of pro-apoptotic BAX, BAD, and BID molecules. (1999) *Cold Spring Harb Symp Quant Biol.* **64:**343-350.

39. Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, Martinou JC. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. (1999) *J Cell Biol.* **144:**891-901.

40. He H, Lam M, McCormick TS, Distelhorst CW. Maintenance of calcium homeostasis in the endoplasmic reticulum by Bcl-2. (1997) *J Cell Biol.* **138:**1219-1228.

41. Bruce-Keller AJ, Begley JG, Fu W, Butterfield DA, Bredesen DE, Hutchins JB, Hensley K, Mattson MP. Bcl-2 protects isolated plasma and mitochondrial membranes against lipid peroxidation induced by hydrogen peroxide and amyloid beta-peptide. (1998) *J Neurochem.* **70:**31-39.

42. Bogdanov MB, Ferrante RJ, Mueller G, Ramos LE, Martinou JC, Beal MF. Oxidative stress is attenuated in mice overexpressing BCL-2. (1999) *Neurosci Lett.* **262:**33-36.

43. Mirkovic N, Voehringer DW, Story MD, McConkey DJ, McDonnell TJ, Meyn RE. Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols. (1997) *Oncogene.* **15:**1461-1470.

44. Decaudin D, Geley S, Hirsch T, Castedo M, Marchetti P, Macho A, Kofler R, Kroemer G. Bcl-2 and Bcl-XL antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. (1997) Cancer Res. **57:**62-67.

45. Rokhlin OW, Guseva N, Tagiyev A, Knudson CM, Cohen MB. Bcl-2 oncoprotein protects the human prostatic carcinoma cell line PC3 from TRAIL-mediated apoptosis. (2001) *Oncogene.* **20:**2836-2843.

46. Memon SA, Moreno MB, Petrak D, Zacharchuk CM. Bcl-2 blocks glucocorticoid- but not Fas- or activation-induced apoptosis in a T cell hybridoma. (1995) *J Immunol.* **155:**4644-4652.

47. Gazitt Y, Shaughnessy P, Montgomery W. Apoptosis-induced by TRAIL AND TNFalpha in human multiple myeloma cells is not blocked by BCL-2. (1999) *Cytokine.* **11:**1010-1019.

48. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. (1993) *Cell.* **75:**229-240.

49. Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Nakayama K, Negishi I, Senju S, Zhang Q, Fujii S, et al. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. (1995) *Science.* **267:**1506-1510.

50. Murphy KM, Streips UN, Lock RB. Bcl-2 inhibits a Fas-induced conformational change in the Bax N terminus and Bax mitochondrial translocation. (2000) *J Biol Chem.* **275:**17225-17228.

51. He L, Perkins GA, Poblenz AT, Harris JB, Hung M, Ellisman MH, Fox DA. Bcl-xL overexpression blocks bax-mediated mitochondrial contact site formation and apoptosis in rod photoreceptors of lead-exposed mice. (2003) *Proc Natl Acad Sci U S A.* **100:**1022-1027.

52. Ito T, Deng X, Carr B, May WS. Bcl-2 phosphorylation required for anti-apoptosis function. (1997) *J Biol Chem.* **272:**11671-11673.

53. Yamamoto K, Ichijo H, Korsmeyer SJ. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. (1999) *Mol Cell Biol.* **19:**8469-8478.

54. Basu A, Haldar S. Identification of a novel Bcl-xL phosphorylation site regulating the sensitivity of taxol- or 2-methoxyestradiol-induced apoptosis. (2003) *FEBS Lett.* **538:**41-47.

55. Nechushtan A, Smith CL, Hsu YT, Youle RJ. Conformation of the Bax C-terminus regulates subcellular location and cell death. (1999) *EMBO J.* **18:**2330-2341.

56. Antonsson B, Montessuit S, Sanchez B, Martinou JC. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. (2001) *J Biol Chem.* **276:**11615-11623.

57. Griffiths GJ, Dubrez L, Morgan CP, Jones NA, Whitehouse J, Corfe BM, Dive C, Hickman JA. Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. (1999) *J Cell Biol.* **144:**903-914.

58. Mandic A, Viktorsson K, Strandberg L, Heiden T, Hansson J, Linder S, Shoshan MC. Calpain-mediated Bid cleavage and calpain-independent Bak modulation: two separate pathways in cisplatin-induced apoptosis. (2002) *Mol Cell Biol.* **22:**3003-3013.

59. Ruffolo SC, Shore GC. BCL-2 selectively interacts with the BID-induced open conformer of BAK, inhibiting BAK auto-oligomerization. (2003) *J Biol Chem.* **278:**25039-25045.

60. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. (2001) Science. **292:**727-730.

61. Deng Y, Lin Y, Wu X. TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. (2002) *Genes Dev.* **16:**33-45.

62. Han J, Goldstein LA, Gastman BR, Rabinovitz A, Wang GQ, Fang B, Rabinowich H. Differential involvement of Bax and Bak in TRAIL-mediated apoptosis of leukemic T cells. (2004) *Leukemia.* **18:**1671-1680.

63. Ruiz-Vela A, Opferman JT, Cheng EH, Korsmeyer SJ. Proapoptotic BAX and BAK control multiple initiator caspases. (2005) *EMBO Rep.* **6:**379-385.

64. Wood DE, Thomas A, Devi LA, Berman Y, Beavis RC, Reed JC, Newcomb EW. Bax cleavage is mediated by calpain during drug-induced apoptosis. (1998) *Oncogene.* **17:**1069-1078.

65. Cao X, Deng X, May WS. Cleavage of Bax to p18 Bax accelerates stress-induced apoptosis, and a cathepsin-like protease may rapidly degrade p18 Bax. (2003) *Blood.* **102:**2605-2614.

66. Letai A. BH3 domains as BCL-2 inhibitors: prototype cancer therapeutics. (2003) *Expert Opin Biol Ther.* **3:**293-304.

67. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. (1997) *Cell.* **91:**231-241.

68. Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB, Greenberg ME. 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. (2000) *Mol Cell.* **6:**41-51.

69. Konishi Y, Lehtinen M, Donovan N, Bonni A. Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery. (2002) *Mol Cell.* **9:**1005-1016.

70. Donovan N, Becker EB, Konishi Y, Bonni A. JNK phosphorylation and activation of BAD couples the stress-activated signaling pathway to the cell death machinery. (2002) *J Biol Chem.* **277:**40944-40949.

71. Dramsi S, Scheid MP, Maiti A, Hojabrpour P, Chen X, Schubert K, Goodlett DR, Aebersold R, Duronio V. Identification of a novel phosphorylation site, Ser-170, as a regulator of bad pro-apoptotic activity. (2002) *J Biol Chem.* **277:**6399-6405.

72. Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B, Alix S, Youle RJ, LaMarche A, Maroney AC, Johnson EM Jr. JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. (2003) *Neuron.* **38:**899-914.

73. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. (2001) *Mol Cell.* **7:**683-694.

74. Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. (2000) *Science.* **288:**1053-1058.

75. Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. (2001) *Mol Cell.* **7:**673-682.

76. Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. (2003) *Proc Natl Acad Sci U S A.* **100:**1931-1936.

77. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. (1998) *Cell.* **94:**491-501.

78. Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. (1999) *J Biol Chem.* **274:**1156-1163.

79. Zha J, Weiler S, Oh KJ, Wei MC, Korsmeyer SJ. Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. (2000) *Science.* **290:**1761-1765.

80. Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. (2002) *Annu Rev Immunol.* **20:**55-72.

81. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. (2002) *Nat Cell Biol.* **4:**E131-E136.

82. Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR. Phosphorylation of c-jun mediated by MAP kinases. (1991) *Nature.* **353:**670-674.

83. Smeal T, Binetruy B, Mercola DA, Birrer M, Karin M. Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. (1991) *Nature.* **354:**494-496.

84. Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, Derijard B, Davis RJ. Selective interaction of JNK protein kinase isoforms with transcription factors. (1996) *EMBO J.* **15:**2760-2770.

85. Yang D, Tournier C, Wysk M, Lu HT, Xu J, Davis RJ, Flavell RA. Targeted disruption of the MKK4 gene causes embryonic death, inhibition of c-Jun NH2-terminal kinase activation, and defects in AP-1 transcriptional activity. (1997) *Proc Natl Acad Sci U S A.* **94:**3004-3009.

86. Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. (2000) *Science.* **288:**870-874.

87. Davis RJ. Signal transduction by the JNK group of MAP kinases. (2000) *Cell.* **103:**239-252.

88. Rubinfeld H, Seger R.The ERK cascade: a prototype of MAPK signaling. (2005) *Mol Biotechnol.* **31:**151-174.

89. Hagemann C, Blank JL. The ups and downs of MEK kinase interactions. (2001) *Cell Signal.* **13:**863-875.

90. Balmanno K, Cook SJ. Sustained MAP kinase activation is required for the expression of cyclin D1, p21Cip1 and a subset of AP-1 proteins in CCL39 cells. (1999) *Oncogene.* **18:**3085-3097.

91. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. (1995) *Science.* **270:**1326-1331.

92. Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. (2005) *Cell Res.* **15:**11-18.

93. Fan M, Chambers TC. Role of mitogen-activated protein kinases in the response of tumor cells to chemotherapy. (2001) *Drug Resist Updat.* **4:**253-267.

94. Faris M, Kokot N, Latinis K, Kasibhatla S, Green DR, Koretzky GA, Nel A. The c-Jun N-terminal kinase cascade plays a role in stress-induced apoptosis in Jurkat cells by up-regulating Fas ligand expression. (1998) *J Immunol.* **160:**134-144.

95. Ito Y, Mishra NC, Yoshida K, Kharbanda S, Saxena S, Kufe D. Mitochondrial targeting of JNK/SAPK in the phorbol ester response of myeloid leukemia cells. (2001) *Cell Death Differ.* **8:**794-800.

96. Maundrell K, Antonsson B, Magnenat E, Camps M, Muda M, Chabert C, Gillieron C, Boschert U, Vial-Knecht E, Martinou JC, Arkinstall S. Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. (1997) *J Biol Chem.* **272:**25238-25242.

97. Fan M, Goodwin M, Vu T, Brantley-Finley C, Gaarde WA, Chambers TC. Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade. (2000) *J Biol Chem.* **275:**29980-29985.

98. Chen YR, Wang X, Templeton D, Davis RJ, Tan TH. The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. (1996) *J Biol Chem.* **271:**31929-31936.

99. Sarkar D, Su ZZ, Lebedeva IV, Sauane M, Gopalkrishnan RV, Valerie K, Dent P, Fisher PB. mda-7 (IL-24) Mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. (2002) *Proc Natl Acad Sci U S A.* **99:**10054-10059.

100. Porras A, Zuluaga S, Black E, Valladares A, Alvarez AM, Ambrosino C, Benito M, Nebreda AR. p38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. (2004) *Mol Biol Cell.* **15:**922-933.

101. Park JG, Yuk Y, Rhim H, Yi SY, Yoo YS. Role of p38 MAPK in the regulation of apoptosis signaling induced by TNF-alpha in differentiated PC12 cells. (2002) *J Biochem Mol Biol.* **35:**267-272.

102. Juretic N, Santibanez JF, Hurtado C, Martinez J. ERK 1,2 and p38 pathways are involved in the proliferative stimuli mediated by urokinase in osteoblastic SaOS-2 cell line. (2001) *J Cell Biochem.* **83:**92-98.

103. Zhang J, Gao JX, Salojin K, Shao Q, Grattan M, Meagher C, Laird DW, Delovitch TL. Regulation of fas ligand expression during activation-induced cell death in T cells by p38 mitogen-activated protein kinase and c-Jun NH2-terminal kinase. (2000) *J Exp Med.* **191:**1017-1030.

104. Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S, Valladares A, Perez L, Klein R, Nebreda AR. Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. (2000) Mol Cell. **6:**109-116.

105. Bulavin DV, Saito S, Hollander MC, Sakaguchi K, Anderson CW, Appella E, Fornace AJ Jr. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. (1999) *EMBO J.* **18:**6845-6854.

106. Qiao L, Studer E, Leach K, McKinstry R, Gupta S, Decker R, Kukreja R, Valerie K, Nagarkatti P, El Deiry W, Molkentin J, Schmidt-Ullrich R, Fisher PB, Grant S, Hylemon PB, Dent P. Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis. (2001) *Mol Biol Cell.* **12:**2629-2645.

107. Giroux S, Tremblay M, Bernard D, Cardin-Girard JF, Aubry S, Larouche L, Rousseau S, Huot J, Landry J, Jeannotte L, Charron J. Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. (1999) *Curr Biol.* **9:**369-372.

108. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. (2001) *Endocr Rev.* **22:**153-183.

109. Lin SJ, Shyue SK, Hung YY, Chen YH, Ku HH, Chen JW, Tam KB, Chen YL. Superoxide dismutase inhibits the expression of vascular cell adhesion molecule-1 and intracellular cell adhesion molecule-1 induced by tumor necrosis factor-alpha in human endothelial cells through the JNK/p38 pathways. (2005) *Arterioscler Thromb Vasc Biol.* **25:**334-340.

110. Tindberg N, Porsmyr-Palmertz M, Simi A. Contribution of MAP kinase pathways to the activation of ATF-2 in human neuroblastoma cells. (2000) *Neurochem Res.* **25:**527-531

111. Janulis M, Silberman S, Ambegaokar A, Gutkind JS, Schultz RM. Role of mitogen-activated protein kinases and c-Jun/AP-1 trans-activating activity in the regulation of protease mRNAs and the malignant phenotype in NIH 3T3 fibroblasts. (1999) *J Biol Chem.* **274:**801-813.

112. Morton S, Davis RJ, Cohen P. Signalling pathways involved in multisite phosphorylation of the transcription factor ATF-2. (2004) *FEBS Lett.* **572:**177-183.

113. Cosenza SC, Yumet G, Soprano DR, Soprano KJ. Induction of c-fos and c-jun mRNA at the M/G1 border is required for cell cycle progression. (1994) *J Cell Biochem.* **55:**503-512.

114. Kovary K, Bravo R. The jun and fos protein families are both required for cell cycle progression in fibroblasts. (1991) *Mol Cell Biol.* **11:**4466-4472.

115. Brown JR, Nigh E, Lee RJ, Ye H, Thompson MA, Saudou F, Pestell RG, Greenberg ME. Fos family members induce cell cycle entry by activating cyclin D1. (1998) *Mol Cell Biol.* **18:**5609-5619.

116. Behrens A, Sibilia M, Wagner EF. Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. (1999) Nat Genet. **21:**326-329.

117. Schreiber M, Kolbus A, Piu F, Szabowski A, Mohle-Steinlein U, Tian J, Karin M, Angel P, Wagner EF. Control of cell cycle progression by c-Jun is p53 dependent. (1999) *Genes Dev.* **13:**607-619.

118. Smeyne RJ, Vendrell M, Hayward M, Baker SJ, Miao GG, Schilling K, Robertson LM, Curran T, Morgan JI. Continuous c-fos expression precedes programmed cell death in vivo. (1993) *Nature.* **363:**166-169.

119. Estus S, Zaks WJ, Freeman RS, Gruda M, Bravo R, Johnson EM Jr. Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. (1994) *J Cell Biol.* **127:**1717-1727.

120. Colotta F, Polentarutti N, Sironi M, Mantovani A. Expression and involvement of c-fos and c-jun protooncogenes in programmed cell death induced by growth factor deprivation in lymphoid cell lines. (1992) *J Biol Chem.* **267:**18278-18283.

121. Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. (2001) *Neuron.* **29:**629-643.

122. Le-Niculescu H, Bonfoco E, Kasuya Y, Claret FX, Green DR, Karin M. Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. (1999) *Mol Cell Biol.* **19:**751-763.

123. Yang DD, Kuan CY, Whitmarsh AJ, Rincon M, Zheng TS, Davis RJ, Rakic P, Flavell RA. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. (1997) *Nature.* **389:**865-870.

124. Shaulian E, Schreiber M, Piu F, Beeche M, Wagner EF, Karin M. The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. (2000) *Cell.* **103:**897-907.

125. Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, Kolesnick RN. Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. (1996) *Nature.* **380:**75-79.

126. Ivanov VN, Bhoumik A, Krasilnikov M, Raz R, Owen-Schaub LB, Levy D, Horvath CM, Ronai Z. Cooperation between STAT3 and c-jun suppresses Fas transcription. (2001) *Mol Cell.* **7:**517-528.

127. Kutuk O, Basaga H. Inflammation meets oxidation: NF-kappaB as a mediator of initial lesion development in atherosclerosis. (2003) *Trends Mol Med.* **9:**549-557.

128. Resink TJ, Tkachuk VA, Bernhardt J, Buhler FR. Oxidized low density lipoproteins stimulate phosphoinositide turnover in cultured vascular smooth muscle cells. (1992) *Arterioscler Thromb.* **12:**278-285.

129. Fyrnys B, Claus R, Wolf G, Deigner HP. Oxidized low density lipoprotein stimulates protein kinase C (PKC) activity and expression of PKC-isotypes via prostaglandin-H-synthase in P388D1 cells. (1997) Adv Exp Med Biol. **407:**93-98.

130. Suc I, Meilhac O, Lajoie-Mazenc I, Vandaele J, Jurgens G, Salvayre R, Negre-Salvayre A. Activation of EGF receptor by oxidized LDL. (1998) *FASEB J.* **12:**665-671.

131. Martens JS, Reiner NE, Herrera-Velit P, Steinbrecher UP. Phosphatidylinositol 3-kinase is involved in the induction of macrophage growth by oxidized low density lipoprotein. (1998) *J Biol Chem.* **273:**4915-4920.

132. Maziere C, Alimardani G, Dantin F, Dubois F, Conte MA, Maziere JC. Oxidized LDL activates STAT1 and STAT3 transcription factors: possible involvement of reactive oxygen species. (1999) *FEBS Lett.* **448:**49-52.

133. Kusuhara M, Chait A, Cader A, Berk BC. Oxidized LDL stimulates mitogen-activated protein kinases in smooth muscle cells and macrophages. (1997) *Arterioscler Thromb Vasc Biol.* **17:**141-148.

134. Maziere C, Djavaheri-Mergny M, Frey-Fressart V, Delattre J, Maziere JC. Copper and cell-oxidized low-density lipoprotein induces activator protein 1 in fibroblasts, endothelial and smooth muscle cells. (1997) FEBS Lett. **409:**351-356.

135. Ricote M, Huang J, Fajas L, Li A, Welch J, Najib J, Witztum JL, Auwerx J, Palinski W, Glass CK. Expression of the peroxisome proliferator-activated receptor gamma (PPARgamma) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein. (1998) *Proc Natl Acad Sci U S A.* **95:**7614-7619.

136. Brambilla G, Sciaba L, Faggin P, Maura A, Marinari UM, Ferro M, Esterbauer H. Cytotoxicity, DNA fragmentation and sister-chromatid exchange in Chinese hamster ovary cells exposed to the lipid peroxidation product 4-hydroxynonenal and homologous aldehydes. (1986) *Mutat Res.* **171:**169-176.

137. Schneider C, Tallman KA, Porter NA, Brash AR. Two distinct pathways of formation of 4-hydroxynonenal. Mechanisms of nonenzymatic transformation of the 9- and 13-hydroperoxides of linoleic acid to 4-hydroxyalkenals. (2001) *J Biol Chem.* **276:**20831-20838.

138. Karlhuber GM, Bauer HC, Eckl PM. Cytotoxic and genotoxic effects of 4-hydroxynonenal in cerebral endothelial cells. (1997) *Mutat Res.* **381:**209-216.

139. Schaur RJ. Basic aspects of the biochemical reactivity of 4-hydroxynonenal. (2003) *Mol Aspects Med.* **24:**149-159.

140. Siems W, Grune T. Intracellular metabolism of 4-hydroxynonenal. (2003) *Mol Aspects Med.* **24:**167-175.

141. Siems W, Carluccio F, Grune T, Jakstadt M, Quast S, Hampl H, Sommerburg O. Elevated serum concentration of cardiotoxic lipid peroxidation products in chronic renal failure in relation to severity of renal anemia. (2002) *Clin Nephrol.* **58:**S20-S25.

142. Grune T, Michel P, Sitte N, Eggert W, Albrecht-Nebe H, Esterbauer H, Siems WG. Increased levels of 4-hydroxynonenal modified proteins in plasma of children with autoimmune diseases. (1997) *Free Radic Biol Med.* **23:**357-360.

143. Siems WG, Capuozzo E, Verginelli D, Salerno C, Crifo C, Grune T. Inhibition of NADPH oxidase-mediated superoxide radical formation in PMA-stimulated human neutrophils by 4-hydroxynonenal--binding to -SH and -NH2 groups. (1997) *Free Radic Res.* **27:**353-358.

144. Liu RM, Gao L, Choi J, Forman HJ. Gamma-glutamylcysteine synthetase: mRNA stabilization and independent subunit transcription by 4-hydroxy-2-nonenal. (1998) *Am J Physiol.* **275:**L861-L869.

145. Dickinson DA, Iles KE, Watanabe N, Iwamoto T, Zhang H, Krzywanski DM, Forman HJ. 4-hydroxynonenal induces glutamate cysteine ligase through JNK in HBE1 cells. (2002) *Free Radic Biol Med.* **33:**974-987.

146. Grune T, Davies KJ. The proteasomal system and HNE-modified proteins. (2003) *Mol Aspects Med.* **24:**195-204.

147. Newton AC. The ins and outs of protein kinase C. (2003) *Methods Mol Biol.* **233:**3-7.

148. Dianzani MU. 4-Hydroxynonenal and cell signalling. (1998) *Free Radic Res.* **28:**553-560.

149. Paola D, Domenicotti C, Nitti M, Vitali A, Borghi R, Cottalasso D, Zaccheo D, Odetti P, Strocchi P, Marinari UM, Tabaton M, Pronzato MA. Oxidative stress induces increase in intracellular amyloid beta-protein production and selective activation of betaI and betaII PKCs in NT2 cells. (2000) *Biochem Biophys Res Commun.* **268:**642-646.

150. Nitti M, Domenicotti C, d'Abramo C, Assereto S, Cottalasso D, Melloni E, Poli G, Biasi F, Marinari UM, Pronzato MA. Activation of PKC-beta isoforms mediates HNE-induced MCP-1 release by macrophages. (2002) *Biochem Biophys Res Commun.* **294:**547-552.

151. Chiarpotto E, Domenicotti C, Paola D, Vitali A, Nitti M, Pronzato MA, Biasi F, Cottalasso D, Marinari UM, Dragonetti A, Cesaro P, Isidoro C, Poli G. Regulation of rat hepatocyte protein kinase C beta isoenzymes by the lipid peroxidation product 4-hydroxy-2,3-nonenal: A signaling pathway to modulate vesicular transport of glycoproteins. (1999) *Hepatology.* **29:**1565-1572.

152. Castello L, Marengo B, Nitti M, Froio T, Domenicotti C, Biasi F, Leonarduzzi G, Pronzato MA, Marinari UM, Poli G, Chiarpotto E. 4-Hydroxynonenal signalling to apoptosis in isolated rat hepatocytes: the role of PKC-delta. (2005) *Biochim Biophys Acta.* **1737:**83-93.

153. Escargueil-Blanc I, Salvayre R, Vacaresse N, Jurgens G, Darblade B, Arnal JF, Parthasarathy S, Negre-Salvayre A. Mildly oxidized LDL induces activation of platelet-derived growth factor beta-receptor pathway. (2001) *Circulation.* **104:**1814-1821.

154. Liu W, Akhand AA, Kato M, Yokoyama I, Miyata T, Kurokawa K, Uchida K, Nakashima I. 4-hydroxynonenal triggers an epidermal growth factor receptor-linked signal pathway for growth inhibition. (1999) *J Cell Sci.* **112:**2409-2417.

155. Kapiotis S, Hermann M, Held I, Seelos C, Ehringer H, Gmeiner BM. Genistein, the dietary-derived angiogenesis inhibitor, prevents LDL oxidation and protects endothelial cells from damage by atherogenic LDL. (1997) *Arterioscler Thromb Vasc Biol.* **17:**2868-2874

156. Soh Y, Jeong KS, Lee IJ, Bae MA, Kim YC, Song BJ. Selective activation of the c-Jun N-terminal protein kinase pathway during 4-hydroxynonenal-induced apoptosis of PC12 cells. (2000) *Mol Pharmacol.* **58:**535-541.

157. Uchida K, Shiraishi M, Naito Y, Torii Y, Nakamura Y, Osawa T. Activation of stress signaling pathways by the end product of lipid peroxidation. 4-hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. (1999) *J Biol Chem.* **274:**2234-2242.

158. Camandola S, Scavazza A, Leonarduzzi G, Biasi F, Chiarpotto E, Azzi A, Poli G. Biogenic 4-hydroxy-2-nonenal activates transcription factor AP-1 but not NF-kappa B in cells of the macrophage lineage. (1997) *Biofactors.* **6:**173-179.

159. Ruef J, Moser M, Bode C, Kubler W, Runge MS. 4-hydroxynonenal induces apoptosis, NF-kappaB-activation and formation of 8-isoprostane in vascular smooth muscle cells. (2001) *Basic Res Cardiol.* **96:**143-150.

160. Ji C, Kozak KR, Marnett LJ. IkappaB kinase, a molecular target for inhibition by 4-hydroxy-2-nonenal. (2001) *J Biol Chem.* **276:**18223-18228.

161. Cornwell T, Cohick W, Raskin I. Dietary phytoestrogens and health. (2004) *Phytochemistry.* **65:**995-1016.

162. Bradamante S, Barenghi L, Villa A. Cardiovascular protective effects of resveratrol. (2004) *Cardiovasc Drug Rev.* **22:**169-188.

163. Cos P, De Bruyne T, Apers S, Vanden Berghe D, Pieters L, Vlietinck AJ. Phytoestrogens: recent developments. (2003) *Planta Med.* **69:**589-599.

164. Hao HD, He LR. Mechanisms of cardiovascular protection by resveratrol. (2004) *J Med Food.* **7:**290-298.

165. Bertelli AA, Giovannini L, Stradi R, Urien S, Tillement JP, Bertelli A. Kinetics of trans- and cis-resveratrol (3,4',5-trihydroxystilbene) after red wine oral administration in rats. (1996) *Int J Clin Pharmacol Res.* **16:**77-81.

166. Karlsson J, Emgard M, Brundin P, Burkitt MJ. Trans-resveratrol protects embryonic mesencephalic cells from tert-butyl hydroperoxide: electron paramagnetic resonance spin trapping evidence for a radical scavenging mechanism. (2000) *J Neurochem.* **75:**141-150.

167. Martinez J, Moreno JJ. Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. (2000) *Biochem Pharmacol.* **59:**865-870.

168. Vinson JA, Teufel K, Wu N. Red wine, dealcoholized red wine, and especially grape juice, inhibit atherosclerosis in a hamster model. (2001) *Atherosclerosis.* **156:**67-72.

169. Ozgova S, Hermanek J, Gut I. Different antioxidant effects of polyphenols on lipid peroxidation and hydroxyl radicals in the NADPH-, Fe-ascorbate- and Fe-microsomal systems. (2003) *Biochem Pharmacol.* **66:**1127-1137.

170. Stivala LA, Savio M, Carafoli F, Perucca P, Bianchi L, Maga G, Forti L, Pagnoni UM, Albini A, Prosperi E, Vannini V. Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. (2001) *J Biol Chem.* **276:**22586-22594.

171. Leonard SS, Xia C, Jiang BH, Stinefelt B, Klandorf H, Harris GK, Shi X. Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. (2003) *Biochem Biophys Res Commun.* **309:**1017-1026.

172. Jang JH, Surh YJ. Protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells. (2001) *Mutat Res*. **496:**181-190.

173. Jang JH, Surh YJ. Protective effect of resveratrol on beta-amyloid-induced oxidative PC12 cell death. (2003) *Free Radic Biol Med.* **34:**1100-1110.

174. Manna SK, Mukhopadhyay A, Aggarwal BB. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. (2000) *J Immunol.* **164:**6509-6519.

175. Bhat KP, Pezzuto JM. Cancer chemopreventive activity of resveratrol. (2002) *Ann N Y Acad Sci.* **957:**210-229.

176. Fontecave M, Lepoivre M, Elleingand E, Gerez C, Guittet O. Resveratrol, a remarkable inhibitor of ribonucleotide reductase. (1998) *FEBS Lett.* **421:**277-279.

177. Kim YA, Choi BT, Lee YT, Park DI, Rhee SH, Park KY, Choi YH. Resveratrol inhibits cell proliferation and induces apoptosis of human breast carcinoma MCF-7 cells. (2004) *Oncol Rep.* **11:**441-446.

178. Hemann MT, Bric A, Teruya-Feldstein J, Herbst A, Nilsson JA, Cordon-Cardo C, Cleveland JL, Tansey WP, Lowe SW. Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants. (2005) *Nature.* **436:**807-811.

179. Tolcher AW. Targeting Bcl-2 protein expression in solid tumors and hematologic malignancies with antisense oligonucleotides. (2005) *Clin Adv Hematol Oncol.* **3:**635-642.

180. Chorna IV, Datsyuk LO, Stoika RS. Expression of Bax, Bad and Bcl-2 proteins under x-radiation effect towards human breast carcinoma MCF-7 cells and their doxorubicin-resistant derivatives. (2005) *Exp Oncol.* **27:**196-201.

181. Tsuruo T, Naito M, Tomida A, Fujita N, Mashima T, Sakamoto H, Haga N. Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal. (2003) *Cancer Sci.* **94:**15-21.

182. Rose LM, Latchman DS, Isenberg DA. Apoptosis in peripheral lymphocytes in systemic lupus erythematosus: a review. (1997) *Br J Rheumatol.* **36:**158-163.

183. Andrikoula M, Tsatsoulis A. The role of Fas-mediated apoptosis in thyroid disease. (2001) *Eur J Endocrinol.* **144:**561-568.

184. Chou CT, Yang JS, Lee MR. Apoptosis in rheumatoid arthritis--expression of Fas, Fas-L, p53, and Bcl-2 in rheumatoid synovial tissues. (2001) *J Pathol.* **193:**110-116.

185. Ekshyyan O, Aw TY. Apoptosis: a key in neurodegenerative disorders. (2004) *Curr Neurovasc Res.* **1:**355-371.

186. LeBlanc AC. The role of apoptotic pathways in Alzheimer's disease neurodegeneration and cell death. (2005) *Curr Alzheimer Res.* **2:**389-402.

187. Reed JC, Pellecchia M. Apoptosis-based therapies for hematologic malignancies. (2005) *Blood.* **106:**408-418.

188. Kunz M, Ibrahim SM. Molecular responses to hypoxia in tumor cells. (2003) *Mol Cancer.* **2:**23-27.

189. Petit F, Arnoult D, Viollet L, Estaquier J. Intrinsic and extrinsic pathways signaling during HIV-1 mediated cell death. (2003) *Biochimie.* **85:**795-811.

190. Endemann DH, Schiffrin EL. Endothelial dysfunction. (2004) *Am Soc Nephrol.* **15:**1983-1892.

191. Robbesyn F, Salvayre R, Negre-Salvayre A. Dual role of oxidized LDL on the NF-kappaB signaling pathway. (2004) *Free Radic Res.* **38:**541-551.

192. Dimmeler S, Zeiher AM. Vascular repair by circulating endothelial progenitor cells: the missing link in atherosclerosis? (2004) *J Mol Med.* **82:**671-677.

193. Stoneman VE, Bennett MR. Role of apoptosis in atherosclerosis and its therapeutic implications. (2004) *Clin Sci (Lond).* **107:**343-354.

194. Kutuk O, Basaga H. Aspirin prevents apoptosis and NF-kappaB activation induced by H_2O_2 in Hela cells. (2003) *Free Radic Res.* **37:**1267-1276.

195. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. (1991) *Free Radic Biol Med.* **11:**81–128.

196. Kowalczyk P, Ciesla JM, Komisarski M, Kusmierek JT, Tudek B. Long-chain adducts of trans-4-hydroxy-2-nonenal to DNA bases cause recombination, base substitutions and frameshift mutations in M13 phage. (2004) *Mutat Res.* **550:**33-48.

197. Zhang H, Dickinson DA, Liu RM, Forman HJ. 4-Hydroxynonenal increases gamma-glutamyl transpeptidase gene expression through mitogen-activated protein kinase pathways. (2005) *Free Radic Biol Med.* **38:**463-471.

198. Alarcon de la Lastra C, Villegas I. Resveratrol as an anti-inflammatory and anti-aging agent: Mechanisms and clinical implications. (2005) *Mol Nutr Food Res.* **49:**405-430.

199. Bradamante, S, Barenghi, L., and Villa, A. Cardiovascular protective effects of resveratrol. *Cardiovasc.* (2004) *Drug Rev.* **22:**169-188.

200. Delmas D, Jannin B, Latruffe N. Resveratrol: Preventing properties against vascular alterations and ageing. (2005) *Mol Nutr Food Res.* **49:**377-395.

201. She QB, Bode AM, Ma WY, Chen NY, Dong Z. Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. (2001) *Cancer Res.* **61:**1604-1610.

202. Dernek S, Ikizler M, Erkasap N, Ergun B, Koken T, Yilmaz K, Sevin B, Kaygisiz Z, Kural T. Cardioprotection with resveratrol pretreatment: improved

beneficial effects over standard treatment in rat hearts after global ischemia. (2004) *Scand Cardiovasc J.* **38:**245-254.

203. Ruef J, Rao GN, Li F, Bode C, Patterson C, Bhatnagar A, Runge MS. Induction of rat aortic smooth muscle cell growth by the lipid peroxidation product 4-hydroxy-2-nonenal. (1998) *Circulation* **97:**1071-1078.

204. Zhang W, He Q, Chan LL, Zhou F, El Naghy M, Thompson EB. Ansari NH. Involvement of caspases in 4-hydroxy-alkenal-induced apoptosis in human leukemic cells. (2001) *Free Radic Biol Med.* **30:**699-706.

205. Liu W, Kato M, Akhand AA, Hayakawa A, Suzuki H, Miyata T, Kurokawa K, Hotta Y, Ishikawa N, Nakashima I. 4-hydroxynonenal induces a cellular redox status-related activation of the caspase cascade for apoptotic cell death. (2000) *J Cell Sci.* **113:**635-641.

206. Gao R, Ball DK, Perbal B, Brigstock DR. Connective tissue growth factor induces c-fos gene activation and cell proliferation through p44/42 MAP kinase in primary rat hepatic stellate cells**.** (2004) *J Hepatol.* **40:**431-438.

207. Tanos T, Marinissen MJ, Leskow FC, Hochbaum D, Martinetto H, Gutkind JS, Coso OA. Phosphorylation of c-Fos by members of the p38 MAPK family. Role in the AP-1 response to UV light. (2005) *J Biol Chem.* **280:**18842-18852.

208. Goldstone SD, Lavin MF. Prolonged expression of c-jun and associated activity of the transcription factor AP-1, during apoptosis in a human leukaemic cell line. *(1994) Oncogene.* **9:**2305-2311.

209. Pruschy M, Shi YQ, Crompton NE, Steinbach J, Aguzzi A, Glanzmann C, Bodis S. The proto-oncogene c-fos mediates apoptosis in murine T-lymphocytes induced by ionizing radiation and dexamethasone. (1997) *Biochem Biophys Res Commun.* **241:**519-524.

210. Cheng JZ, Singhal SS, Sharma A, Saini M, Yang Y, Awasthi S, Zimniak P, Awasthi YC. Transfection of mGSTA4 in HL-60 cells protects against 4-hydroxynonenal-induced apoptosis by inhibiting JNK-mediated signaling. (2001) *Arch Biochem Biophys.* **392:**197-207.

211. Camandola S, Poli G, Mattson MP. The lipid peroxidation product 4-hydroxy-2,3-nonenal increases AP-1-binding activity through caspase activation in neurons. (2000) *J Neurochem.* **74:**159-168.

212. Kikuta K, Masamune A, Satoh M, Suzuki N, Shimosegawa T. 4-hydroxy-2, 3-nonenal activates activator protein-1 and mitogen-activated protein kinases in rat pancreatic stellate cells. (2004) *World J Gastroenterol.* **10:**2344-2351;

213. Kreuzer T, Zarkovic N, Grube R, Schaur RJ. Inhibition of HeLa cell proliferation by 4-hydroxynonenal is associated with enhanced expression of the c-fos oncogene. (1997) *Cancer Biother Radiopharm.* **12:**131-136.

214. Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M, Rubin LL. A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. (1995) *Neuron.* **14:**927-939.

215. Yasumoto H, Kim S, Zhan Y, Miyazaki H, Hoshiga M, Kaneda Y, Morishita R, Iwao H. Dominant negative c-jun gene transfer inhibits vascular smooth muscle cell proliferation and neointimal hyperplasia in rats. (2001) *Gene Ther.* **8**:1682-1689.

216. Bossy-Wetzel E, Bakiri L, Yaniv M. Induction of apoptosis by the transcription factor c-Jun. (1997) *EMBO J.* **16:**1695-1709.

217. Wang N, Verna L, Hardy S, Zhu Y, Ma KS, Birrer MJ, Stemerman MB. c-Jun triggers apoptosis in human vascular endothelial cells. (1999) *Circ Res.* **85:**387-393.

218. Schaeffer HJ, Weber MJ. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. (1999) *Mol Cell Biol.* **19:**2435-2444.

219. Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. (1997) *Curr Opin Cell Biol.* **9:**180–186.

220. Wang X, Martindale JL, Liu Y, Holbrook NJ. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. (1998) *Biochem J.* **333:**291-300.

221. Parola M, Robino G, Marra F, Pinzani M, Bellomo G, Leonarduzzi G, Chiarugi P, Camandola S, Poli G, Waeg G, Gentilini P, Dianzani, MU. HNE interacts directly with JNK isoforms in human hepatic stellate cells. (1998) *J Clin Invest.* **102:**1942-1950.

222. Usatyuk PV, Natarajan V. Role of mitogen-activated protein kinases in 4-hydroxy-2-nonenal-induced actin remodeling and barrier function in endothelial cells. (2004) *J Biol Chem.* **279:**11789-11797.

223. Bruckner SR, Estus S. JNK3 contributes to c-jun induction and apoptosis in 4-hydroxynonenal-treated sympathetic neurons. (2002) *J Neurosci Res.* **70:**665-670.

224. Tsukagoshi H, Kawata T, Shimizu Y, Ishizuka T, Dobashi K, Mori M. 4-Hydroxy-2-nonenal enhances fibronectin production by IMR-90 human lung fibroblasts partly via activation of epidermal growth factor receptor-linked extracellular signal-regulated kinase p44/42 pathway. (2002) *Toxicol Appl Pharmacol.* **184:**127-135.

225. Kruman I, Bruce-Keller AJ, Bredesen D, Waeg G, Mattson MP. Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. (1997) *J Neurosci.* **17:**5089-5100.

226. Urabe T, Hattori N, Yoshikawa M, Yoshino H, Uchida K, Mizuno Y. Colocalization of Bcl-2 and 4-hydroxynonenal modified proteins in microglial cells and neurons of rat brain following transient focal ischemia. (1998) *Neurosci Lett.* **247:**159-162.

227. Haynes RL, Brune B, Townsend AJ. Apoptosis in RAW 264.7 cells exposed to 4-hydroxy-2-nonenal: dependence on cytochrome C release but not p53 accumulation. (2001) *Free Radic Biol Med.* **30:**884-894.

228. Ji C, Amarnath V, Pietenpol JA, Marnett LJ. 4-hydroxynonenal induces apoptosis via caspase-3 activation and cytochrome c release. (2001) *Chem Res Toxicol.* **14:**1090-1096.

229. Laurora S, Tamagno E, Briatore F, Bardini P, Pizzimenti S, Toaldo C, Reffo P, Costelli P, Dianzani MU, Danni O, Barrera G. 4-Hydroxynonenal modulation of p53 family gene expression in the SK-N-BE neuroblastoma cell line. (2005) *Free Radic Biol Med.* **38:**215-225.

230. Hallmann-Mikolajczak A. [Ebers Papyrus. The book of medical knowledge of the 16th century B.C. Egyptians] (2004) *Arch Hist Filoz Med.* **67:**5-14.

231. Newsom SW. Pioneers in infection control. Ignaz Philipp Semmelweis. (1993) *J Hosp Infect.* **23:**175-187.

232. Exner M, Hartemann P, Kistemann T. Hygiene and health - the need for a holistic approach. (2001) *Am J Infect Control.* **29:**228-231.

APPENDIX A

CHEMICALS

(in alphabetical order)

Name of Chemical	Supplier Company	Catalog Number
Acetic acid	Sigma, Germany	A9967
Acridine Orange	Sigma, Germany	A6014
Acrylamide/Bis-acrylamide	Sigma, Germany	A3699
	Biorad Inc., USA	161-0158
Agarose low EO	Applichem, Germany	A2114
Ammonium persulfate	Sigma, Germany	A3678
Ampicillin	Sigma, Germany	A9518
Antibiotic solution	Sigma, Germany	P3539
Anti-β-actin Ab	Santa Cruz Biotech, USA	sc-1616
Anti-c-Jun Ab	Santa Cruz Biotech, USA	sc-044

Anti-p-c-Jun Ab	Santa Cruz Biotech, USA	sc-046
Anti-c-Fos Ab	Santa Cruz Biotech, USA	sc-052
Anti-JNK Ab	Cell Signal Tech., USA	9252
Anti-p-JNK Ab	Cell Signal Tech., USA	9251
Anti-ERK 1/2 Ab	Cell Signal Tech., USA	9102
Anti-p-ERK 1/2 Ab	Cell Signal Tech., USA	9101
Anti-p38 Ab	Cell Signal Tech., USA	9212
Anti-p-p38 Ab	Cell Signal Tech., USA	9211
Anti-caspase 9 Ab	Cell Signal Tech., USA	9505
Anti-caspase 3 Ab	Cell Signal Tech., USA	9665
Anti-cytochrome *c*	Cell Signal Tech., USA	4272
Anti-CoxIV Ab	Cell Signal Tech., USA	4844
Bradford solution	Biorad Inc., USA	500-0001
Chloroform	Merck, Germany	102431
CM-H_2DCF-DA	Molecular Probes, USA	C-6827
Coomassie Brilliant Blue	Merck, Germany	115444

Crystal Violet	Sigma, Germany	C3886
Dulbecco's MEM (DMEM)	Sigma, Germany	D5546
DMSO	Sigma, Germany	D2650
DTT	Sigma, Germany	D9779
EDTA	Riedel-de Haén, Germany	27248
Ethanol	Riedel-de Haén, Germany	32221
Ethidium Bromide	Merck, Germany	OCO28942
Foetal Bovine Serum	Sigma, Germany	F2442
Glycerol	Riedel-de Haén, Germany	15523
Glycine	Amnesa, USA	0167
HCl	Merck, Germany	100314
Hepes	Sigma, Germany	H7006
Hoechst dye	Sigma, Germany	B2883
4-Hydroxynonenal	Calbiochem, USA	393204
Hyperfilm ECL	Amersham Biosciences, UK	RPN2114K
IPTG	Promega, Germany	V39517

Isopropanol	Riedel-de Haén, Germany	24137
KCl	Fluka, Switzerland	60129
KH_2PO_4	Riedel-de Haén, Germany	04243
KOH	Riedel-de Haén, Germany	06005
Liquid nitrogen	Karbogaz, Turkey	
Lipofectamine	Invitrogen, Germany	18324020
Luria Agar	Sigma,Germany	L-3147
Luria Broth	Sigma, Germany	L-3022
2-Mercaptoethanol	Sigma, Germany	M370-1
Methanol	Riedel-de Haén, Germany	24229
$MgCl_2$	Sigma, Germany	M9272
Milk Diluent concentrate	KPL, USA	50-82-00
NaCl	Riedel-de Haén, Germany	13423
$NaO_2C_2H_3.3H_2O$	Riedel-de Haén, Germany	25022
NaOH	Merck, Germany	106462
$NaPO_4H_2$	Riedel-de Haén, Germany	04269

NP-40	Sigma, Germany	I3021
PD98059	Calbiochem, USA	513000
Phenol	Applichem, Germany	A1153
Phenol/chloroform /isoamylalcohol	Applichem, Germany	A0889
Phosphate buffered saline	Sigma, Germany	P4417
PMSF	Sigma, Germany	P7626
Poly dI/dC	Sigma, Germany	P4929
Propidium iodide	Sigma, Germany	P4170
Resveratrol	Sigma, Germany	R5010
SB203580	Calbiochem, USA	559389
SP600125	Calbiochem, USA	420123
Sodium Dodecyl Sulphate	Sigma, Germany	L4390
Sucrose	Sigma, Germany	S0389
TEMED	Sigma, Germany	T7029
Triton X-100	Applichem, Germany	A1388

Tris	Fluka, Switzerland	93349
Tween® 20	Merck, Germany	822184

APPENDIX B

MOLECULAR BIOLOGY KITS

(in alphabetical order)

Name of Kit	Supplier Company	Catalog Number
ECL Advance Chemiluminescence Detection Kit	Amersham Biosciences, UK	RPN2135
Cell Proliferation Kit I (MTT)	Roche, Germany	1465007-001
Cell Death Detection ELISA^{PLUS}	Roche, Germany	11774425-001
Qiaprep® Spin Miniprep Kit (250)	Qiagen, Germany	27106
QIAGEN® Plasmid Midi Kit (100)	Qiagen, Germany	12145
QIAGEN® Plasmid Maxi Kit (500)	Qiagen, Germany	12165

APPENDIX C

OTHER MATERIALS

(in alphabetical order)

Name of material	**Supplier Company**	**Catalog Number**
Hybond-P membrane (PVDF)	Amersham Biosciences, UK	RPN2020F
Hyperfilm ECL	Amersham Biosciences, UK	RPN2103K
Mass Ruler DNA Ladder,Mix	Fermentas, Germany	#SM0403

(Agarose gel photograph and MW values of bands are provided below)

Protein MW Marker	Fermentas, Germany	#SM0431

(gel photograph and MW values of bands are provided below)

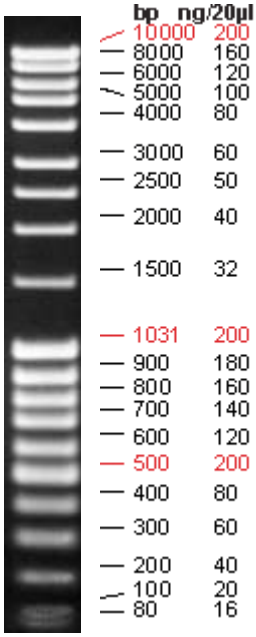
Prestained Protein MW Marker	Fermentas,Germany	#SM0441

(gel photograph and MW values of bands are provided below)

RNase A	Roche, Germany	1119915

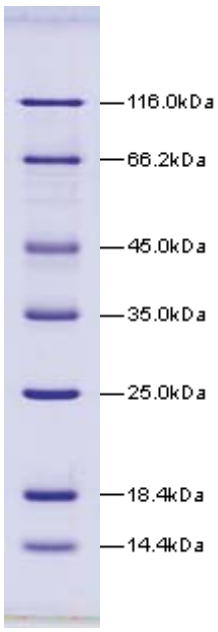
T4 kinase	Promega, Germany	M4103
T4 DNA kinase Buffer (10X)	Promega, Germany	M4103-B
TOP10	Invitrogen, Germany	C4040-03

Mass Ruler DNA LadderMix

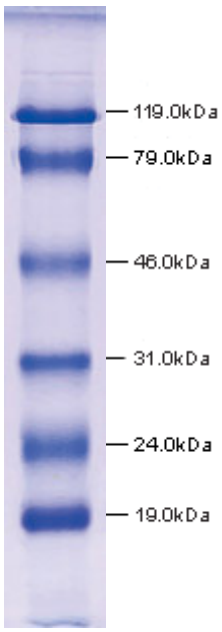


1.0% agarose

Protein MW Marker

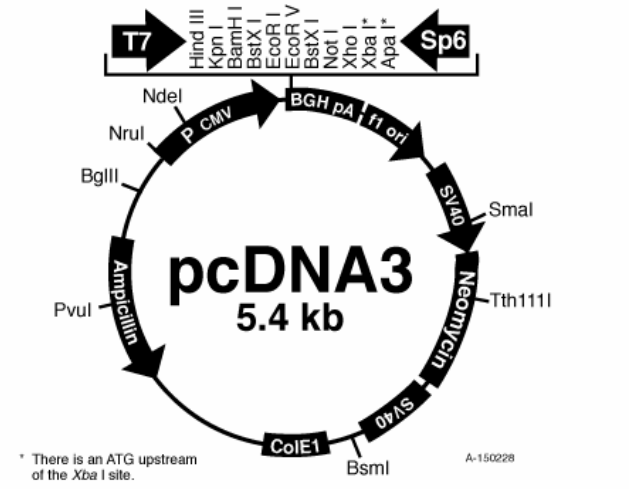


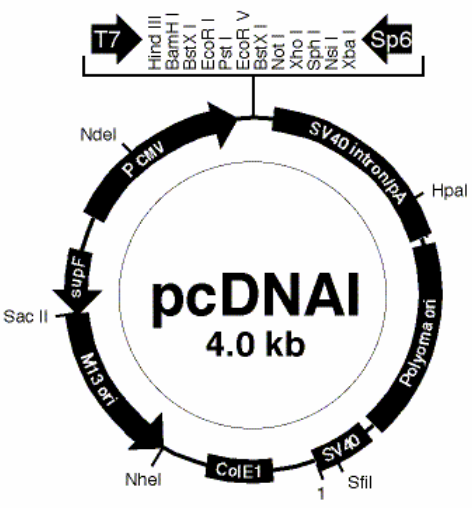
Prestained Protein Molecular Weight Marker



12% SDS-PAGE, Coomassie Brilliant Blue R-250 stained

Plasmids





APPENDIX D

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA
Balance:	Sartorius, BP211D, GERMANY
	Sartorius, BP221S, GERMANY
	Sartorius, BP610, GERMANY
	Schimadzu, Libror EB-3200 HU, JAPAN
Blot Module	X Cell II ™ Blot Module, Novex, USA
Centrifuge:	Eppendorf, 5415C, GERMANY
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Kendro Lab. Prod., Heraeus Multifuge 3L, GERMANY
	Hitachi, Sorvall RC5C Plus, USA
	Hitachi, Sorvall Discovery 100 SE, USA
Deepfreeze:	-70^{o} C, Kendro Lab. Prod., Heraeus Hfu486 Basic, GERMANY

	-20^{o} C, Bosch, TÜRKİYE
Distilled Water:	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA
	X Cell SureLock ™ Electrophoresis Cell, Novex USA
Gel Documentation:	UVITEC, UVIdoc Gel Documentation System, UK
	Biorad, UV-Transilluminator 2000, USA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
Laminar Flow:	Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY
Microliter Pipette:	Gilson, Pipetman, FRANCE
	Mettler Toledo, Volumate, USA

Microwave Oven:	Bosch, TÜRKİYE
pH meter:	WTW, pH540 GLP MultiCal®, GERMANY
Power Supply:	Biorad, PowerPac 300, USA
	Wealtec, Elite 300, USA
Refrigerator:	+4^{o} C, Bosch, TÜRKİYE
Shaker:	Forma Scientific, Orbital Shaker 4520, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., Innova™ 4330, USA
	C25HC Incubator shaker New Brunswick Scientific, USA
Sonicator	Vibracell 75043, Bioblock Scientific,FRANCE
Spectrophotometer:	Schimadzu, UV-1208, JAPAN
	Schimadzu, UV-3150, JAPAN
	Secoman, Anthelie Advanced, ITALY
Speed Vacuum:	Savant, Speed Vac® Plus Sc100A, USA
	Savant, Refrigerated Vapor Trap RVT 400, USA
Thermocycler:	Eppendorf, Mastercycler Gradient, GERMANY

Vacuum:	Heto, MasterJet Sue 300Q, DENMARK
Water bath:	Huber, Polystat cc1, GERMANY

Claims to original research and publications arising from this thesis

1. First characterization of HNE-induced apoptosis signaling cascades in 3T3 fibroblasts, including cytochrome c release, caspases, MAP kinases, AP-1 and Bcl-2 family proteins.

2. First demonstration that JNK-c-Jun/AP-1 pathway is involved in HNE-induced apoptosis.

3. First direct evidence that resveratrol could protect againts lipid peroxidation-induced ROS production.

4. First demonstration of the protective effect of resveratol against HNE-induced apoptosis through modulation of MAP kinases, caspases and Bcl-2 proteins.

5. First evidence that transcriptional activity of c-Jun is involved in HNE-induced apoptosis.

Refeered publications arising from this thesis:

1. **Kutuk, O.,** Adli, M., Poli, G., Basaga, H. Resveratrol protects against 4-HNE induced oxidativestress and apoptosis in Swiss 3T3 fibroblasts. Biofactors, Vol 20, Issue 1, 1-10, 2004.

2. **Kutuk, O.,** Poli, G., Basaga H. 4-Hydroxynonenal induces apoptosis through activation of JNK and c-Jun/AP-1: protection by resveratrol, Toxicological Sciences, Vol 90, Issue 1, 120-132, 2006.