ROLE OF BCL-2 FAMILY MEMBERS IN 4-HNE INDUCED APOPTOSIS

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Abstract

In this study we have investigated the role of Bcl-2 protein family members in response to 4-HNE induced apoptosis by using endothelial cells and monocytes as models for atherosclerosis.

Cell viability and Annexin-V staining experiments demonstrated that 4-HNE was cytotoxic for both human umbilical vein endothelial cells (huv-ec-c`s) and human leukemic monocyte lymphoma cell line (U937 cells) and induced apoptosis in a dose dependent manner. In order to gain further insight into the apoptotic mechanism, the protein levels of Bcl-2 family members were investigated by immunoblot analysis.

In 20 μM 4-HNE treated huv-ec-c`s, Bcl-$_{XL}$ and Bax were upregulated within 4 hours of treatment, followed by a sharp decrease in the prosurvival Bcl-2 protein. Among proapoptotic members of the Bcl-2 family; Bid truncation occurred prior to Bcl-2 down-regulation.

In 20μM of 4-HNE treated U937 cell line for 12 hours, Bcl-$_{XL}$ was downregulated at an early time point of 2 hours, then restored back to control level at the 4th hour, whereas prosurvival Bcl-2 had a sharp decrease in a time dependent manner. Contrary to Bcl-2 and Bcl-$_{XL}$, the protein level of Mcl-1 did not change in 12 hours time after 4-HNE treatment. The proapoptotic member of Bcl-2 family, Bax was upregulated however Bak, responsible for pore formation on the mitochondria membrane, was downregulated after the 4-HNE treatment in 2 hours time.

In 10μM of 4-HNE treated U937 cell line for 24 hours, Bcl-$_{XL}$ down-regulation took place between 2 to 8 hours and Bcl-2 was downregulated at 2 to 12 hours then Bcl-$_{XL}$ and Bcl-2

protein levels were reestablished at 12 hours and 24 hours respectively. Mcl-1 and Bak protein levels did not change. Bax was also upregulated similar to high dose treatment. Protein levels of Bim and Bid were also studied by immunoblotting and were not found to change

In our experimental system, several cell lines (huv-ec-c's and U937 cells) and several Bcl-2 family proteins` levels (Among prosurvival; Bcl-2, Bcl-$_{XL}$ and Mcl-1, among proapoptotics; Bax, Bak, Bid, Bim) were studied in response to 4-HNE treatment. Shortly, Bcl-2 proteins mainly responded in cell specific manner. Among these proteins, in huv-ec-c`s Bcl-$_{XL}$ and Bax were upregulated, Bcl-2 was downregulated. In U937 cells, Bcl-$_{XL}$ and Bcl-2 were downregulated at early time points, Mcl-1 level remained the same whereas Bax was upregulated. Only Bak protein responded in dose dependent manner in monocytes. In order to enlighten the role of Bak in the apoptotic mechanism, further investigations are necessary.

Finally, these results indicate the involvement of mitochondrial pathway and sequential activation of Bcl-2 family proteins in high and low doses of 4-HNE induced apoptosis in two different cell lines.

4-HNE İLE İNDÜKLENEN APOPTOZDA BCL-2 PROTEİN AİLESİNİN ROLÜ

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Anahtar kelimeler: Bcl-2 protein ailesi bireyleri, 4-HNE, apoptoz, huv-ec-c, U937 hücre hattı

Özet

Bu çalışmada, 4-HNE ile indüklenen apoptozda Bcl-2 protein ailesi üyelerinin rolü, ateroskleroza model teşkil eden endotel ve monosit hücreleri kullanılarak araştırılmıştır.

Hücre canlılığı ve Annexin-V boyama deneyleri, 4-HNE uygulamasının hem insan göbek bağı damar içi endotel hücrelerinde (huv-e-c-c) hem de insan lösemi monosit lenfoma hücre hattında (U937 hücreleri) sitotoksik olduğunu ve doza bağlı olarak apoptozu teşvik ettiğini göstermiştir. Apoptoz mekanizması hakkında daha ayrıntılı bilgi sahibi olmak amacıyla, Bcl-2 protein ailesi bireylerinin protein miktarları immunoblot analizleri ile incelenmiştir.

20 μM 4-HNE ile muamele edilen huv-ec-c'lerde, Bcl-$_{XL}$ ve Bax proteinlerinin seviyesi 4 saat içinde yükselmesinin ardından Bcl-2 proteinin seviyesinde ani bir azalma gerçekleşmiştir. Apoptoza indükleyen Bcl-2 protein ailesi üyelerinden, Bid'in kesilmesi de Bcl-2 proteinin azalmasından önce gözlenmiştir.

12 saat boyunca 20 μM 4-HNE ile muamele edilen U937 hücre hattında, Bcl-$_{XL}$ proteinin miktarı 2 saatte azalıp, bunu mütakiben 4. saatte kontrol miktarına geri dönerken, Bcl-2 protein miktarı zaman içerisinde belirgin bir azalma göstermeye devam etmiştir. Bcl-2 ve Bcl-$_{XL}$' den farklı olarak Mcl-1 protein seviyesinde 12 saat süren muamele esnasında herhangi bir değişiklik gözlenmemiştir. Apoptoza indükleyen Bcl-2 protein ailesi üyelerinden Bax seviyesi artarken, mitokondri zarındaki por oluşumunda sorumlu diğer önemli protein olan Bak'ın protein seviyesinde 4-HNE ile muamelesinden 2 saat sonrasından başlayan bir azalma gerçekleşmiştir.

24 saat boyunca 10 μM 4-HNE ile muamele edilen U937 hücre hattında ise Bcl-$_{XL}$ protein miktarı 2 ve 8 saat arasında azalırken Bcl-2 protein miktarı 2 ve 12 saat aralığında azalmıştır. Bcl-$_{XL}$ ve Bcl-2 proteinlerinin miktarlarının hücre içinde yeniden temini sırasıyla 12 ve 24. saatlerde gerçekleşmiştir. Mcl-1 ve Bak protein miktarlarında değişme gözlenmemiştir. Bir önceki yüksek doz sonucuna benzer olarak Bax protein seviyesindeki artış burada da görülmüştür. Bim ve Bid proteinlerinin seviyeleri de immunoblot yöntemiyle çalışılmış ancak herhangi bir değişim bulunmamıştır.

Deneysel sistemizde, farklı hücreler (huv-ec-c ve U937 hücre hatları) ve farklı Bcl-2 protein ailesi bireylerinin (yaşam destekleyici proteinlerden; Bcl-2, Bcl-$_{XL}$ ve Mcl-1; apoptoz destekleyici proteinlerden Bax, Bak, Bid ve Bim) seviyeleri 4-HNE muamelesi sonucu tetkik edilmiştir. Kısaca Bcl-2 proteinleri temel olarak hücreye spesifik cevap vermiştir. Bu proteinlerden huv-ec-c'lerde Bcl-$_{XL}$ ve Bax artarken Bcl-2 azalmıştır. U937 hücre hattında ise Bcl-$_{XL}$ ve Bcl-2 erken saatlerde azalırken, Mcl-1 seviyesi aynı kalmış ve Bax seviyesi artmıştır. Sadece Bak proteini monosit hücrelerinde farklı 4-HNE dozlarına farklı cevap vermiştir ve Bak proteininin apoptoz mekanizmasındaki rolünü aydınlatmak için daha ayrıntılı çalışmalar gerekmektedir.

Bu sonuçlar, düşük ve yüksek doz 4-HNE ile teşvik edilen apoptozda ve 2 farklı hücre hattında, mitokondrial yolağın katılımını ve Bcl-2 ailesi proteinlerinin sırasıyla aktivasyonunu göstermiştir.

"To my family"

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4-HNE	4-Hydroxynonenal
huv-ec-c	Human umbilical vein endothelial cell
U937	Human leukemic monocyte lymphoma cell line
TRADD	TNFRSF1A-associated via death domain
FADD	Fas-Associated protein with Death Domain
Daxx	A Death-Domain-Associated Protein
RIP	Ribosome inhibiting protein
RAIDD	A caspase recruitment domain (CARD) containing molecule, interacts with procaspase-2 in a CARD-dependent manner
FLIP	FLICE-inhibitory protein
DISC	Death-inducing signaling complex
UV	Ultraviolet
IAP	Inhibitor of apoptosis family of proteins.
BH	Bcl-2 Homology Domain
FOXO3A	Forkhead box O3A
CEBPα	CCAAT-Enhancer Binding Protein
CHOP	CCAAT/enhancer binding protein (C/EBP) epsilon
ERK	Extracellular signal-regulated kinase
JAK-STAT	Janus kinase-Signal Transducers and Activator of Transcription protein
AIF	Apoptosis Inducing Factor
DDP	Deafness dystonia protein
GST	Glutathione *S*-transferase
RLIP76	RalA binding protein
PUFA	Polyunsaturated fatty acids
L-OOH	Lipid hydroxyperoxides

L-OH	Lipid hydroxides
ROS	Reactive oxygen species
K562	Human immortalized myelogenous leukemia line
HLEB-3	Human lens epithelial cell line
JNK	C-Jun N-terminal kinases
ASK	Apoptosis signal-regulating kinase
MAPK	Mitogen-activated protein kinases
HSP	Heat shock protein
CEM-C7	Human leukemic cell line
ZVAD-fmk	A pan-caspase inhibitor
YVAD	Caspase-1 inhibitor
VDVAD	Caspase-2 inhibitor
DEVD	Caspase-3 inhibitor
IETD	Caspase-8 inhibitor
RKO	Colocteral carcinoma cells
PARP	Poly (ADP-ribose) polymerase
OxLDL	Oxidized low density lipoprotein
ECGS	Endothelial cell growth supplement

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1 INTRODUCTION

Role of 4-Hydroxynonenal (4-HNE) which is one of the major membrane lipid peroxidation product in cellular metabolism is becoming more important day by day. Its involvement in signaling pathways related with inflammation-related events, regulation of cell growth, differentiation and different cell death mechanisms such as apoptosis and necrosis have been declared in various studies. Its modulation of these signaling pathways are associated with the reactions of 4-HNE with related proteins, lipids, vitamins, cofactors and even with nucleic acids because of the high reactivity of this molecule. [1]

As a result of formation of adducts between 4-HNE and cellular and tissue proteins, advanced lipid peroxidation end products deposit that may actively play roles in signal transduction depending on cellular detoxifying mechanisms. It`s been clarified that 4-HNE has been metabolised by conjugation with cellular GSH catalysed by GST`s and transferred to the extracellular environment by RLIP 76. 4-HNE modified proteins are removed from the environment by proteolytic systems via degradation. [2]

It has been discovered that 4-HNE involved in fatty streak formation and 4-HNE detection within the atherosclerotic plaque is a mark in development of atherosclerosis. [3] In many studies, it has been claimed that programmed cell death of lesional macrophages, smooth muscle cells and endothelial cells took place. [4]

Apoptosis is programmed of cell death taking place in many processes such as normal cell turnover, embryonic development, hormone-dependent atrophy and chemical induced cell death. Inappropriate apoptosis either too much or too little may generate various disorders like; neurodegenerative disorders, cancer, atherosclerosis, autoimmune disorders and ischemic damage.

Apoptosis is executed mainly by 2 pathways; extrinsic and intrinsic pathways. Extrinsic pathway consists of a transmembrane receptor (Fas and TNF receptors), related adaptor molecules (FADD, TRADD) and caspases. Caspases are cysteine dependent aspartic acid specific proteases and classified as executioner and initiator.

Initiators are mainly related with extrinsic pathway however executioner caspases involve both in extrinsic and intrinsic pathway since these two pathways diverge at a point to execute the apoptosis.

Intrinsic pathway is triggered by a non-receptor mediated stimuli that cause pore formation on mitochondrial membrane and a decraese in mitochondrial membrane potential. Various proteins such as Smac/DIABLO, cytochrome c, HtrA2/Omi, AIF, endonuclease G and CAD are released from mitochondria to perform their tasks in apoptosis like apoptosome formation or DNA fragmentation. The control and regulation of the mitochondrial events are carried by Bcl-2 protein family members. Bcl-2 protein family members contain one of the BH (Bcl-2 Homology) domains out of four BH domains. According to their functions and homology domain they are classified in three groups; multidomain prosurvival proteins (Bcl-2, Bcl-$_{XL}$, Bcl-W, A1, Mcl-1, Bcl-Rambo, Boo/ Diva/ Bcl-B/ Bcl2L10, Bcl-G), multidomain proapoptotic proteins (Bax, Bak, Bok/ Mtd), BH3 only proapoptotic proteins (Bid, Bim/Bod, Bad, Bmf, Noxa, Hrk/Dp5, Puma/Bbc3, Bik/ Blk/ Nbk, Mule). [5]

Since 4-HNE induced apoptosis is related with atherosclerosis progression, to develop and apply novel therapeutics which will alleviate this disease via cell death mechanism, detailed molecular mechanism has to be understood. In this study we aim to identify the detailed mechanism of apoptosis triggered by 4-HNE in endothelial cells and monocytes.

2 OVERVIEW

2.1 Apoptosis

Apoptosis is defined as an essential and evolutionary conserved physiological process of programmed cell death. The term of apoptosis was first announced by Kerr, Wyllie and Currie in 1972 in order to describe a morphologically distinct form of cell death.

It is a cellular self destruction mechanism and necessary for variety of cellular events via eliminating selectively unwanted cells (normal embryogenesis, organ development, tissue homeostasis and elimination of deleterious cells from multicellular organisms). Apoptotic mechanism helps the organism to maintain tissue homeostasis during development and aging. It also acts as a defence mechanism in immune reactions or when the cells are destructed by a disease or a noxious agent. [6]

Inappropriate functioning of apoptotic machinery may be the reason of various diseases. Inhibition of apoptosis may cause cancer and autoimmune diseases where as enhancement of apoptosis may cause neurodegenerative diseases, AIDS, hematological disorders and ischemic damage.

The characteristic morphological patterns of apoptosis are chromatin condensation, nuclear fragmentation, cytoplasmic shrinkage, membrane blebbing, formation of apoptotic vesicles and phagocytosis of the apoptotic cell by the immune cells. [7] During the early stages of apoptosis cell shrinkage and pyknosis can be visualized by light microscopy. [8] Cell shrinkage results with a decrease in size of the cell, increase in the density of the cytoplasm and tightly packing of the organelles. Pyknosis which is the most characteristic feature of apoptosis concludes with chromatin condensation. Plasma membrane blebbing is continued with karyorrhexis and separation of cell fragments into apoptotic buddies that this process is called as budding. The apoptotic bodies contain tightly packed organelles with or without a nuclear fragment

and cytoplasm. The bodies are phagocytesed by macrophages, parenchymal cells or neoplastic cells and further degraded with phagolysosomes. [9]

Neither in the process of apoptosis nor in the removal of the apoptotic bodies there is inflammation since the cellular constituents of the apoptotic cells are not released, the apoptotic bodies are quickly removed from the environment by phagocytosis and the engulfing cells do not produce inflammatory cytokines.[10,11]

Various proapoptotic signals initiate the apoptotic signal transduction pathway from seperate signaling pathways then eventually those signals converge into a unique mechanism done by a family of cysteine proteases called caspases. [12, 13]

2.2 Apoptotic Pathways

Caspases, cysteine dependent aspartic acid specific proteases, are the main executioners of the apoptotic machinery. Up to date there have been at least 14 distinct mammalian caspases that 11 of them belonging to human. [5] Caspases are classified as effector, initiator and inflammatory caspases. In mammals, caspase-3, -7 and -6 are effector caspases; caspases -8, -9, -10 and -2 are initiator caspases and caspase -1, -4 and -5 are inflammatory caspases. [14, 15]

They are expressed as catalically inactive zymogens and have to undergo proteolytic activation during the apoptosis. The activation of effector procaspases occur by the cleavage of them by another caspase. Caspase-3 that is an effector caspase is activated by caspase-9 through cleavage at a specific internal aspartic acid residue. Despite the effector caspases, the initiator caspases are usually auto activated. The activation of caspase 9 via the formation of apoptosome which contains procaspase-9 with APAF-1 and cytochrome c is an example of auto activation. Once initiator caspases are activated, they can activate other procaspases forming a caspase cascade. The activation of effector caspases result with cleavage of broad spectrum of targets triggering the cell death. Via the caspase cascade the signal is amplified that leads to a rapid cell death.

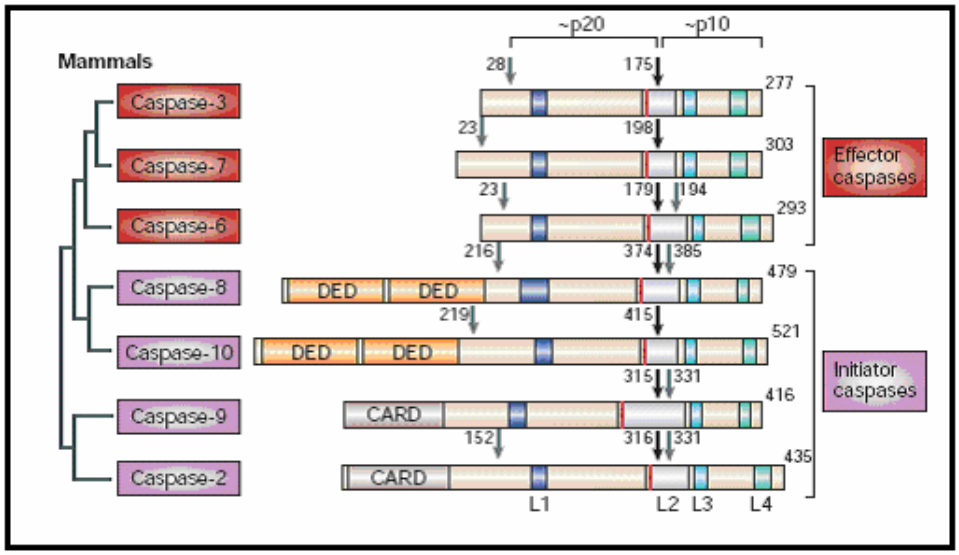

Figure 2.2 1: Effector and initiator caspases and their structures in apoptotic mechanism. [16]

It is indicated that the apoptotic signaling pathway consist of two major parts; extrinsic and intrinsic pathways. Extrinsic pathway involves direct initiation of caspase cascade that is triggered by death receptors on the cell surface. Intrinsic pathway includes mitochondria and is triggered by intracellular death signals. Today there is much evidence that both of the pathways are in a communication and one of the proteins in one pathway can influence another protein in the other pathway.[17] Both extrinsic and intrinsic pathways converge on the same execution pathway that is initiated by cleavage of caspase 3. Activation of the caspase 3 continues with DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross linking of proteins, apoptotic bodies' formation, recognition and uptake of the apoptotic bodies by phagocytic cells.

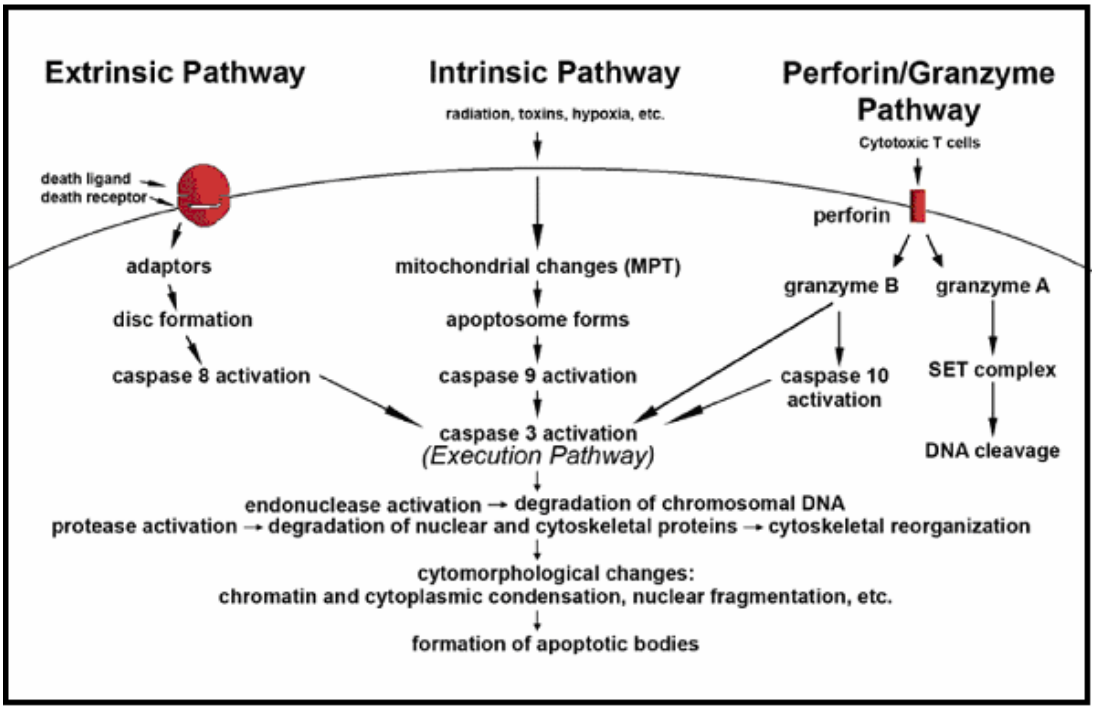

Figure 2.2 2: Mechanism of apoptosis via extrinsic, intrinsic or perforin/granzyme pathway involvement [9]

2.2.1 Extrinsic Pathway

Extrinsic pathway is initiated by an extracellular proapoptotic signal that is recognized by specific death receptors. The death receptor family members are CD95/Fas/Apo, DR3–6 and TNF-R I-II. They have similar cysteine rich extracellular domains and death domain in their cytoplasmic portion as a protein-protein interaction module. [18]

Binding of specific ligands to the receptor leads to the multimerization of the receptors. The ligand-receptor complex recruits various adaptor proteins such as TRADD, FADD, Daxx, RIP, RAIDD and FLIP. [14] The complex is than called DISC that is an oligomeric death inducing signaling complex. The complex then interacts with caspases and activates them. FADD interacting with caspase 8 results with oligomerization of caspase-8 in the DISC and the autocatalytic activation of caspase-8. [19, 20, 21]

2.2.2 Intrinsic Pathway

The intrinsic pathway is initiated by an intracellular death signal such as deprivation of growth factor, hormones or cytokines, DNA damaging agents, UV and gamma ray radiation, hypoxia, hyperthermia, viral infections and free radicals. [22, 23, 24] These stimuli produce intracellular signals and initiate mitochondrial events.

The signals cause changes in inner mitochondrial membrane and mitochondrial permeability transition pores in open form. The mitochondrial transmembrane potential decreases and eventually related proteins are released from intermembrane space of mitochondria to the cytosol. [25] Cytochrome c, Smac/DIABLO and serine protease HtrA2/Omi are 3 of the released proteins. [26, 27] They activate the caspase dependent mitochondrial pathway. For instance cytochrome c binds Apaf-1 forming the apoptosome complex there by activating caspase-9. [28, 29] Smac/DIABLO and HtrA2/Omi that inhibit IAP activity are also released from mitochindria. [30, 31] From the last three proteins released from mitochondria, AIF and endonuclease G work in a caspase independent way. AIF has a role in DNA fragmentation and condensation of peripheral nuclear chromatin. [32] Endonuclease G produces oligosomal DNA fragments. [33] CAD activates caspase 3 after translocation to nucleus and resulting more pronounced DNA fragmentation and chromatin condensation. [34]

It is known that extrinsic pathway may intersect with the intrinsic pathway which might be called mitochondrial amplification loop. In this way caspase-8 cleaved a protein called Bid that is a BH3 only domain containing pro apoptotic member of Bcl-2 protein family. The cleavage of the C terminal of Bid turns it into an active form and truncated Bid translocates from cytosol to mitochondria, there oligomerizes with Bax and Bak and leads to the cytochrome c release from the mitochondria. [35, 36]

The mitochondrial apoptotic machinery is regulated by the Bcl-2 protein family members. They are responsible for mitochondrial membrane permeability and work in a preventive or provocative manner depending of the nature of the proteins.

According to the homology shared within four conserved BCL-2 homology domains (BH1-4) and functions of the protein in the case of the apoptotic stimuli, the Bcl-2 family members are grouped as

1. Multidomain Prosurvival Proteins: Bcl-2, Bcl-$_{XL}$, Bcl-W, A1, Mcl-1, Bcl-Rambo, Boo/ Diva/ Bcl-B/ Bcl2L10, Bcl-G

2. Multidomain Proapoptotic Proteins: Bax, Bak, Bok/ Mtd
3. BH3 only Proapoptotic Proteins: Bid, Bim/Bod, Bad, Bmf, Noxa, Hrk/Dp5, Puma/Bbc3, Bik/ Blk/ Nbk, Mule

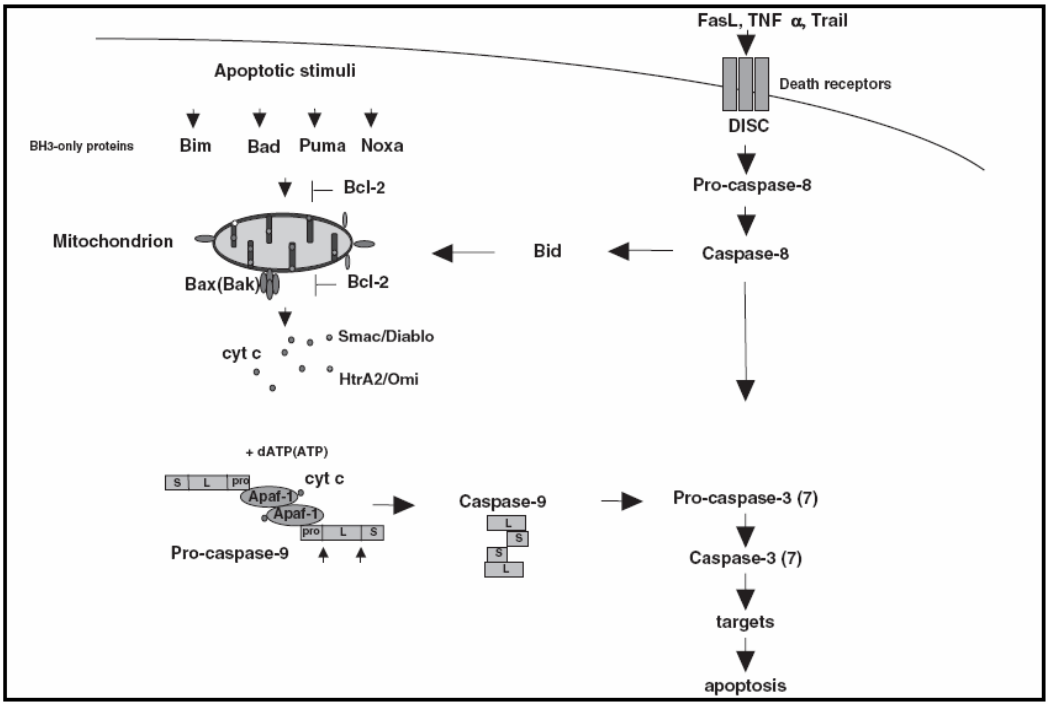

Figure 2.2.2 1: A more detailed mechanism of apoptosis in extrinsic and intrinsic pathway, Bcl-2 family proteins and caspase interface [37]

2.2.2.1 The Structure of Bcl-2 Protein Family Members

The multidomain anti apoptotic proteins including Bcl-2, Bcl-$_{XL}$, Bcl-W, A1, Mcl-1, Bcl-Rambo, Boo/ Diva/ Bcl-B/ Bcl2L10, Bcl-G display sequence conservation between BH1-4. Proapoptotics are subdivided into two more groups, the multidomain proteins including Bax, Bak and Bok having homology in BH 1-3 and the others called BH3 only proteins having homology only in BH3 domain.

The 3D structures of many of the prosurvivalproteins Bcl-2 [38], Bcl-$_{XL}$ [39], Bcl-W [40, 41] and Mcl-1[42] have been resolved. The common point of their structures is that both of them having a similar overall helical fold centered on a core hydrophobic helix with the BH 1-3 domains arranged to have a hydrophobic groove on

the molecule. This hydrophobic groove is important and required to bind proapoptotic partners. [43]

Bax [44], Bak [45] , and Bid [46] show similarity between four members of Bcl-2 prosurvival proteins (Bcl-$_{XL}$, Bcl-2, Bcl-1 and Mcl-1); however they promote apoptosis rather than opposing it. They are helical bundles with a hydrophobic helix turn helix hairpin that is flanked on both sides of amphipathic helices.

Except Bid, other BH3 only proteins have different 3D structures than the core Bcl-2 family members. BH3 domain has four conserved hydrophobic residues that can insert themselves into a distinct pocket in the hydrophobic groove of the target prosurvivalprotein. Mutations in these conserved amino acids reduce the binding affinity of the BH3 only proteins. [47] Thus through the BH3 domain they are able to bind to and regulate other Bcl-2 family members.

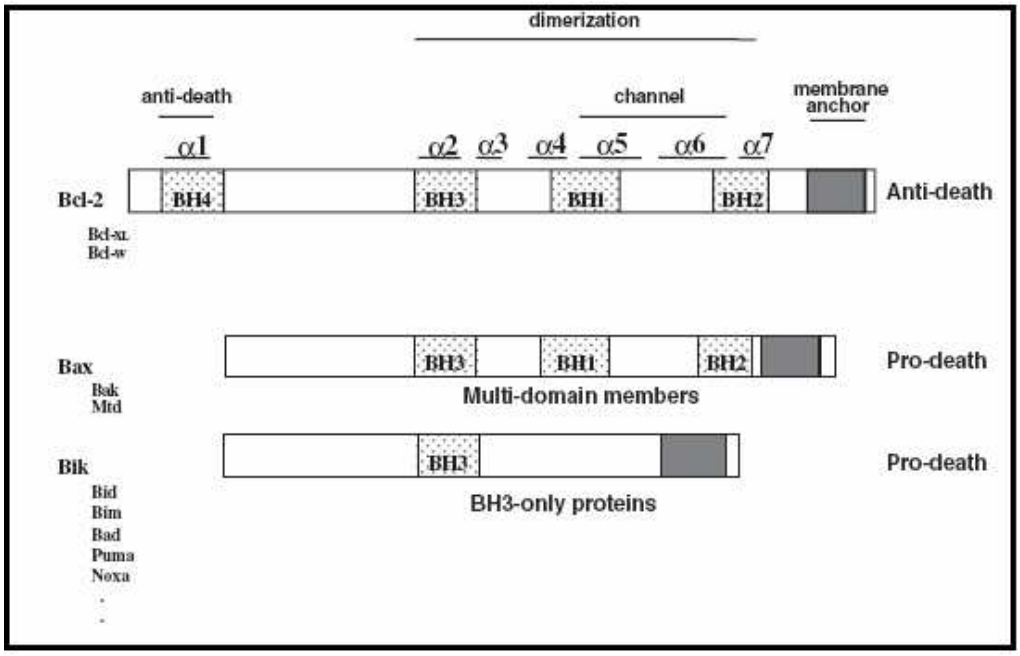

Figure 2.2.2.1 1: Structures of Bcl-2 family members; BH domains of proapoptotics, prosurvival and BH3 only proteins [37]

2.2.2.2 Activation of Bcl-2 Protein Family Members

BH3 only proteins respond various intracellular deaths signals. Their activations occur in different ways such as by transcriptional factors, a post-translational modification, a cleavage, phosphorylation or dephoshorylation event. For instance Noxa and Puma are induced by p53 due to DNA damage. [47, 48, 49] The transcription of Bim depends on FOXO3A [50] in the case of growth factor deprivation or on CEBPα or CHOP in the case of endoplasmic reticulum stress. [51] Bad is post-translationally modified by dephosphorylation in response to growth factor deprivation. [52] Activation of Bid occurs when full length Bid is truncated with active caspase-8. [53, 54] Bim may be activated either by the release from dynein motor complex [55] or phosphorylation by ERK. [56, 57]

Expression levels of prosurvival Bcl-2 family members are also regulated. Bcl-$_{XL}$ level can be changed through the JAK-STAT pathway in response to growth factor. [58] Mcl-1 level is decreased with cytokine deprivation since it is rapidly degraded by ubiquitin-proteasome pathway. [59, 60] Bax and Bak seem to be constitutively expressed nearly constant levels but post translationally regulated by other Bcl-2 proteins. [61]

2.2.2.3 Localization of Bcl-2 Protein Family Members

Bcl-2 protein is located in the endoplasmic reticulum, the nuclear envelope and the outer mitochondrial membrane with its C terminal domain and most of its amino acids are in cytosol. [62, 63] Bax is mainly in cytosol as a monomer rather than being bound to another Bcl-2 member and its hydrophobic C terminal membrane anchor domain is in its BH3 binding pocket in the absence of death stimulus. [64, 65] With an apoptotic signal, Bax oligomerizes to produce large complexes, translocates to mitochondria, undergoes conformational change and with the help of its C terminal membrane anchor domain inserts itself into outer mitochondrial membrane. [66, 67, 68] Similar to Bax, Bok also translocates to the mitochondria. [69] Despite Bax and Bok, Bak constitutively integrated on the outer mitochondria membrane in healthy cells but in the case of apoptotic stimuli its conformation changes and oligomeric complex

formation occurs. [70, 71] According to crosslinking studies it is found that Bak and Bax complexes are homo oligomers but in an interaction with each other in apoptotic cells. [72]

The Bcl-2 protein family members that are in an interaction with outer mitochondrial membrane regulate the apoptosis through their impact in outer mitochondrial membrane. With Bax translocation and Bak oligomerization, outer mitochondrial membrane become permeable and soluble intermebrane space proteins; cytochrome c, Smac/DIABLO, adenylate kinase, Serine protese Omi, AIF (Apoptosis Inducing Factor), DDP (Deafness dystonia protein), endonuclease G and a cleaved form of OPA1, are released from mitochondrial intermembrane space into the cytosol. [73, 74, 75]

2.2.2.4 Models Proposed to Explain Bax/Bak Activation

It is known that cells lacking Bax and Bak have resistant to tested intrinsic death signals. [76, 77] Although it is known that BH3 only proteins require Bax and Bak, it is not certainly found that if they can control Bax and Bak activation directly or indirectly.

The first model, direct binding model, depends on the similarity of Bax to prosurvivalproteins so that BH3 only proteins can directly bind to Bax. [78] According to this model Bim, tBid and Puma termed as “direct activators”, have the ability to directly activate Bax and Bak. [79, 80, 81] Except Bim, tBid and Puma, other BH3 only proteins, called as “sensitizers” or “derepressors” and bind to prosurvivalproteins thereby releasing direct activators ready to bind Bax and Bak. This model is in consistency with the fact that BadBH3 peptide has a greater affinity for Bcl-2 than BidBH3 has. BadBH3 peptide can displace Bcl-2 bound BidBH3 peptide from Bcl-2 but not the other way can occur. [82]

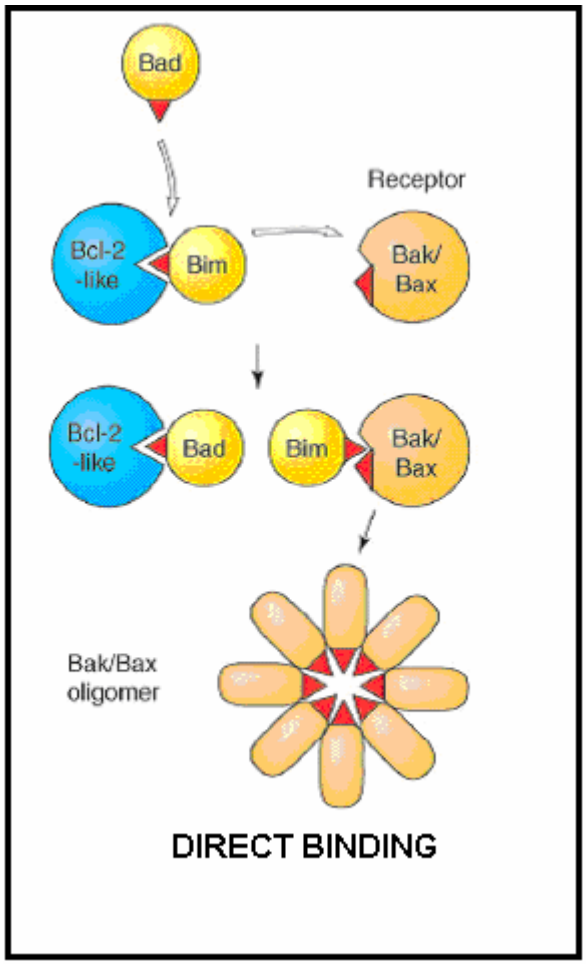

Figure 2.2.2.4 1: Direct binding model for how BH3-only proteins activate Bax and Bak. BH3-only proteins are classified as sensitizers and activators. Sensitizers are assumed to liberate activators from prosurvival ones so they can activate Bax and Bak for oligemerization. [83]

The second model, displacement model, relies on the fact that some of the BH3 only proteins, Bim, tBid and Puma have a high binding affinity to all prosurvivalproteins whereas Bad and Noxa bind only certain prosurvivalproteins. [81, 82, 79, 84]

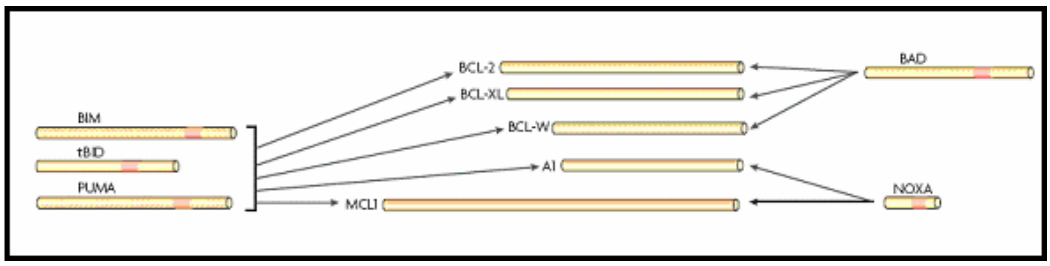

Figure 2.2.2.4 2: Binding affinities of BH3-only proteins to Bcl-2 homologues prosurvival members. [61]

Bim, tBid and Puma are accepted as more potent inducers of apoptosis whereas Noxa and Bad are accepted as less potent inducers. In the direct binding model the default is survival and in the displacement model the default is death.

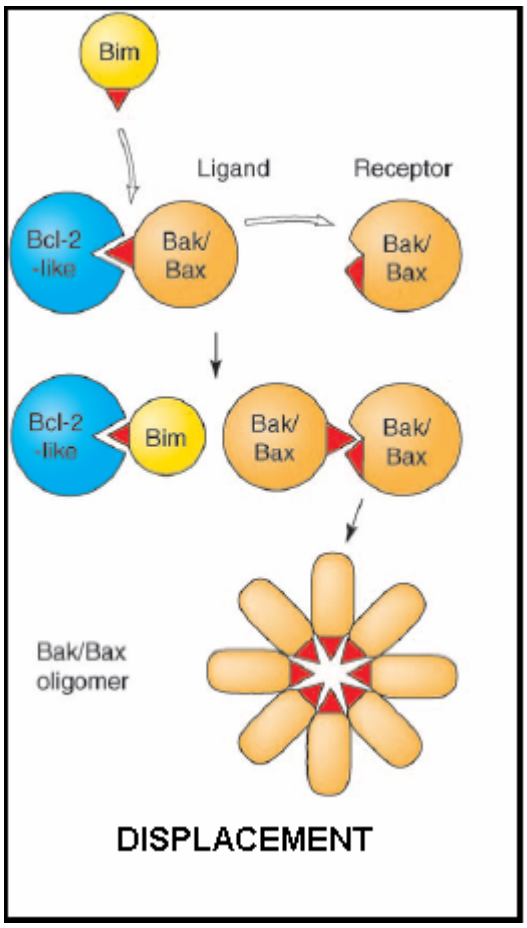

Figure 2.2.2.4 3: Displacement model for how BH3-only proteins activate Bax and Bak. Certain BH3-only proteins are assumed to be affinity to prosurvival proteins. According to the model because of the affinity the interaction between Bax or Bak and anti-apoptotics are disrupted with BH3-only proteins thus leading to Bax and Bak oligomerization. [83]

2.3 4-Hydroxynonenal

4-hydroxynonenal (4-HNE) is known to be a major aldehyde that is produced as a result of peroxidation of ω6 polyunsaturated fatty acids like linoelic acid and arachidonic acid. 4-HNE is an amphiphilic compound with a 156 g/mol molecular weight. It is slightly soluble in water; its lipophilic properties are much stronger.

Because of this lipophilicity, it tends to concentrate in biomembranes. Phospholipids and proteins in biomembranes like transporters, ion channels and receptors react readily with 4-HNE.

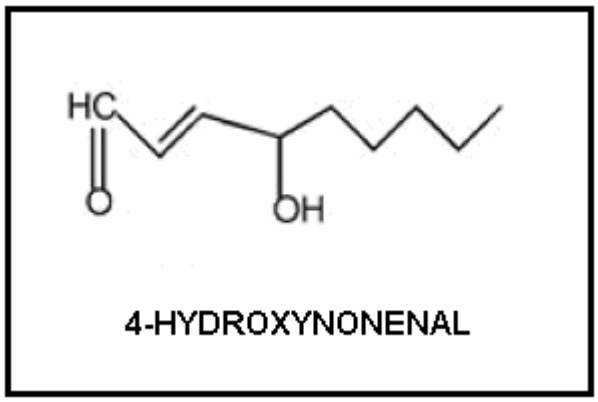

Figure 2.3 1: Structure of 4-HNE

4-HNE has three important functional groups, a carbonyl group, a C=C double bond and an hydroxyl group, that all of them contribute to the high reactivity of HNE. The positive charge on carbon 3, produced by the carbonyl group and C=C double bond is increased with the hydroxyl group. Thus a nucleophilic attack may occur primarily at carbon 3 then secondly at carbonyl carbon 1.

Strong nucleophiles such as thiols and amino groups cause mainly two kinds of reactions especially between 4-HNE and proteins or peptides. These reactions are called Micheal addition of thiols and Schiff base formation.

2.3.1 Reactions of 4-HNE:

2.3.1.1 Micheal Addition

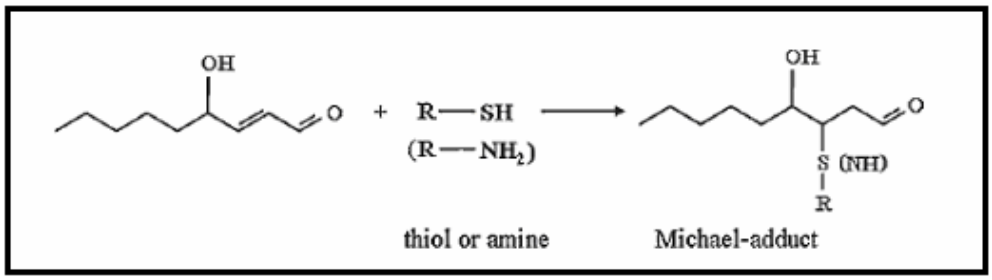

Figure 2.3.1.1 1: Micheal addition of thiols with 4-HNE

2.3.1.2 Schiff Base Formation

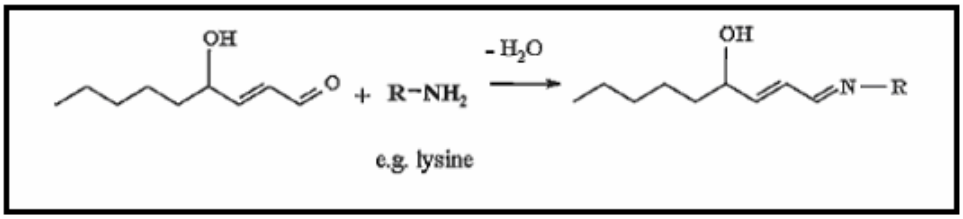

Figure 2.3.1.2 1: Schiff Base Formation of 4-HNE

It has been established that 4-HNE has susceptibility to three amino acids cysteine, histidine and lysine. The reactivity of these amino acids in the order of HNE/ amino acid ratio is Cys (0.6) > His ($1x10^{-3}$) > Lys ($3x10^{-4}$). [85]

It has been showed that 4-HNE makes adducts many groups of proteins. Many glutathione-S- transferases involve in 4-HNE conjugation. An isoenzyme named GSTA4-4 found in the human liver mitochondria has high affinity to 4-HNE. This is accepted as the evidence that GSTs have role in the protection of mitochondrial oxidative damage dependent on 4-HNE. [86] It has been shown that 4-HNE has an influence on many enzymes` activity via the adduct formation. For instance, 4-HNE disrupts the active site of lipoxygenase-1 and thereby causes its loss of function. [87, 88]

Not only with proteins and peptides, 4-HNE also reacts with lipids, cofactors, vitamins and nucleic acids. 4-HNE reacts with vitamin C in a Micheal-type conjugation that can be detected in detectable micromolar concentrations in healthy donors' plasma. This might be relevant with the elimination of 4-HNE. [89] It is known that 4-HNE reacts with all four DNA bases but with different efficiency: G>C>A>T. [90]

2.3.2 Metabolism of 4-HNE

4-HNE is the major 4-hydroxyalkenal formed in cells due to peroxidative breakdown of n-6-PUFA as linoleic acid, arachidonic acid and γ-linolenic acid. While it has been established as a toxic end product and contributor to the deleterious effects of oxidative stress, it is now declared as an important signaling molecule. In various studies it has been showed that it has affects on apoptosis, differentiation, cell growth and signal transduction pathways such as MAPK pathway in concentration dependent manner. [91] It is found that in a healthy human blood serum under basal conditions, 4-HNE concentration varies between 0.05-0.15 μM. [92] The regulation of intracellular concentration of 4-HNE is crucial for the concentration dependent mechanism of 4-HNE since 4-HNE is generated by uncontrolled nonenzymatic reactions.

4-HNE is primarily metabolised by its conjugation to glutathione (GSH) through reactions catalyzed by glutathione S-transferases (GSTs). [93, 94] The majority of conjugated 4-HNE with GSH (GS-HNE) is transported to the extracellular environment in an ATP- dependent process that is catalyzed by RLIP76. [95] GS-HNE may also be further metabolized to mercapturic acid or the aldeyde group may be reduced to alcohol by aldose reductase. [96]

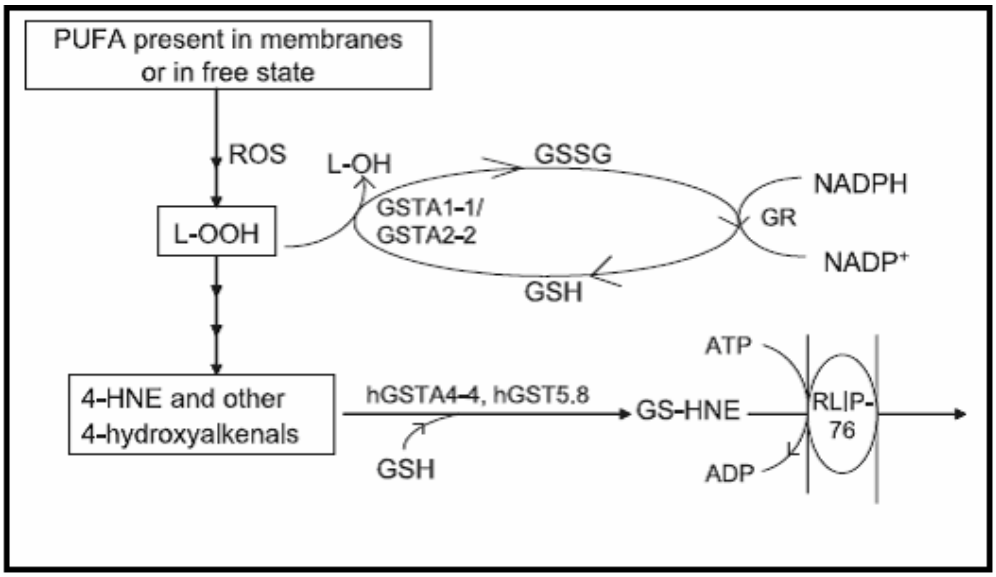

Figure 2.3.2 1: GST`s and their roles in intracellular metabolism of 4-HNE and other lipid peroxidation products [97]

GST Isozymes Known to Be Involved in Regulation of 4-HNE in Cells			
Enzyme name	**Species**	**Class**	**Preferred substrate**
hGSTA1-1	Human	alpha	Phospholipid hydroxyperoxides, Fatty acid hydroxyperoxides
hGSTA2-2	Human	alpha	Phospholipid hydroxyperoxides, Fatty acid hydroxyperoxides
hGSTA4-4	Human	alpha	4-HNE
hGST5.8	Human	alpha	4-HNE
mGSTA4-4	Mouse	alpha	4-HNE
rGSTA4-4	Rat	alpha	4-HNE

Table 2.3.2 1: Main well known GST Isozymes in lipid peroxidation products`s regulation [97]

Because GSTs have roles in the regulation of intracellular concentration of 4-HNE, GSTs indirectly affects 4-HNE mediated signaling pathways. In recent works, it is concluded that transfection of hGSTA1-1 and A2-2 to K562 and HLEB-3 cells lipid peroxidation is suppressed. Due to the suppression of lipid peroxidation, when these transfected cells are treated with H_2O_2+$FeSO_4$ to induce lipid peroxidation, 4-HNE levels still remained lower than the basal levels present in untreated wild type cells or cells transfected with an empty vector. However in treated wild type cells or cells

transfected with an empty vector, 4-HNE amount increases 3 fold of observed in untreated controls. [98] The confirmation of regulation of intracellular 4-HNE levels by hGSTA1-1 and A2-2 overexpression, is done by showing the resistance of these transfected cells due to the cytotoxicity of H_2O_2 or oxidant xenobiotics. [99]Since it is known that H_2O_2 is not the substrate of hGSTA2-2, the protection by hGSTA2-2 against H_2O_2 induced apoptosis has to be provided by reducing lipid hydroxyperoxides.

2.3.3 4-HNE Induced Apoptosis

It has been shown that 4-HNE involve in induction of apoptosis in various cell types. The recent studies focus on especially the mechanistic approach of 4-HNE induced apoptosis. The mechanistic approaches in 4-HNE induced apoptosis mainly deal its role in signaling for apoptosis, regulation of gene expression, proliferation and its interaction with signaling components in membranes, cytoplasm and nucleus.

4-HNE induced apoptosis in PC12 cells with MAPK proteins involvement. c-Jun N-terminal protein kinase (JNK) activation is observed within 15-30 min after the treatment then it returned to control level at 1 hour post-treatment with 25 μM 4-HNE. The upstream kinases of JNK, stress-activated protein kinase (SEK1) and ASK1 are also activated before JNK activation. However extracellular regulated kinase (ERK) and p38 MAPK remained unchanged at their basal levels after the treatment. In conclusion 4-HNE may activate ASK1-SEK1-JNK sequentially in order to give an apoptotic response in PC12 cells. [100]

In a study done in our laboratory, it is showed that 4-HNE induced oxidative stress and apoptosis in Swiss 3T3 fibroblast cells, the oxidative and apoptotic effects are reversed with resveratrol treatment before 4-HNE addition. [101] Later experiments give an insight about the mechanistic view of 4-HNE induced apoptosis in Swiss 3T3 fibroblasts. MAPK and caspase activation pathways are followed after 20μM 4-HNE treatment. 4-HNE induced JNK and p38 activation between 15-30 minutes, whereas the basal activity of ERK1/2 is down regulated. AP-1 activation is also confirmed by EMSA and increase in c-Jun level. Cytochrome c release, caspase-9 and -3 activation is observed at a later than MAPK proteins` activation. Pretreatment of resveratrol not only inhibits apoptosis but also prevents JNK, p38, caspase-9, -3 activation, cytochrome c

release and AP-1 binding to DNA. One of the most important things in this study is the ability of resveratrol to increase DNA binding activity of antioxidant response element (ARE). [102]

In 4-HNE induced apoptosis, the extrinsic pathway and related receptors are also studied. In a recent work, the correlation between intracellular levels of 4-HNE and expression of Fas, a receptor belonging to TNF-α family, is studied in HLEB-3 cells. 4-HNE induces Fas expression, activation of JNK and caspase 3 in time and dose dependent manner. In order to confirm their result, the experiments are done with mGsta4 null mice and transiently transfected cells with hGSTA4. Tissues belonging to mGsta4 mice have high levels of 4-HNE that causes Fas induction and JNK activation. hGSTA4 transiently transfected cells suppress Fas. Fas deficient HLEB-3 cells stably transfected with hGSTA4 are resistant to apoptosis. As well as Fas deficient cells, HLEB-3 cells that Fas is silenced by siRNA have also resistance to 4-HNE induced apoptosis. It is concluded that 4-HNE mediated Fas expression does not related with FADD, FasL and procaspase 8 according to immunoprecipitation experiments. It is able to precipitate Fas with Daxx however it is not certain that Fas and Daxx association is specific or not due to the lack of ASK1 activation. As a conclusion it is declared that in 4-HNE induced apoptosis in HLEB-3 cells, Fas at least has a partial role independent of FADD, Fas L, procaspase 8 and DISC formation. [103]

In a study related with the previous one, 4-HNE causes the translocation of Daxx from nucleus to the cytoplasm and binding of Daxx to Fas in 4-HNE induced DISC independent apoptosis in Jurkat cells. Moreover the binding of Daxx to Fas inhibits 4-HNE induced apoptosis through the inhibition of the signaling from Fas to ASK1, JNK and caspase 3. This is shown by the sensitivity of Jurkat cells that Daxx is silenced, to not only 4-HNE induced apoptosis but also apoptosis triggered by agonistic Fas antibodies. Despite Fas mediated extrinsic pathway, p53 mediated intrinsic pathway also contribute to 4-HNE induced apoptosis. In caspase 8 mutant Jurkat cells, CRL2571, dose dependent increase in p53 expression both in nuclear and cytoplasmic fraction and Bax upregulation are observed cells treated with 4-HNE. [104] It is suggested that 4-HNE can self regulate Fas mediated apoptosis by using the inhibitory role of Daxx.

The interactions between heat shock proteins (HSP`s) and 4-HNE is demonstrated by Marnett *et al.* According to the study it is shown that 4-HNE induces the nuclear translocaion of heat shock factor 1 (HSF1) and thus the expression of related heat shock proteins; Hsp40 and Hsp70-1. It also disrupts the inhibitory interaction between the

HSF1 and Hsp70-1. The lack of heat shock proteins ends with an increase in 4-HNE induced apoptosis. Silencing HSF1 expression promotes the activation of JNK proapoptotic signaling and decrease in expression of prosurvivalBcl-2 family member, Bcl-$_{XL}$. Overexpression of Bcl-$_{XL}$ in HSF1 silenced cells attenuates in 4-HNE induced apoptosis. [105]

Both studies showing that 4-HNE self limits Fas mediated apoptosis and 4-HNE induces heat shock proteins suggest that 4-HNE has an important protective role in cells under stress conditions.

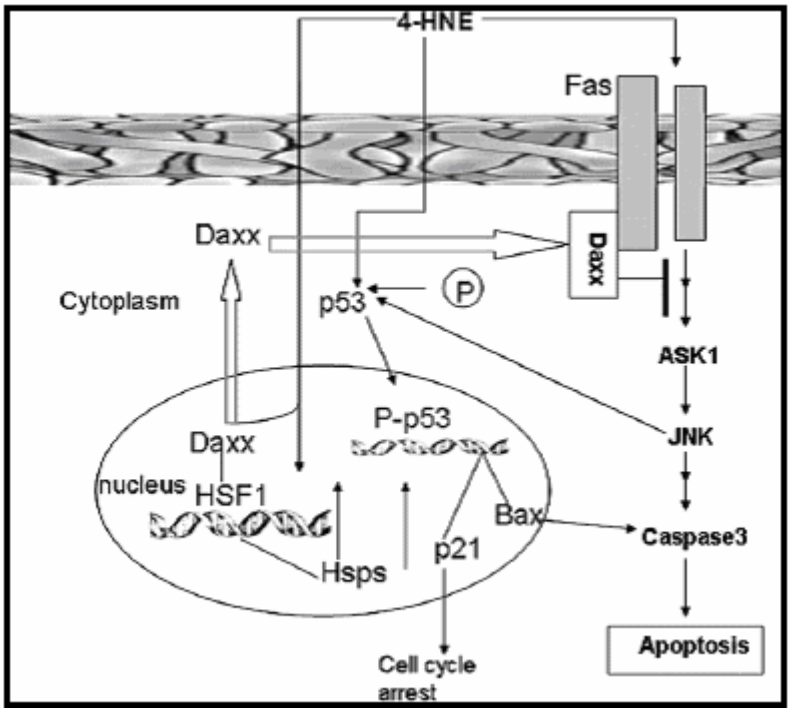

Figure 2.3.3 1: Proposed mechanism of 4-HNE induced signaling according to Awasthi *et al.* 2008[104]

Many studies show that 4-HNE induced apoptosis in a caspase dependent manner. In one of early studies dealing with caspase involvement in 4-HNE induced apoptosis, it is showed that caspase-2, caspase-3 and caspase-8 have roles in 4-HNE induced apoptosis in human leukemic cell line (CEM-C7). Apoptosis is triggered by 4-HNE in micro molar concentrations and in a time and a dose dependent manner. The effect of caspase involvement in apoptosis is monitored by using specific fluorogenic substances and irreversible caspase inhibitors.

The caspase inhibitors used in the research are ZVAD-fmk (a pan-caspase inhibitor), YVAD (caspase-1 inhibitor), VDVAD (caspase-2 inhibitor), DEVD (caspase-3 inhibitor), IETD (caspase-8 inhibitor). Apoptosis is completely inhibited with ZVAD-fmk and caspase-8 inhibitor IETD. Caspase-1 inhibitor YVAD

demonstrates 28% inhibition, caspase-2 inhibitor VDVAD provides 60-80 % inhibition and caspase-3 inhibitor DEVD provides 85 % inhibition. The results show that caspase-1 lacks involvement in apoptosis from the inhibition profile and time course profile of activation of caspases, the initiator caspases-2 and -8, and executioner caspase-3 are involved in the apoptosis induced by 4-HNE. According to these results a caspase cascade is involved in 4-HNE induced apoptosis in CEM-C7 cells. [106]

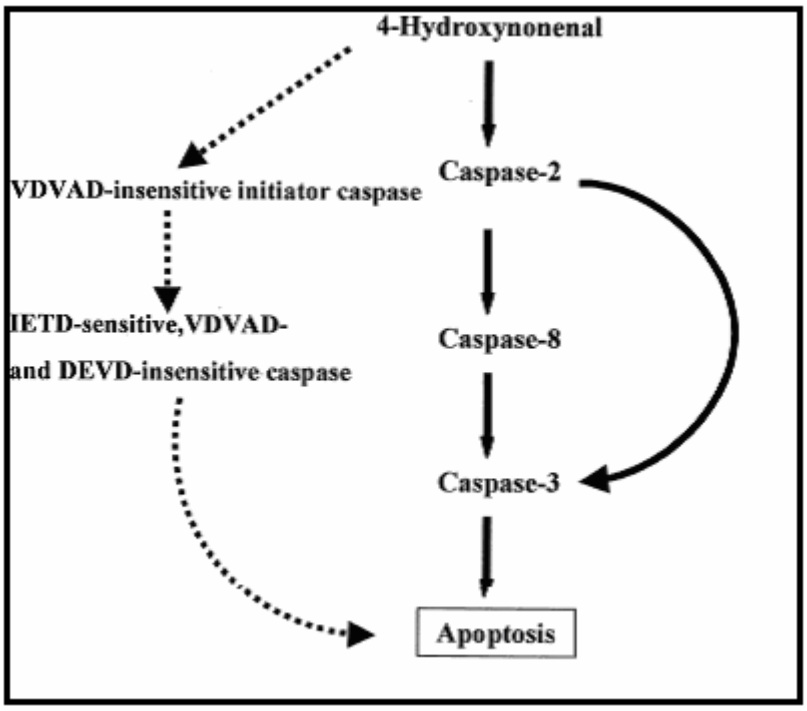

Figure 2.3.3 2: Caspase cascade in the presence of 4-HNE according to Zhang *et al.* 2001[106]

In another literature work 4-HNE induces apoptosis via the mitochondrial pathway. It is seen that treatment of human colocteral carcinoma cells (RKO) with 4-HNE induces PARP cleavage, DNA fragmentation and the activation of caspase-2,-3,-8 and -9 in a time and dose dependent manner. Caspase-3 might be related with the induction of PARP cleavage and DNA fragmentation so the caspase activation is measured by using a specific caspase-3 substrate (DEVD-pNA) in 4-HNE treated RKO cells. From the time analysis of the process and from the activity assay of caspase-3, it is showed that caspase-3 is a key event in the induction of apoptosis. These results are confirmed by using caspase-3 inhibitor and a general caspase inhibitor and thus inhibiting procaspase-3 and PARP cleavage. Using the overexpression studies they concluded that the apoptotic cascade caspase-3, caspase-2 and PARP cleavage can be inhibited by the overexpression of a prosurvivalprotein Bcl-2. However overexpression of Bcl-2 protein cannot block caspase-8 activation in 4-HNE treated RKO cells

overexpressing Bcl-2. Thus they concluded caspase-2 and -8 are induced by 4-HNE with different mechanisms. Cytochrome c release and activation of caspase-9 induced are also diminished by the Bcl-2 overexpression. These results showed that 4-HNE triggers cell death through promoting cytochrome c release and caspase cascade in a mitochondrial dependent pathway and the apoptosis is blocked by overexpressing the prosurvivalprotein Bcl-2. [107]

2.3.4 Atherosclerosis, 4-HNE and Apoptosis

It has been long time that the importance of macrophages for intracellular lipid accumulation and foam cell formation has been established in atherosclerosis. Monocytes on peripheral blood migrate into the arterial intima and differentiate into macrophages in atherosclerotic lesions in response to various stimuli such as cytokines, chemotactic factors and macrophage growth factors. Those stimuli are produced by vascular endothelial cells, smooth muscle cells and infiltrated cells. Monocytes chemoattractant protein-1 is one of the most important stimuli for monocyte migration and macrophage colony-stimulating factor is one of the crucial stimuli for monocyte/macrophage differentiation. Once the monocytes migrate and differentiate into macrophages, they begin to express a variety of receptors including scavenger receptors for the uptake of modified lipids such as OxLDL, β-very low-density lipoprotein and enzymatically degraded low-density lipoprotein. The modified lipid uptake results with the accumulation of cholesterol esters in the cytoplasm of macrophages and leads them to turn into foam cell formation in the atherosclerotic lesion development. [108]

Apoptosis occur in most of the foam cells in the loco of atherosclerotic lesions both in the two phase of atherosclerosis. The first phase is the long lasting and subclinical one. In this phase because of the presence of macrophages, vascular smooth muscle cells and extracellular matrix, subendothelial space enlarges and the lumen is not notably occluded. In this step the apoptosis of the foam cell is desired since apoptotic cells are rapidly and safely cleared by phagocytosis by the neighbouring phagocytes. This process is called efferocytosis which prevents the contribution to the

plaque construction, secondary cellular necrosis and leads to decrease in lesion cellularity.

When the apoptosis in the first phase is not sufficient, a critical step evolved with the development of a central area of necrosis. In this second phase of atherosclerosis, plaque disruption, fibrous cap rupture or endothelial cell erosion take place. In this condition the apoptotic cell death macrophages contribute to plaque necrosis because of the insufficient efferocytosis. [109]

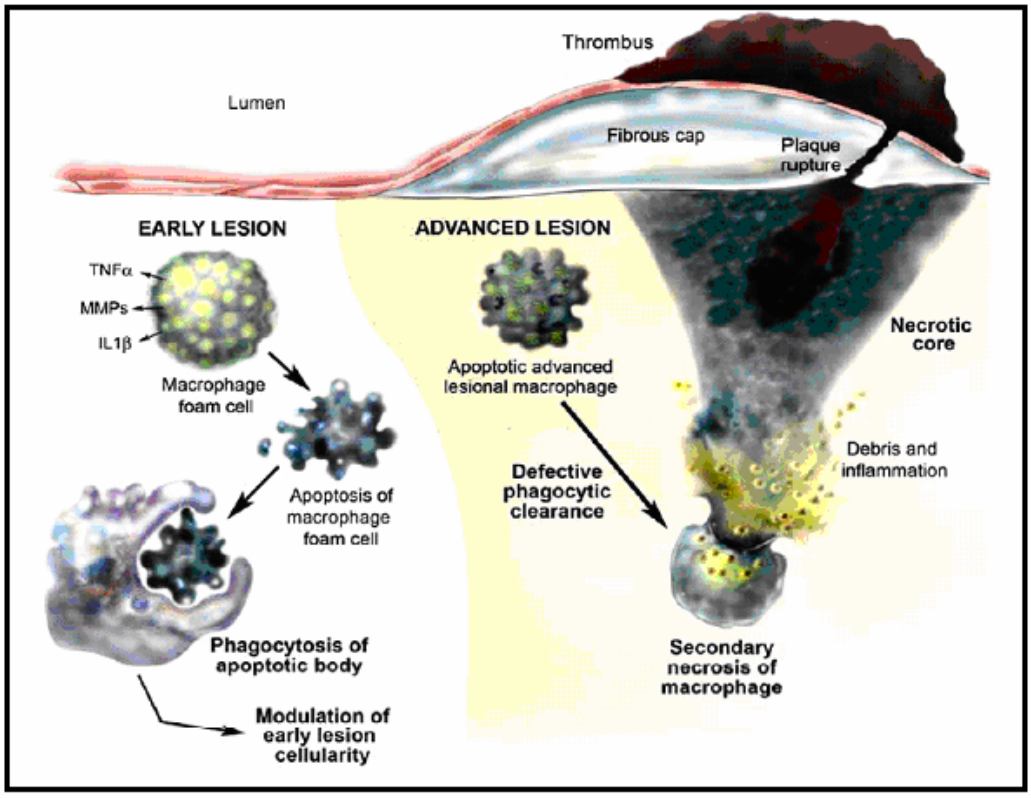

Figure 2.3.4 1: Proposed macrophages apoptosis in early and advanced stages of atherosclerosis [109]

It has been explained that as well as lesional macrophages, smooth muscle cells and endothelial cells also undergo programmed cell death. It has been introduced that there is a hypothesis about the apoptosis of endothelial cells that it plays a role in the minority of disrupted plaques involving erosion rather than fibrous cap rupture. [110]

In this study, our aim was to identify the 4-HNE induced apoptosis signalling pathways in two different cell lines; huv-ec-c's and U937 cell line. 4-HNE induced apoptosis signalling in endothelial cells and monocytes was assumed to clearify the mechanisms in atherosclerosis.

3 MATERIAL AND METHODS

3.1 Materials

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 cell viability and protein analysis are listed in Appendix B.

3.1.3 Equipments

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

3.1.4 Buffers and Solutions

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

10 mM 4-HNE:

64mM 4-HNE solution was further diluted in pure ethanol in 1 to 6.4 proportions.

Annexin V-FITC incubation buffer:

10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$ were dissolved in 500 ml of ddH_2O

10X PBS (Phosphate Buffered Saline):

80 g NaCl, 2.25 g KCl, 23.27 g $Na_2HPO_4.12H_2O$ and 2.05 KH_2PO_4 were dissolved in 1L ddH_2O of and pH was adjusted to 7.4.

1X PBS-T:

10X PBS was diluted, 0.2% Tween-20 was added and pH was adjusted to 7.4

10X Running Buffer:

250 mM Tris base, 1.92 M glycine and 1% (w/v) SDS were dissolved in 500 ml ddH_2O.pH was adjusted to 8.5.

Buffer for SDS polyacrylamide gel electrophoresis:

1X running buffer was prepared from 10X and was used for polyacrylamide gel electrophoresis.

4X Tris-Cl/SDS pH 6.8:

0.25M Tris and 0.2% SDS (w/v) were dissolved in 50 ml ddH_2O.pH was adjusted to 6.8

4X Tris-Cl/SDS pH 8.8:

0.75M Tris and 0.2% SDS (w/v) were dissolved in 50 ml ddH_2O.pH was adjusted to 8.8.

10X Transfer Buffer:

0.25 M Tris Base and 1.92 M glycine was dissolved in 500 ml of ddH_2O.

Buffers for Western Blotting:

1X transfer buffer was prepared by dilution from 10 X and 20% methanol addition prior usage for the blotting the proteins into PVDF membrane. The membranes were blocked with blocking solution, 5% milk powder in 1X PBS-T and washed with washing buffer 1X PBS and 1X PBS-T. The antibodies were diluted in 5% milk diluent in 1X PBS-T.

Stripping Buffer

62.5 mM Tris-HCl and 2% SDS (w/v) were dissolved in 500 ml ddH_2O and pH was adjusted to 6.7. Prior to use 352.1 µl of β-mercaptoethanol was added for each 50 ml of solution.

NP-40 Lysis Buffer

150 mM NaCl, 1% NP-40 and 50 mM Tris-HCl were dissolved in dd H_2O. pH was adjusted at 8, buffer was stored at -20^oC till usage.

3.2 Methods

3.2.1 Cell Culture

Human umbilical vein endothelial cells (huv-ec-c) were obtained from ATCC Cell Biology Collection(Cell Culture Collection Catalog number: CRL-1730). These cells were cultured in F-12K Medium supplemented with 0.1 mg/ml heparin; 0.03-0.05 mg/ml endothelial cell growth supplement (ECGS); adjusted to a final concentration of 10% foetal bovine serum (FBS) (PAN Biotech). Cells were maintained in 37^oC in a humidified 5% CO_2 atmosphere in 25 or 75 cm^2 flasks as an attached monolayer. When confluency has been reached, cells were washed with 1X cell culture grade phosphate buffer saline (PBS) before trypsinization, were detached by 1X cell culture grade trypsin (PAN Biotech) and one flask was passaged to two flasks. Cells were seeded in 96-well culture plates ($1x10^4$), 6-well culuture plates ($8x10^4$) and 60 mm culture dishes ($1.3x10^6$) in complete medium before experiments.

Human leukemic monocyte lymphoma cell line, U937, was a kind gift of Prof. Dr. Poli from Department of Clinical and Biological Sciences, University of Turin in Italy. These cells were cultured in RPMI 1640 (Biological Industries) supplemented with 1% penicillin and streptomycin solution (Biological Industries), adjusted to a final concentration of 10% FBS (PAN Biotech). Cells were maintained in 37^oC in a humidified 5% CO_2 atmosphere in 25 or 75 cm^2 flasks as suspension. When confluency

has been reached, cells were centrifuged and resuspended in complete medium and passaged. Cells were seeded in 96-well culture plates ($5x10^4$ in 100 µL medium), 12-well culture plates ($5x10^5$ in 1 ml medium) and 60 mm culture dishes ($2.5x10^6$ in 5 ml medium) in serum free medium before experiments.

Both huv-ec-c`s and U937 cell line were resuspended and frozen in freezing mix containing heat inactivated FBS with 10% DMSO after centrifiguation at 300g. The resuspended cells in freezing mix were transfered into cryovials and were kept at -80^oC for 24 hours and then stored in liquid nitrogen to remain until thawing. These stocks were thawed in 37^oC in a short time to avoid damage from crystallization.

3.2.2 4-HNE Treatment

Huv-ec-c`s were seeded in complete medium 24 hours before the treatment. 24 hours after the seeding, complete medium is removed and 4-HNE is treated with serum free medium at the indicated concentration. Control cells were treated with serum free medium, since the ethanol amount, even in the highest concentration of 4-HNE treatment is below 0.3 % (v/v).

U937 cells were seeded in serum free medium 24 hours before the treatment for synchronization. 24 hours after the seeding 4-HNE is delivered to the serum free medium at the indicated concentration. Control cells were treated with serum free medium.

3.2.3 MTT Assay

Cell death was determined with an MTT assay kit, according to the manufacturer`s protocol. Huv-ec-c`s and U937 cells were seeded in 96-well plate and treated as indicated above.After cells were incubated with 4-HNE for 24 hours, 10 µl of MTT labeling solution was added to each well and each well was incubated with this labeling solution for 4 hours. Then 100 µl of the solubilization solution was introduced to the each well and incubated for overnight. The absorbance was measured with a microtiter plate reader at a wavelength of 550 nm and a reference 655 nm. Percent of

relative cell viability was calculated as (OD of drug treated sample x 100)/ (OD of control sample).

3.2.4 Annexin V Staining Assay

Apoptotic cell death was analyzed by AnnexinV-FITC labeling. Huv-ec-c`s and U937 cells were seeded in 6-well plate and 12-well plate respectively treated as indicated above. After cells were incubated with 4-HNE for 24 hours, cells were removed by trypsinization for huv-ec-c`s or taking the medium for U937, transfered to flow-cytometer tubes and centrifuged at 300g for 5 min. Supernatant was removed in order to discard the medium. Cells were washed with 1ml of 1X PBS then centrifuged again.Once cells were pelleted, PBS was discarded and cells were resuspended in 102 µl of labeling solution consisting of 100 µl of incubation buffer and 2µl of AnnexinV-FITC. Cells were incubated in labeling solution for 15 minutes in dark at room temperature. Then 500 µl of incubation buffer was added to stop the reaction. The measurement was immediately done by a flow cytometer using lasers and filters required for FITC detection.

3.2.5 Total Protein Isolation

Protein isolation was carried on 2, 4, 8, 12 and 24 hours after 4-HNE treatment as it is explained below.

Treated and control huv-ec-c`s were washed with ice cold 1X PBS. Again 1 ml of ice cold 1X PBS was added and cells were scraped from the plates. The scraped cells were put in eppendorf rubes and centrifuged for 13200 rpm for 30 seconds at +4^{o}C. Cells were lysed on ice in NP 40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl) containing 0.1 mM PMSF and protease inhibitor cocktail for 45 minutes. After cells were lysed, cell debris was removed from the lysate by centrifugation for 13200 rpm for 15 minutes at +4^{o}C. Supernatant was transferred to a clean pre-labeled tube.

Treated and control U937 cells were collected with removal of medium from the dishes. Cells were centrifuged at 300g for 5 minutes. Medium was discarded and cells

were washed with 1 ml of ice cold 1X PBS. Cells were centrifuged at 13200 rpm for 30 seconds at +4^{o}C. PBS was discarded and lysis buffer containing the ingredients above was added to the cells. Cells were lysed in lysis buffer on ice for 45 minutes and lysate was centrifuged 13200 rpm for 15 minutes at +4^{o}C for removal of debris. Supernatant was transferred to a clean pre-labeled tube.

3.2.6 Protein Content Assay

Protein concentrations were determined by DC Assay (BioRad) for equal loading to SDS gel elecrophoresis. Different concentrations (0.1, 0.25, 0.5, 0.75, 1.0, 1.5 μg/μl) of BSA standards were obtained by serial dilutions. 5 μl from BSA standards were put in 96-well plates in triplicates. 4μl of ddH_2O and 1μl from the protein was put in 96-well plates in triplicates. 25 μl from solution A and 200 μl from solution B were added to each well. The absorbance of each sample and standards were measured in a microtiter plate reader at a wavelength 655nm. From the BSA standards, a standard curve was obtained and unknown concentrations of samples were obtained by the use of this graph and its equation.

3.2.7 SDS-PAGE Gel Electrophoresis

Biorad mini protean gel systems were used as protein eletrophoresis. 12% SDS-PAGE was prepared by mixing 6.00 ml of 30% acrylamide / 0.8% bisacrylamide, 3.75 ml of 4x Tris-Cl/SDS at pH 8.8, 5.25 ml of ddH_2O, 50 μl of 10% ammonium per sulphate (APS) freshly prepared and immediately 10 μl of TEMED in the given order for the running gel. The gels were overlaid with isopropanol to initiate polymerization and to block the association of the gel with the air. After the gel was polymerized, the isopropanol was discarded and the above of the gel was washed with water and dried with a tissue. The stacking gel was prepared with mixing sequentially 0.65 ml of 30% acrylamide / 0.8% bisacrylamide, 1.25 ml of 4x Tris-Cl/SDS at pH 6.8, 3.05 ml of ddH_2O, and 25 μl of 10% APS (freshly prepared) and immediately 5 μl of TEMED. Then the combs were placed for the formation of the wells. 30-40 μg proteins were

pipetted for the sample preparation and mixed with the same volume of 2x Laemli loading dye in eppendorf tubes. Proteins were denatured in a 95^{o}C for 5 minutes. Equal amounts of proteins were loaded to SDS-PAGE gel. The proteins were seperated on the gels at constant voltage 70V for 2 hours at room temperature.

3.2.8 Immunoblotting

After the proteins were seperated, they were blotted on a polyvinylidene fluoride (PVDF) membrane. PVDF membrane was washed in methanol for 30 seconds, sponges and Watman papers were washed with transfer buffer (10X transfer buffered diluted to 1X and with the addition of 20% methanol) before immunoblotting. After the gel was placed in the tank, proteins were blotted onto PVDF membrane via the application of 60V constant voltage for 70 minutes.

After the blotting procedures, the blot was blocked in 5% dried milk in 1X PBS-Tween20 (20 %) for 2 hours in room temperature. The primary antibody was diluted in 5% dried milk in 1X PBS-Tween20 (20 %) in 1 to 2000 proportion. The primary antibody incubation was carried for overnight at +4^{o}C. After the primary antibody incubation, the blot was washed with 1X PBS-Tween20 (20%) for 2 times during 15 minutes. The blot was further blocked in 5% dried milk in 1X PBS-Tween20 (20%) for 15 minutes in room temperature. The secondary antibody was diluted in 5 % dried milk in 1X PBS-Tween20 (20%) in 1 to 10000 proportion and incubation was carried in room temperature for $2^{1/2}$ hours. The blot was washed with 1X PBS-Tween20 (20%) for 3 times each having 15 minutes duration. Finally the last washing was done with 1X PBS for 15 minutes.

The proteins were at last analyzed using an enhanced chemiluminescence detection system and expose to Hyperfilm-ECL (GE Biosciences).

3.2.9 ImageJ

An image processing and analysis program Image J was used for densitometric analysis of western blots. Densitometric measurements were made for each band and normalized according to their corresponding β-actin controls.

3.2.10 Statistical Analysis

Data are given as means ± SEM. For statistical comparison t-test was employed. *P* values smaller than 0.05 was considered to be * statistical significant, smaller than 0.001 was considered to be ** statistical significant.

4 RESULTS

It is well established that apoptosis of macrophages, smooth muscle cells and endothelial cells take place throughout the process of atherosclerosis. [105] Since Bcl-2 proteins involve in 4-HNE (4-Hydroxynonenal) induced signaling of apoptosis and the apoptosis of foam cells is introduced as a very critical step in progression of atherosclerosis, Bcl-2 proteins are studied by using endothelial cells and monocytes as models. The reason to study the signalling in monocytes is that they are the precursor of macrophages in the case of a triggering signal such as specific differentiation cytokines and factors. Human umbilical vein endothelial cells (huv-ec-c`s) and human leukemic monocyte lymphoma cell line (U937) were chosen as cell lines to be studied in this study.

4.1 The apoptotic response of 4-HNE treated endothelial cells (huv-ec-c's)

4.1.1 Determination of 4-HNE induced cytotoxicity in endothelial cells

In order to determine the cytotoxicity of 4-HNE in huv-ec-c`s, the cells were treated with 1, 5, 10, 15 and 20 μM of 4-HNE in serum free medium for 24 hours then the cell viability was checked with MTT assay. MTT assay is a standard colorimetric assay for the activity of reductase enzymes in mitochondria to reduce yellow tetrazole to formazan giving a purple color. Insoluble purple formazan product is dissolved by a solubilization buffer containing sodium dodecy sulphate in diluted hydrochloric acid. The absorbance of the colored solution is measured by a spectrophotometer. This assay is usually used to determine the cytotoxicity of a material. During the experiment, it is important to perform the treatment in serum free medium to eliminate the possible

reactions that 4-HNE can make with the ingredient proteins in complete medium, because the reactions between 4-HNE and serum proteins might attenuate the effect of 4-HNE given to the media. Serum, heparin and endothelial cell growth supplement were omitted from the medium, during the treatment. According the cell viability assay, the cytotoxic effect of 4-HNE could not be observed untill a dose of 10 μM of 4-HNE. The cell death began at 15 μM of 4-HNE and only 55 % of cells were viable after 20 μM of 4-HNE treatment for 24 hours as shown at Figure 4.1.1 1.A.

To be sure that 20 μM of 4-HNE is the most suitable dose that could be studied, higher dose treatments were also carried on in MTT assay. It was seen in Figure 4.1.1 1B that the cell viability decreased below 50 % of total cells, which might cause difficulties in studying the signaling responses in apoptosis.

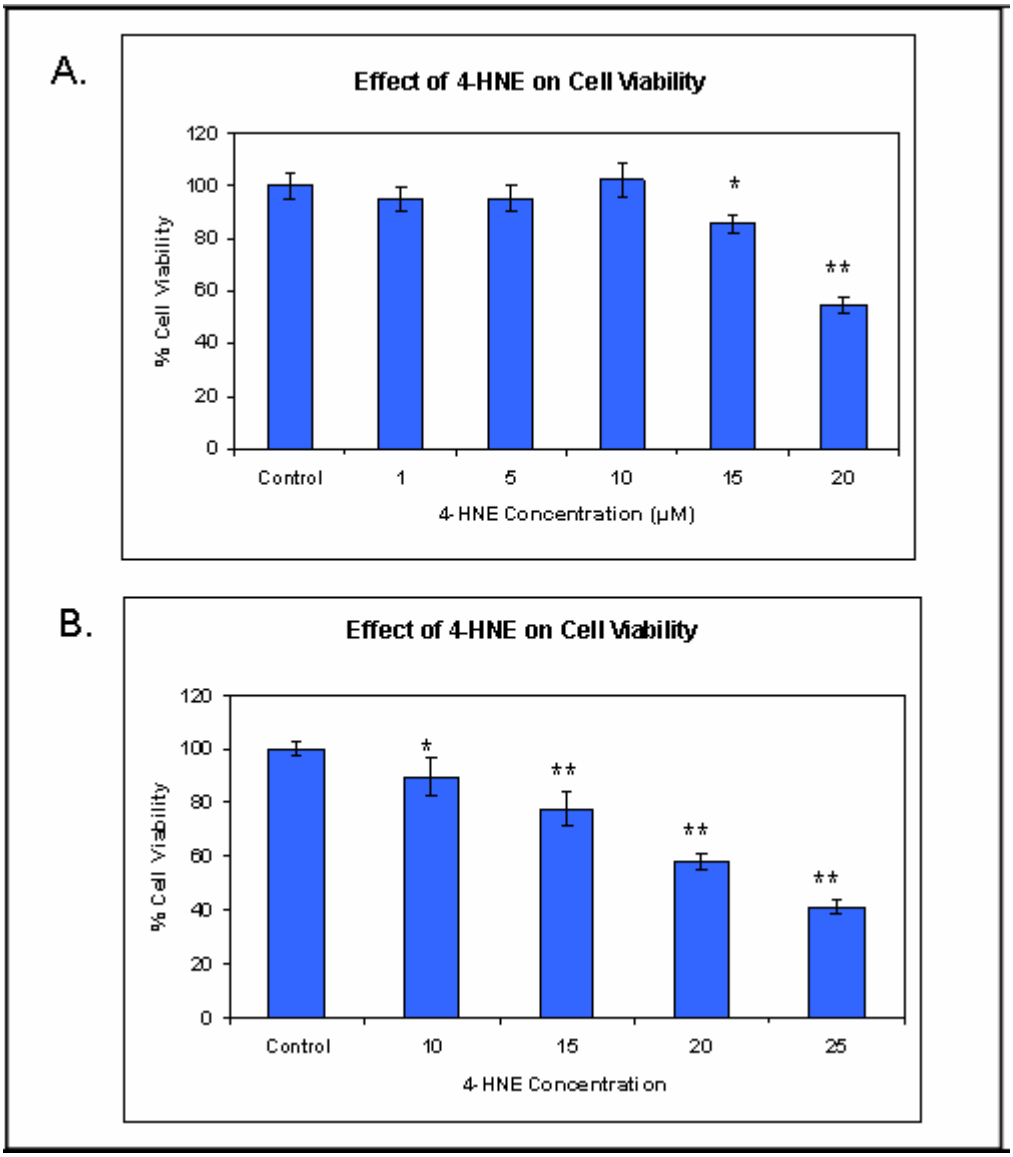

Figure 4.1.1 1: 4-HNE induced cell death in huv-e-c`s. 10000 cells are seeded in 96 well-plate in 100 µl complete medium. 24 hours later, complete medium is removed and huv-ec-c's are treated with indicated concentrations of 4-HNE for 24 hours in serum free medium. A: Concentration between 1 to 20 µM of 4-HNE. B: Concentration between 10 to 25 µM of 4-HNE. The data are expressed as mean ± SEM % of untreated control and representative of six experiments. * $P < 0.05$, ** $P < 0.01$

The apoptotic cell death was presented by Annexin-V staining assay that detected the exposure of the phosphatidylserine residues to the external cellular environment during the apoptosis. The cells were seeded in complete F12K medium. 24 hours after seeding the cells, the complete medium was removed and 20 µM of 4-HNE was added to cells in serum free medium followed by an incubation at 37 ^{0}C for 24 hours. Cells

were detached by trypsin, washed with 1X PBS and stained with Annexin-V FITC that was having high affinity to phoshatidylserine. AnnexinV-FITC positive cells were analyzed by flow cytometric analysis. A significant amount of apoptotic cell death was present in control cells that were incubated in serum free medium for 24 hours, due to serum, heparin and endothelial cell growth supplement depletion. The apoptotic cell death induced by 4-HNE treatment is approximately 44% of the total cell population.

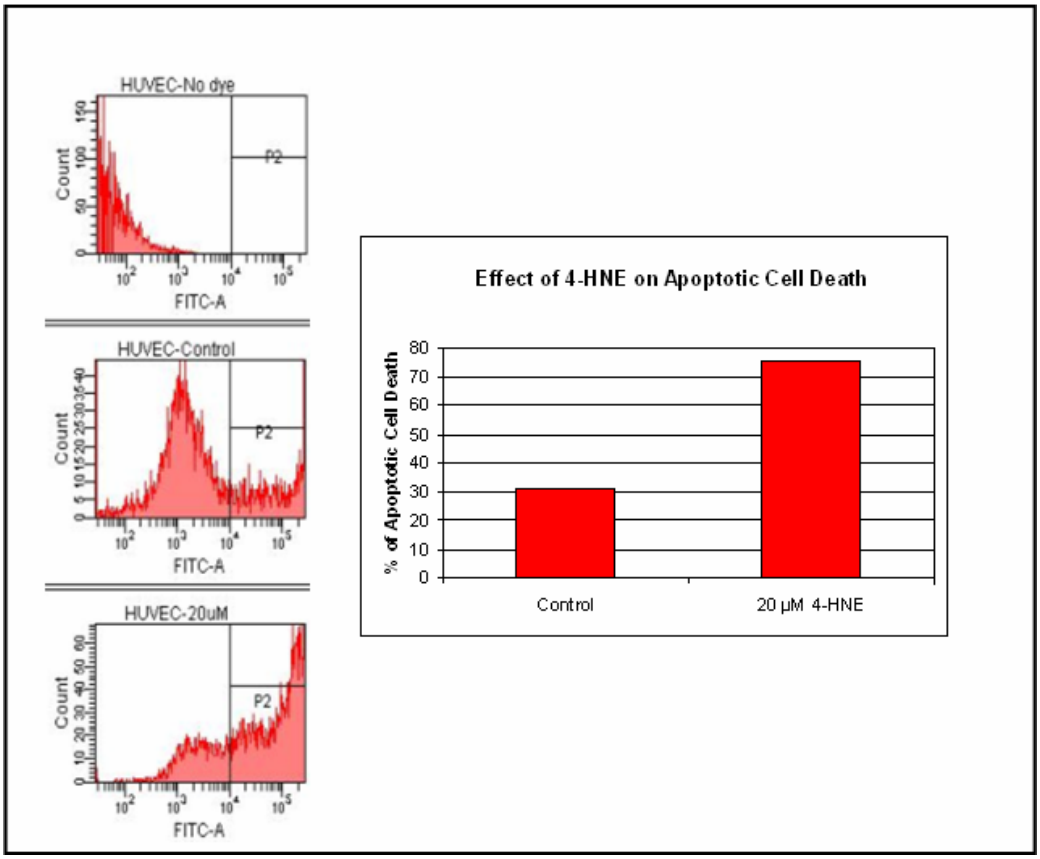

Figure 4.1.1 2: Apoptotic cell death is determined by Annexin V staining assay in response to 20 µM 4-HNE treatment in huv-ec-c's for 24 hours. 80000 cells are seeded in 6 well-plate in 2ml complete medium. 24 hours later complete medium is removed and 4-HNE is given in serum free medium.

Because Annexin-V staining assay was a more sensitive method than MTT assay and because huv-ec-c cells underwent apoptotic cell death without any 4-HNE treatment, the apoptotic cell death in the control group was subtracted from the apoptotic cell death in the 4-HNE treated group. Then both the MTT results and Annexin V staining results were in correlation with each other. As a result, the apoptotic cell death due to 4-HNE treatment in huv-ec-c cells was shown by Annexin-V staining assay.

Once we verified the apoptotic dose of 4-HNE in huv-ec-c`s, we treated the cells with 20 μM 4-HNE prior to protein isolation with 0 to 24 hours to understand the expression profiles of Bcl-2 family members in 4-HNE induced apoptosis. From the protein isolation, 20 μg of total protein is loaded to SDS-PAGE gel and western blotting was performed for specific Bcl-2 family members. The following profiles were obtained for Bcl-$_{XL}$, Bcl-2, Bax and Bid.

4.1.2 Bcl-2 proteins in 4-HNE induced apoptosis in endothelial cells

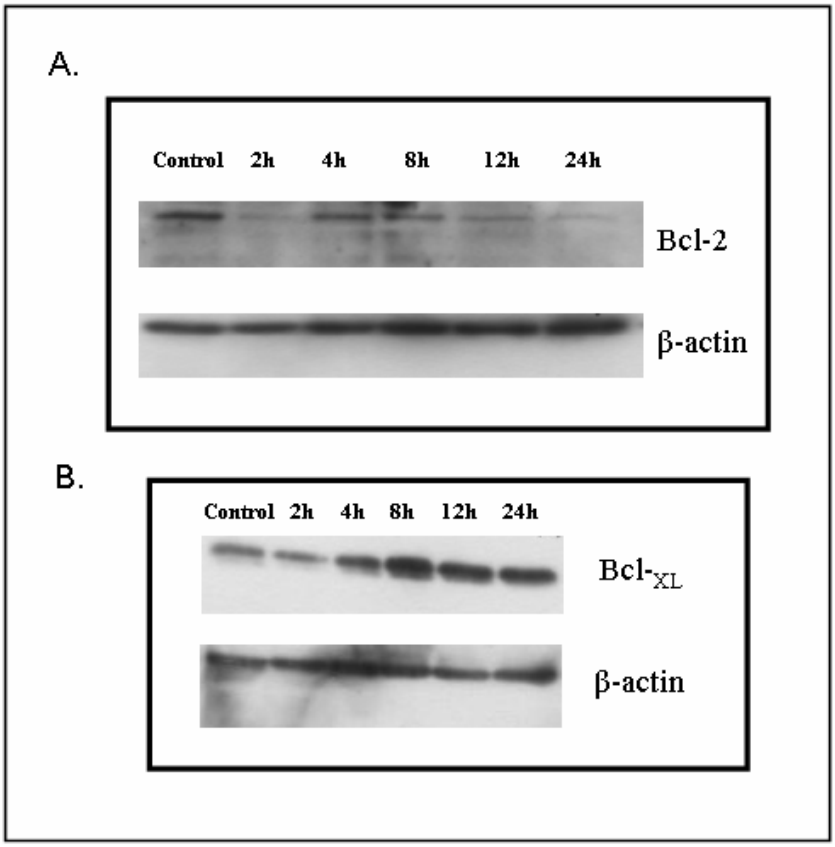

Figure 4.1.2 1: 4-HNE-induced A: down-regulation of Bcl-2 and B: up-regulation of Bcl-$_{XL}$ in huv-ec-c's.. Cells from passage 10 were grown on 60 mm culture dishes (1.3x 10^6 cells/dish) and treated with 20 μM 4-HNE for 0-24 h. The up-regulation of Bcl-$_{XL}$ and down-regulation of Bcl-2 were evaluated by immunoblot analysis. For immunoblot analysis antibodies against Bcl-$_{XL}$ and Bcl-2 were used and β-actin was probed as a loading control.

As shown in Figure 4.1.2 1, Bcl-$_{XL}$ levels remained same within 2 hours and then increased compared to the control level within 24 hours whereas, the protein level of Bcl-2 decreased after 8 hours in a time dependent manner.

The proapoptotic member Bax also has pronounced increase after 4 hours. Interestingly full length Bid is truncated or downregulated after the 4-HNE treatment in 2 hours time.

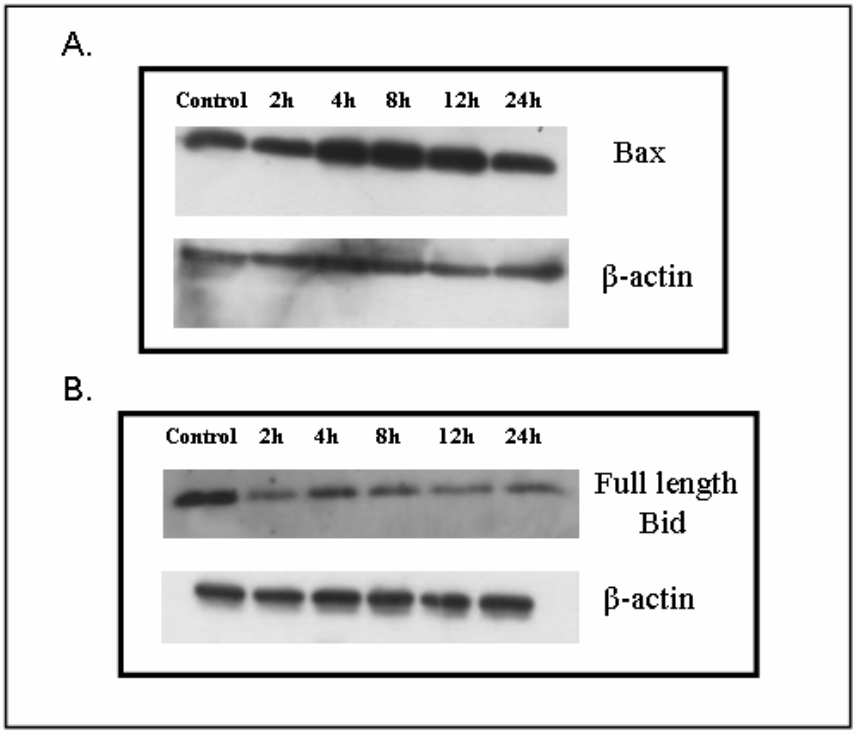

Figure 4.1.2 2: 4-HNE-induced A: up-regulation of Bax and B: the response of full length Bid to the treatment in huv-ec-c's. Cells from passage 10 were grown on 60 mm culture dishes (1.3x 10^6 cells/dish) and treated with 20 μM 4-HNE for 0-24 h. The up-regulation of Bax and Bid's response were evaluated by immunoblot analysis. For immunoblot analysis antibodies against Bax and Bid were used and β-actin was probed as a loading control

4.2 The apoptotic response of 20 µM 4-HNE treated monocytes (U937)

Since it is known that OxLDL and many other stimuli such as monocyte chemoattractant proteins (MCPs) lead to the migration of monocytes into the arterial intima then monocyte/macrophage differentiation and macrophage foam cell transformation in atherosclerosis, monocytic cell line U937 was used in these experiments to determine the apoptotic response of macrophages in atherosclerotic lesions.[108]

U937 cell line was seeded and synchronized in serum free medium RPMI 1640 followed by incubation at 37^0C for 24 hours. 4-HNE was delivered to the cells in serum free medium for 24 hours and cell viability was determined by MTT Assay. Cell viability data could not be obtained because of an experimental drawback in lower 4-HNE doses (1-20 µM), however cell death could easily be detected at higher doses (<20 µM 4-HNE) in MTT Assay. According to the experiment result, 20 µM of 4-HNE treatment decreased the cell viability up to 48% of total cell population. Cell death increased in dose dependent manner.

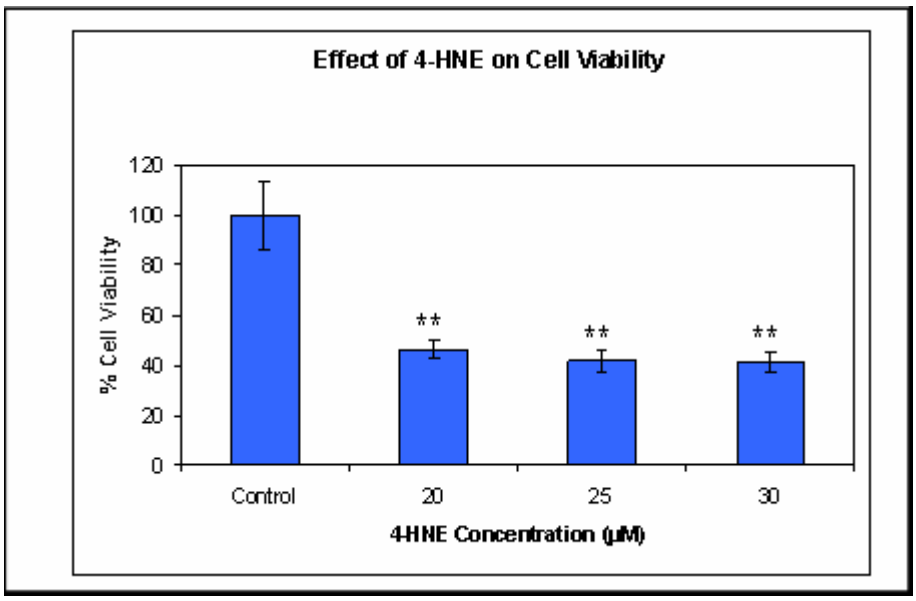

Figure 4.2.1 1: 4-HNE induced cell death in U937 cell line. 30000 cells are seeded in 96 well-plate in 100 µl serum free medium. 24 hours later, U937 cells are treated with indicated concentrations of 4-HNE for 24 hours in serum free medium. The data are expressed as mean ± SEM % of untreated control and representative of six experiments. * $P < 0.05$, ** $P < 0.01$

Dose determination experiments were further continued by confirming apoptotic cell death by Annexin-V staining in flow cytometer. According to the Annexin-V staining result, approximately 33 % of the total cell population underwent apoptosis in 20 μM 4-HNE treatment.

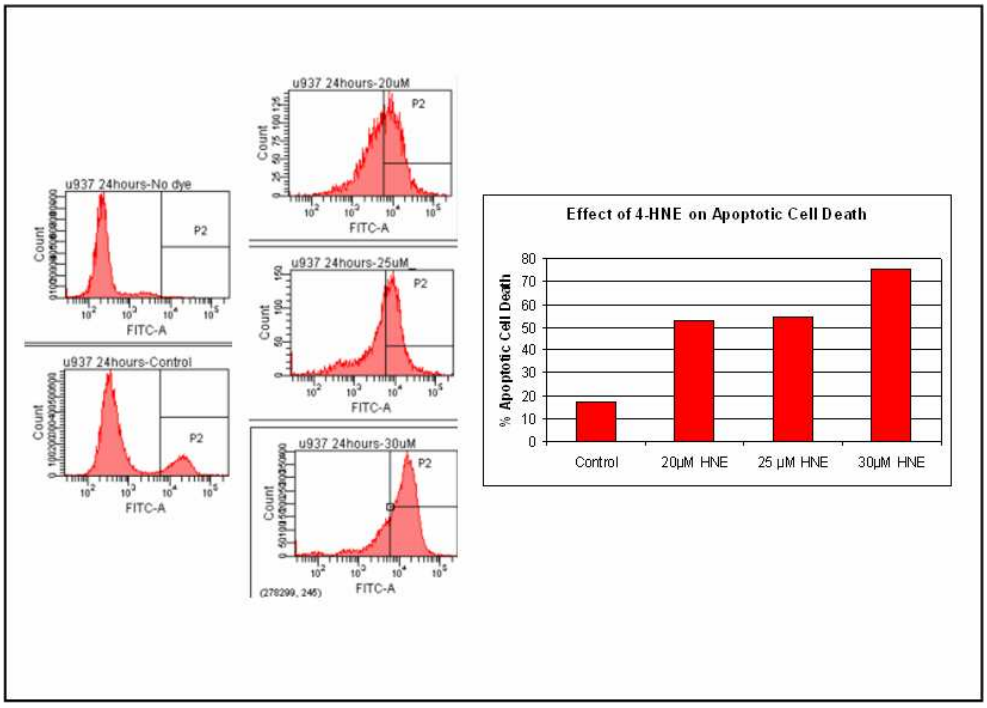

Figure 4.2.1 2: Apoptotic cell death is determined by Annexin V staining assay in response to 20, 25 and 30μM 4-HNE treatment in U937 cell line for 24 hours. 300000 cells are seeded in 6 well-plate in 2ml serum free medium. 24 hours indicated concentrations of 4-HNE is given in serum free medium

4.2.1 Bcl-2 proteins in 20 μM 4-HNE induced apoptosis in monocytes

After the dose determination by MTT and Annexin-V staining assays, the total endogeneous levels of prosurvival Bcl-2 proteins; Bcl-$_{XL}$, Bcl-2 and Mcl-1 and the proapoptotic Bcl-2 proteins; Bak and Bak were evaluated by means of immunoblot analysis using total protein lysates to investigate the effect of 4-HNE on Bcl-2 protein family members. Because of the excess of cell death at 24th hours, the immunoblotting experiments were carried with lysates obtained in 12 hours of 4-HNE treatment.

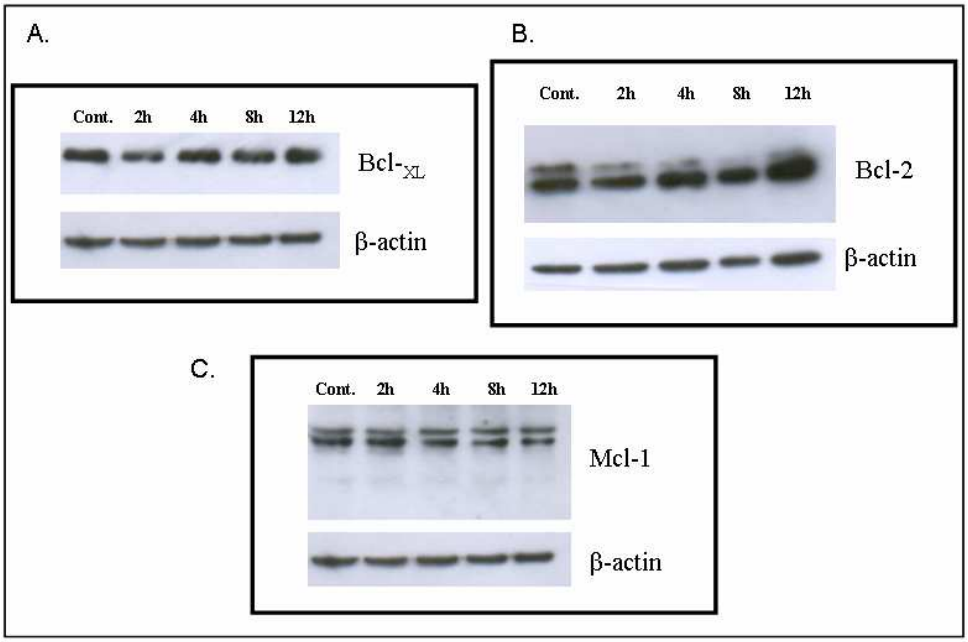

Figure 4.2.2 1: 4-HNE-induced response of prosurvivalBcl-2 proteins. A: Bcl-$_{XL}$, B: Bcl-2, C: Mcl-1. U937 cells were grown on 60 mm culture dishes (3x 10^5 cells/dish) and treated with 20 μM 4-HNE for 0-12 h. The effects of Bcl-$_{XL}$, Bcl-2 and Mcl-1 against 4-HNE treatment were evaluated by immunoblot analysis. For immunoblot analysis antibodies against Bcl-$_{XL}$, Bcl-2 and Mcl-1 were used and β-actin was probed as a loading control.

As shown in Figure 4.2.2 1A, the endogenous level of Bcl-$_{XL}$ in response to 4-HNE treatment showed minor decrease in 2 hours that was followed by the reestablishment of Bcl-$_{XL}$ levels in 12 hours. (Confirmed by Dr Bodur, unpublished result).

In all of the Bcl-2 immunoblots obtained with U937 cells` lysate, double band was observed interestingly. This double band could not be observed in immunoblots carried on with lysates of any cell lines in our laboratory. According to literature Bcl-2 gene can potentially encode 26 and 22 kDa proteins by the alternative splicing mechanism. It is included that the 26kDa form of Bcl-2 protein is far more important and 22 kDa form of the protein is rarely observed in cells. [116] Since the bigger spliced form was the most pronounced form according to the literature, the upper band was taken into consideration. Time dependent decrease in protein level of Bcl-2 could was observed in the upper band.

In Figure 4.2.2 1C, Mcl-1 immunoblot also had double band that each having a close molecular weight so that their exact molecular weights could not be determined by using the marker. However when the other Mcl-1 blots were taken into consideration,

the upper band was generally the more pronounced one. The band having higher molecular weight did not have a major change at 12 hours.

After screening the total protein levels of prosurvival Bcl-2 family members, Bax and Bak protein levels were also investigated by immunoblotting technique in order to understand the response of proapototic multidomain Bcl-2 proteins. Bax had an increase at 8 hours and 12 hours. Interestingly the Bak levels had a marked decrease in 2 hours and did not return to the control levels in any time points checked.

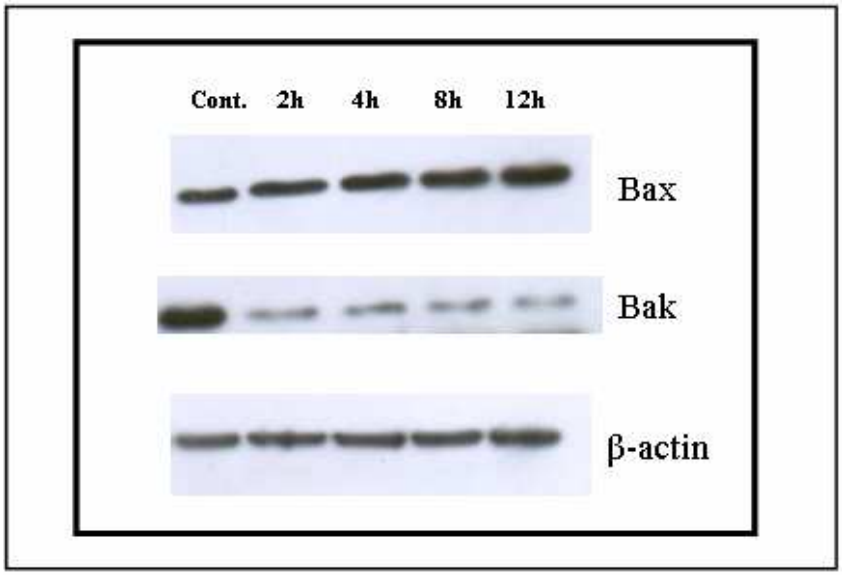

Figure 4.2.2 2: 4-HNE-induced response of A: Bax and B: Bak. U937 cells were grown on 60 mm culture dishes (3x 10^5 cells/ml) and treated with 20 μM 4-HNE for 0-12 h. The effects of Bax and Bak against 4-HNE treatment were evaluated by immunoblot analysis. For immunoblot analysis antibodies against Bax and Bak were used and β-actin was probed as a loading control

4.3 The apoptotic response of 10 μM 4-HNE treated monocytes (U937)

Since there was problem to isolate the protein lysates at 24 hours, we tried to decrease the dose and increase the treatment time. Thereby it was enabled to screen the response of Bcl-2 family members in a lower dose treatment through a longer time. MTT cell viability and Annexin-V staining assays were repeated in order to determine a lower dose to study the signaling effect of 4-HNE.

4.3.1 Determination of 4-HNE induced cytotoxicity in monocytes

According to our experiments, 10 μM 4-HNE treatment was chosen to study the signaling effect of the lower dose. The cell viability was between 70-80 % of the total population and the apoptotic cell death resulting from the treatment was about 12% as shown in Figure 4.3.1 1 and Figure 4.3.1 2 respectively. Cell viability value was higher and the apoptotic cell death value was lower than the values obtained with 20 μM 4-HNE treatment, which leaded to study signalling effect easier.

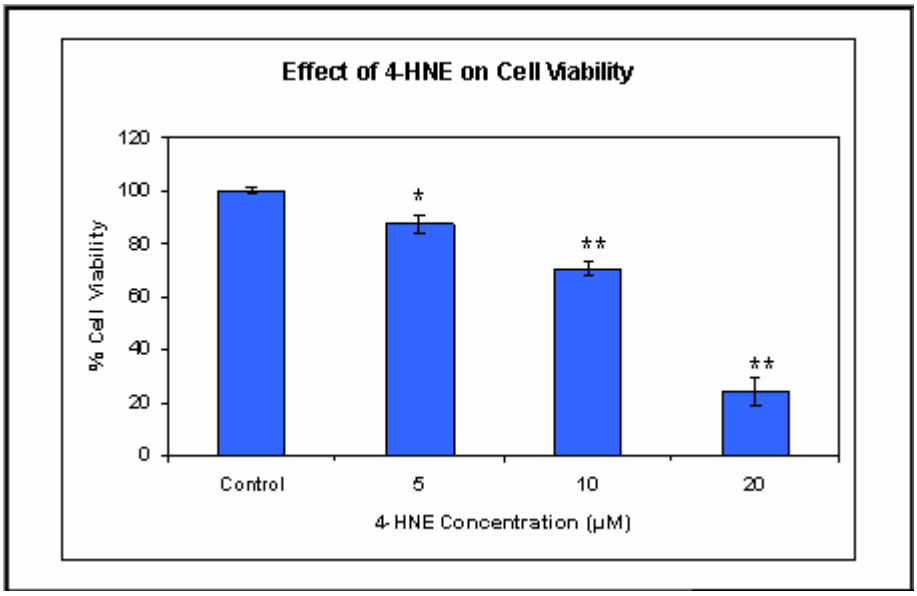

Figure 4.3.1 1: 4-HNE induced cell death in U937 cell line. 50000 cells are seeded in 96 well-plate in 100 μl serum free medium. 24 hours later, U937 cells are treated with indicated concentrations of 4-HNE for 24 hours in serum free medium. The data are expressed as mean ± SEM % of untreated control and representative of six experiments. * $P < 0.05$, ** $P < 0.01$

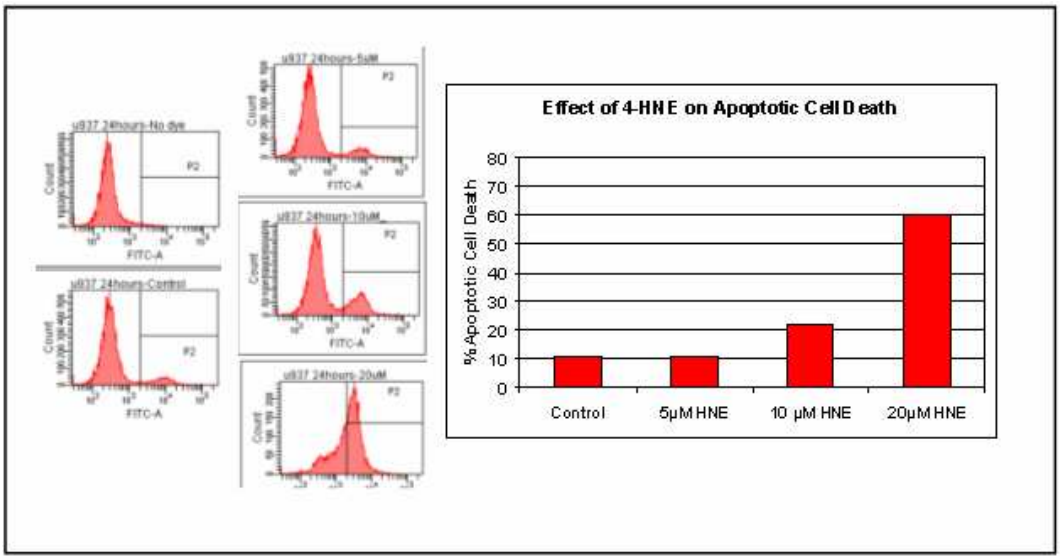

Figure 4.3.1 2: Apoptotic cell death is determined by Annexin V staining assay in response to 5, 10 and 20µM 4-HNE treatment in U937 cell line for 24 hours. 500000 cells are seeded in 12 well-plate in 1ml serum free medium. 24 hours indicated concentrations of 4-HNE is given in serum free medium.

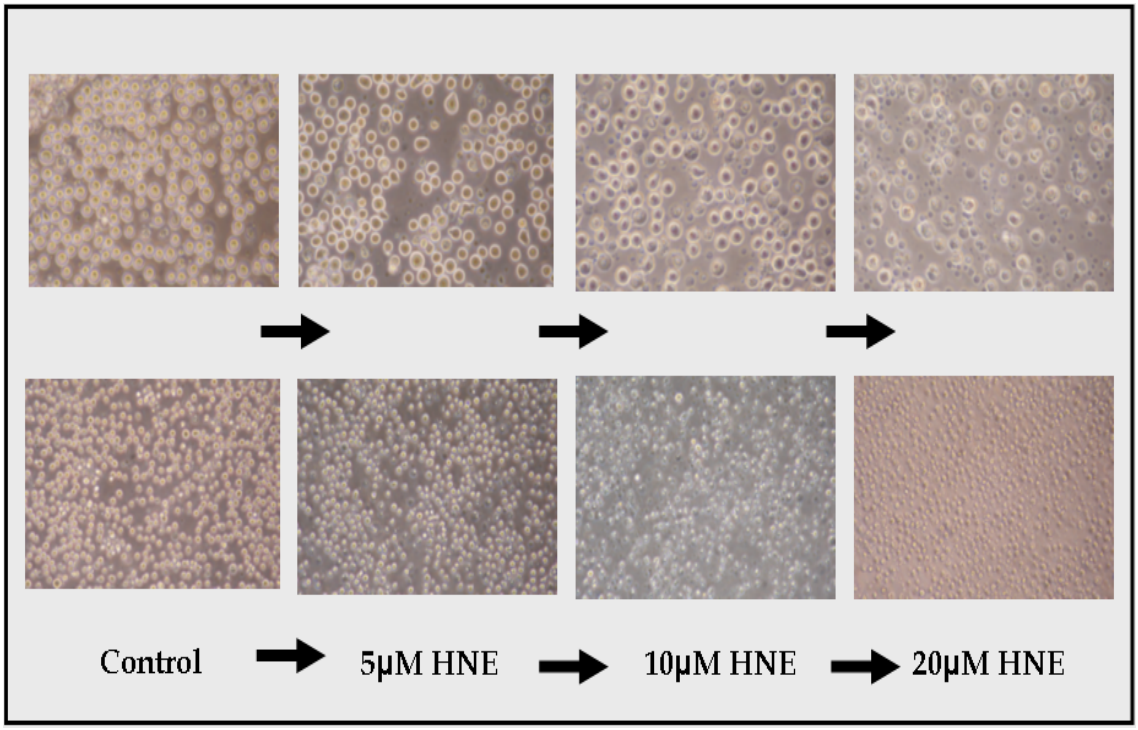

Figure 4.3.1 3: Morphological changes in U937 cells undergoing apoptosis in different doses of 4-HNE treatment. The images were observed under light microscope.

The morphological appearance of U937 cells treated with different concentrations of 4-HNE, was observed under light microscope. With the increasing concentrations of

4-HNE, the cell shrinkage, membrane blebbing and apoptotic body formation were easily observed under the light microscope.

4.3.2 Bcl-2 proteins in 10 μM 4-HNE induced apoptosis in monocytes

The signaling effect of 10μM 4-HNE was monitored by isolating protein lysates of U937 cells through 0-24 hours then immunoblotting Bcl-2 family members. Endogenous Bcl-$_{XL}$ level in response to 4-HNE treatment had a decrease in 2 and 4 hours then the level was restored beginning from 8 hours till to 24 hours.

In Bcl-2 immunoblot, upper band belonging to 26 kDa Bcl-2 had a decrease in 4 hours and stayed at low levels till to 12 hours. However at 24 hours there was a pronounced increase.

The Mcl-1 immunoblot had also double in this treatment. However in this blot it was seen that the bands were highly separated from each other. The upper one was considered as Mcl-1 since it was on the expected molecular weight according to marker and the lower band could not be defined. According to the loading control (since the loading was not nice according to the loading control), Mcl-1 level did not change after 4-HNE treatment.

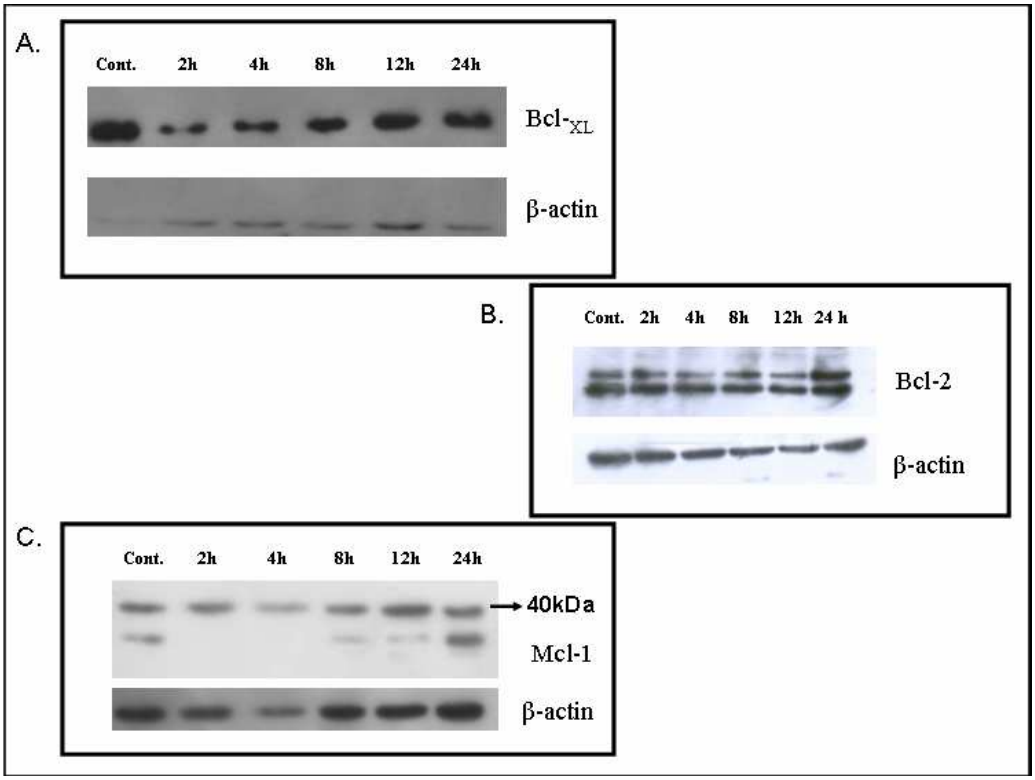

Figure 4.3.2 1: 4-HNE-induced response of A: Bcl-$_{XL}$, B: Bcl-2 and C: Mcl-1. U937 cells were grown on 60 mm culture dishes (0.5x 10^6 cells/dish) and treated with 10 μM 4-HNE for 0-24 h. The effects of Bcl-$_{XL}$, Bcl-2 and Mcl-1 against 4-HNE treatment were evaluated by immunoblot analysis. For immunoblot analysis antibodies against Bcl-$_{XL}$ Bcl-2 and Mcl-1were used and β-actin was probed as a loading control.

Proapoptotic multidomain Bcl-2 proteins; Bak and Bax were also examined by immunoblotting. According to Bax and Bak immunoblots, Bax had a more clear response than Bak had. Similar to the previous Bax blot, the increase of Bax`s protein level was observed in 8 hours time. Contrary to the previous Bak blot, the levels of Bak did not change.

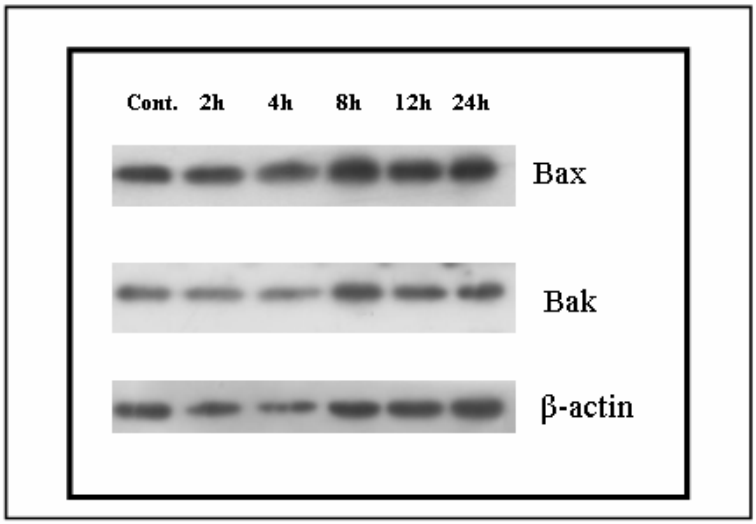

Figure 4.3.2 2: 4-HNE-induced response of Bax and Bak respectively. U937 cells were grown on 60 mm culture dishes (5x 10^5 cells/dish) and treated with 10 μM 4-HNE for 0-24 h. The effects of Bax and Bak against 4-HNE treatment were evaluated by immunoblot analysis. For immunoblot analysis antibodies against Bax and Bak were used and β-actin was probed as a loading control.

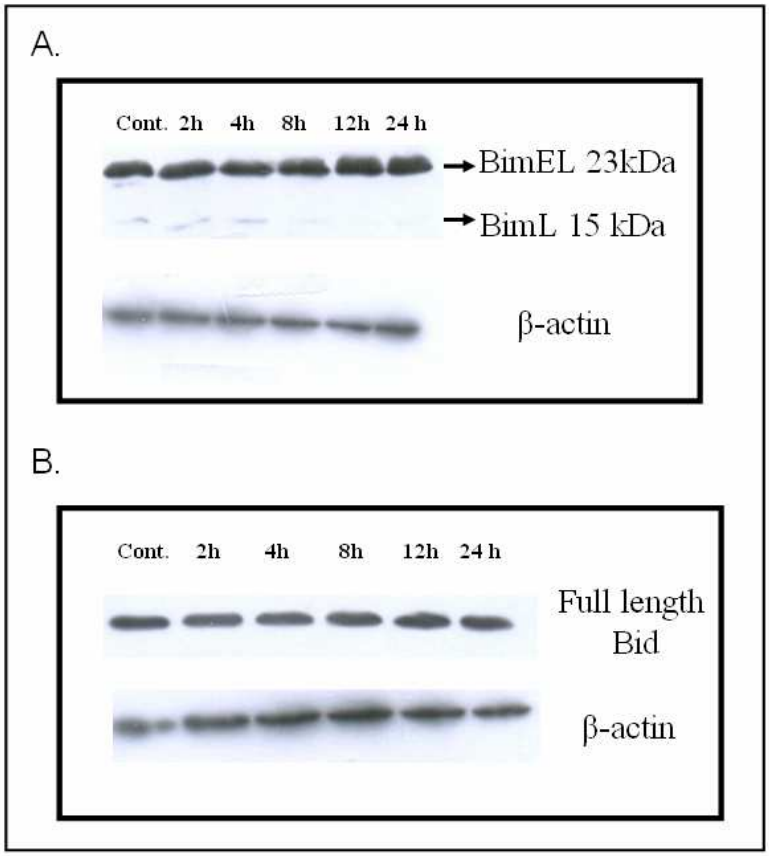

Figure 4.3.2 3: 4-HNE-induced response of A: Bim and B: Bid. U937 cells were grown on 60 mm culture dishes (5x 10^5 cells/dish) and treated with 10 μM 4-HNE for 0-24 h. The effects of Bim and Bid against 4-HNE treatment was evaluated by immunoblot analysis. For immunoblot analysis antibodies against Bim and Bid were used and β-actin was probed as a loading control.

The levels of Bim and Bid proteins were also evaluated by immunoblotting since they were important BH3 only proteins. They were classified as activators according to direct method, thus the change in their endogenous level might enlighten how Bax and Bak were activated. The level of extra long isoform of Bim and fullength Bid were not change however a decrease in long isoform of Bim was observed after 4 hours.

5 DISCUSSION

In the present study, we investigated the response of huv-ec-c and U937 cells in 4-HNE induced apoptosis and further identified the role of Bcl-2 protein family members in this event. The differences in Bcl-2 protein levels triggered by 4-HNE treatment in huv-ec-c and U937 cells are reviewed in the table below.

	Bcl-$_{XL}$	**Bcl-2**	**Mcl-1**	**Bax**	**Bak**	**Bim**	**Bid**
Huv-ec-c`s *20 μM 4-HNE*	Up-regulation (4-24 hrs)	Down-regulation (8-24 hrs)	-------	Up-regulation (4-24 hrs)	-------	-------	Truncation (2-24 hrs)
U937 *20 μM 4-HNE*	Down-regulation at 2 hrs Restore (4-12 hrs)	Down regulation (2-12 hrs)	No change	Up-regulation (8-12 hrs)	Down-regulation (2-12 hrs)	-------	-------
U937 *10 μM 4-HNE*	Down-regulation (2-4 hrs) Restore (8-24 hrs)	Down-regulation (2-12hrs) Restore (12-24 hrs)	No change	Up-regulation (2-24hrs)	No change	No change	No change

Table 5 1: The response of Bcl-2 proteins in 4-HNE induced apoptosis in huv-ec-c`s and U937 cell line.

We showed that incubation of huv-ec-c cells with higher than 10 μM doses of 4-HNE, resulted a decrease in cell viability in 24 hours as shown in Figure 4.1.1 1. 10 μM or lower than 10 μM of 4-HNE treatment did not induce cell death in huv-ec-c`s as it was expected from literature studies claiming about low levels of 4-HNE promoting proliferation where as at higher concentration inducing differentiation and apoptosis. [90] The experiment showed that the cell death was mainly apoptosis from the detection of phosphatidyl serine residues by Annexin-V as shown in Figure 4.1.1 2. When the cell

death resulting from the serum deprivation was eliminated, MTT and Annexin-V results were in correlation with each other.

From the immunoblotting results in Figure 4.1.2 1B, it was seen that Bcl-$_{XL}$ had a clear increase in a time dependent manner. This might be related with the resistance to apoptosis because; in earlier studies, nuclear factor-κB binding sequences have been characterized in the promoter of human and murine Bcl-X gene. [111, 112, 4] That might suggest the ability of the cells to up-regulate Bcl-$_{XL}$ expression under the stress condition. In a study done by C.M. Luetjens, similar results were obtained. According to their results, prooxidants such as hydrogen peroxides (3-100 μM) and ferric ions (1-10μM) increased the expression of Bcl-$_{XL}$ in PC12 neuronal cells and human neuroblastoma SH-SY5Y cell line. They also related the resistance to apoptosis with the high levels of Bcl-$_{XL}$, by showing the overexpression of Bcl-$_{XL}$ mildly protected from cell death however, failure of caspase activation occurred. [113] Also in another work, the protective response, mediated by the activation of HSF1 and stabilization of Bcl-$_{XL}$, was emphasized as the contributor to the cellular biology of lipid peroxidation. [105]

Bcl-2 had a different pattern than Bcl-$_{XL}$ had. It was seen in Figure 4.1.2 1A that Bcl-2 protein level decreased after 8 hours. In a previous study, it was argued that when human umbilical vein endothelial cells were treated with arachidonic acid (>25μM) that was the reactant of peroxidation reaction resulting with 4-HNE formation, typical morphological changes of apoptosis were observed by Giemsa stain and transmission electron microscopy. The western blots showed that apoptosis induced by arachidonic acid was associated with the downregulation of Bcl-2. [114]

The level of proapoptotic protein Bax, responsible for the mitochondrial pore formation, increased after 4 hours and went on increasing in time dependent manner as seen in Figure 4.1.2 2A. According to Bid immunoblot in Figure 4.1.2 2B, Bid was downregulated or truncated just after 4-HNE treatments in 2 hours then maintained its level. Since the truncated portion of the protein could not be observed, a certain response of the protein could not be known. However, it was assumed that fullength Bid was truncated; since the decrease was sudden, in a short time and not in a time dependent manner. It is well known that truncated Bid is produced by caspase 8 activation and caspase 8 activation is related with Fas dependent extrinsic pathway. The possible truncation of the protein indicated 4-HNE induced apoptosis in huv-ec-c`s might take place with the involvement of Fas pathway. Fas related apoptosis triggered by 4-HNE in HLE B-3 cells was explained before by Awasthi *et al*. [103]

The protein levels of Bcl-$_{XL}$ and Bax in endothelial cell apoptosis were in similar manner interestingly as it was seen in Figure 4.1.2 1A and Figure 4.1.2.2A. In a recent study the relation between Bax and Bcl-$_{XL}$ was described in an unexpected way. Andrews *et al*. studied the mechanisms of two proteins by using full length recombinant proteins and measuring permeabilization of liposomes and mitochondria in vitro. It was demonstrated that functionally Bcl-$_{XL}$ and Bax were similar and both of them did not bind to the membrane alone. However in the presence of an activator, in this case tBid was used, the activator activated both of them to immobilize them in membrane. Bcl-$_{XL}$ competed with Bax for the activation of soluble monomeric Bax through interaction with membrane or tBid or activated Bax by tBid. By competition, Bcl-$_{XL}$ inhibited Bax from binding to membrane, oligomerization and membrane permeabilization. Finally it was proposed that Bcl-$_{XL}$ functioned like a dominant negative Bax in the membrane permeabilization process [115]. The similar pattern of Bcl-$_{XL}$ and Bax that we observed in this study, might be explained in terms of the competitive relation between Bcl-$_{XL}$ and Bax during an apoptotic stimulus.

Since macrophage apoptosis is very critical for atherosclerosis progression, the experiments were also carried out by a different cell line, U937. Initially 20 μM was chosen as the apoptotic dose. As in the huv-ec-c`s case, the apoptotic cell death in the control cells due to serum deficiency was also monitored, even though the extend of cell death was not as high as in huv-ec-c`s as shown in Figure 4.2.1 2.

Within 12 hours, cells were incubated with 4-HNE, total proteins were isolated at defined time points and the immunoblotting experiments were carried out. A slight decrease in 2 hours was observed in Bcl-$_{XL}$ immunoblot in Figure 4.2.2 1A. After 2 hours, the endogenous level of the protein was restored back to the control levels. In general, prosurvival proteins decrase in response to an apoptotic stimulus however in this case Bcl-$_{XL}$ protein levels had a different response to apoptosis.

In Bcl-2 immunoblot shown in Figure 4.2.2.1B, double band with similar molecular weights were observed. Upper one was considered as Bcl-2 according to the marker and it was seen that there was a decrease in Bcl-2 protein level in response to 4-HNE induced apoptosis. In a study done by Redd *et al.* in 1993, it was postulated that two isoforms of Bcl-2, 26 kDa and 22 kDa were potentially encoded by an alternative splicing mechanism. According to the work, the larger of these proteins has been shown to prolong cell survival by blocking apoptosis however, the shorter one did not extend the survival of 32D cells in the absence of IL-3 when they were expressed exogenously.

[116] If we looked to the situation from their point of view, the larger one, claimed as prosurvival protein, decreased and the shorter one, claimed as proapoptotic protein, increased in a time dependent manner.

In Figure 4.2.2 2C, the upper one was accepted as the Mcl-1 band. In the immunoblot, it was seen that Mcl-1 protein level stayed constant during apoptosis.

Proapoptotic Bax protein level shown in Figure 4.2.2 lane 1, had a marked increase similar to the 4-HNE triggered apoptosis in huv-ec-c`s. In contradiction to level of Bax protein, the protein level of Bak had a dramatic decrease in a short time as shown in Figure 4.2.2 lane 2. The decrease in protein level of Bak and the increase in protein level of Bax were meaningful since in various cell types such as HCT-116, clear apoptotic sensitivities existed in the order of $Bax^+/Bak^+ > Bax^+/Bak^- >> Bax^-/Bak^+ >> Bax^-/Bak^-$. Bax had a preferential role in apoptosis. [117] Even in the absence of Bak, the increase in Bax`s protein levels leaded U937 cells to undergo to apoptosis.

The decrease in Bak`s level and the constant Mcl-1 level might be related in terms of a work done to identify the ability of six human prosurvival Bcl-2 proteins(Bcl-$_{XL}$, Bcl-2, Bcl-W, Bfl-1, Mcl-1 and Bcl-B)to suppress apoptosis induced by overexpression of Bax or Bak. They demonstrated that Mcl-1 was bound to Bak and Mcl-1 suppressed apoptosis induced by overexpression of Bak but not with Bax [118].The behaviour of Mcl-1 and Bak by not taking place pronouncedly in apoptotic mechanism was more meaningful since they were related from this aspect.

The immunoblotting analyze was also done with lysates of U937 cells treated with 10 μM 4-HNE in 24 hours. In this experimental set up, similar to the results obtained with 20 μM of 4-HNE treatment, as seen in Figure 4.3.2 1A and B respectively, Bcl-$_{XL}$ and Bcl-2 had decreasing patterns at early hours then restored themselves after 24 hours. Bcl-2 had a more pronounced decrease at 20 μM of 4-HNE treatment; however in 10 μM of 4-HNE treatment, the decrease pattern was smoother. Interestingly at 24 hours it was seen that protein level of Bcl-2 reestablished to the control levels which could not be observed in 20 μM of 4-HNE treatment due to the lack of 24th hour's protein lysate in that experimental set up.

As well as Bcl-$_{XL}$ and Bcl-2; in 10 μM of 4-HNE treatment, Mcl-1 showed similar pattern with 20 μM of 4-HNE treatment and did not had a significant change in protein levels as seen in Figure 4.3.2 1C. The steady levels of Mcl-1 protein in monocytes treated with both 10 -20 μM of 4-HNE, might also be associated with the cell type. It has been accomplished that Mcl-1 was a very important protein which was upregulated

in numerous hematological and solid tumor malignancies and became a factor in the resistance of some types of cancer. [119] Because U937 cells were cancerous and hematologic, Mcl-1 level might be already upregulated and might not be abolished by 4-HNE treatment.

In Figure 4.3.2 2, it was seen that Bax's protein level increased and Bak's protein level did not change but also did not decrease as it was monitored in 20 μM of 4-HNE treatment. Because it was not possible to be sure about the involvement of Bak in apoptosis by looking the endogenous level of the protein, further experiments have to be done about its oligomerization or interaction with other proteins.

Interestingly in both of the cell lines, prosurvival members of Bcl-2 family somehow regenerated themselves after a transient decrease, although in huv-ec-c`s Bcl-$_{XL}$ increase in protein level was observed. In recent studies, the resistance against 4-HNE induced apoptosis was emphasized. For instance, it was concluded that 4-HNE triggered heat shock genes` expression, in which the lack of them resulted with an increase in apoptosis. [105] In another work, it was showed that 4-HNE exposure caused the induction of both Fas and Daxx in Jurkat leukaemia cells. It promoted export of Daxx from nucleus to the cytosol and in cytosol; Daxx bound to Fas and inhibited apoptosis. Also translocation of Daxx from nucleus to the cytosol was related with the activation of heat shock factor 1 (HSF1) which was responsible for the activation of heat shock proteins. [103] Thus; besides triggering apoptosis, 4-HNE also induced some of the defence mechanism opposed to apoptosis.

It is well established that the metabolism of 4-HNE is very important and we assume that the intracellular concentration of 4-HNE might decrease within time points checked due to removal of 4-HNE by GST`s and RLIP 76.

Both of these discussed defense mechanisms and metabolism of 4-HNE showed that a resistance was built against 4-HNE by the cellular machinery. The reestablishment of 2 important prosurvival proteins might be related with that resistance mechanism however, detailed investigation has to be carried on in order to relate Bcl-2 proteins with the figured resistance mechanisms.

One of the other interesting point in this study, is the difference of Bak`s response in different doses of 4-HNE treatment. In a higher dose, 20 μM of 4-HNE treatment, its level decreased sharply after the treatment. However, in 10 μM 4-HNE treatment, major change in Bak's level could not be detected. In a recent article, it was argued that Bak was a key molecule in determining the mitochondrial morphology in apoptosis. They

said that the mitochondrial network collapse into short punctuate fragments occurred by two opposing processes, fission and fussion. The regulation of this mechanism was done by Bak through the interaction with mitofusins; Mfn1 and Mfn 2. Mitochondrial fragmentation took place in the presence of Bak, and Bax was not necessary for this process according to their work. It was also concluded that filamentous mitochondria was preserved in Bak deficient cells and Bak might function to inhibit fusion.[120] This unique role of Bak might be the starting point to discuss the different response of Bak in different doses of 4-HNE treatment. Still, detailed investigation has to be done to make these two concepts related with each other and to find a well established response of Bak. The established interactions between Bak and Mfn 1/2 could be investigated for this aim in 4-HNE induced apoptosis in monocytes.

6 CONCLUSION

In conclusion, we report the involvement of intrinsic pathway and Bcl-2 protein family members in 4-HNE triggered apoptosis in 2 different cell lines; huv-ec-c and U937 cells.

In huv-ec-c`s, 4-HNE induced apoptosis takes places by the marked increase of Bcl-$_{XL}$ and Bax, decrease of Bcl-2 and possible truncation of full length Bid. In huv-ec-c`s, as well as intrinsic pathway, extrinsic pathway is assumed to be involved according to full length Bid truncation by Fas receptor activation followed by caspase 8 activation in 4-HNE apoptosis. However in order to be sure about the extrinsic pathway involvement, further experiments have to be done by showing Fas receptor activation, FADD association, DISC formation and caspase 8 activation.

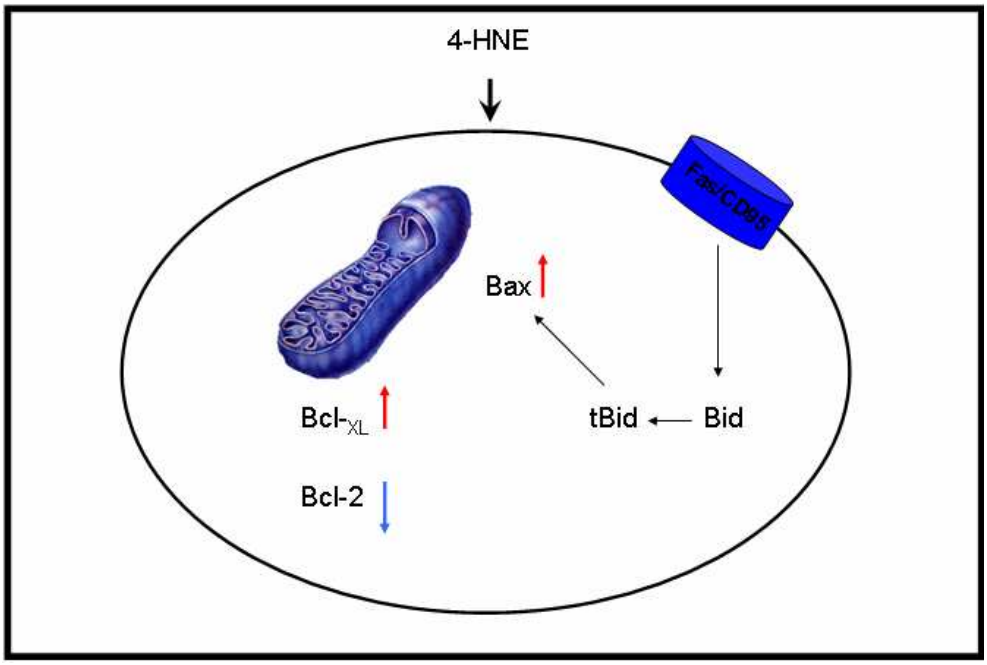

Figure 6 1: Proposed mechanism of Bcl-2 proteins involvement in 4-HNE induced apoptosis in huv-ec-c`s.

In U937 cells, Mcl-1 and Bak do not contribute to the apoptosis as major players where as decrease in Bcl-$_{XL}$ and Bcl-2 protein levels occur at early time points with a apparent increase of Bax. After 24 hours, both Bcl-2 and Bcl-$_{XL}$ somehow restore themselves which may be related with a resistance mechanism.

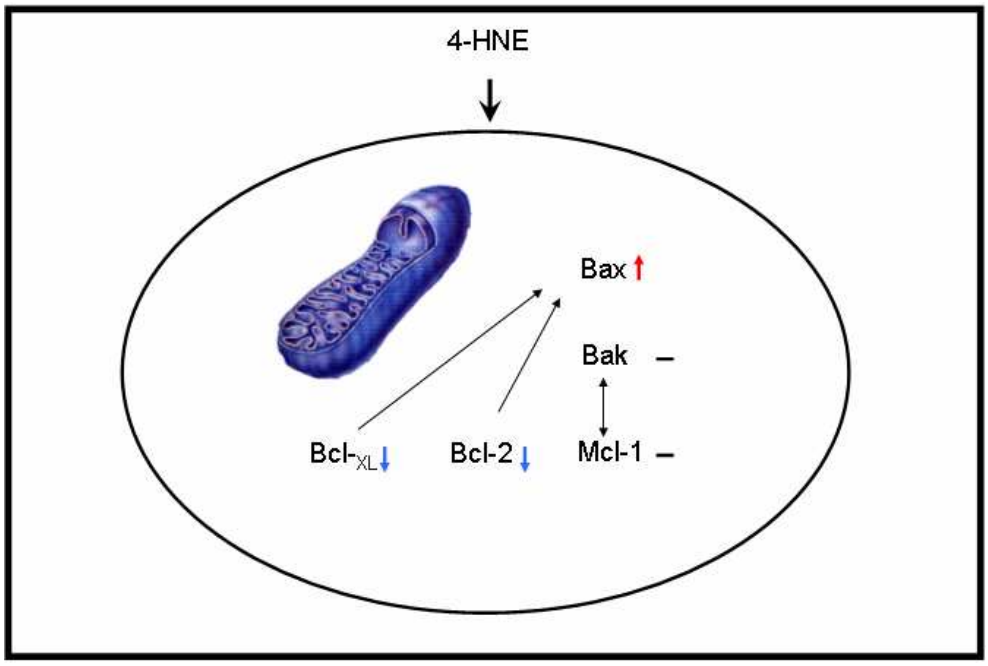

Figure 6 2: Proposed mechanism of Bcl-2 proteins involvement in 4-HNE induced apoptosis in U937 cell line.

When huv-ec-c and U937 cells are compared, two major differences attract attention. One of them is the possible involvement of Fas depedent pathway in huv-ec-c but not in U937 cells. Another one is the different response of the two cell types in resistance manner. In huv-ec-c`s, Bcl-$_{XL}$ protein level increases whereas in U937 cells Bcl-$_{XL}$ and Bcl-2 protein levels decrease and their levels are somehow restored to the control levels.

When U937 cells treated with 10 and 20 μM of 4-HNE, are compared, the only difference results from the response of Bak protein.

In order to have a detailed understanding, further studies has to be carried. Offered studies are listed below:

- In order to be sure about the fas pathway involvement in huv-ec-c`s, Fas receptor activation, FADD association, DISC formation and caspase 8 activation have to be controlled. The results may further be controlled by the caspase 8 inhibitor or Fas receptor inhibitor.

- In order to have a reliable information about Bak, Bax and Mcl-1 involvements in 4-HNE triggered apoptosis in U937 cells; oligomerization and interaction of Bax and Bak, cleavage and post-translational regulation of Mcl-1 have to be studied.
- The interaction between the proposed resistance mechanisms (metabolism of 4-HNE by GST`s, HSP and inhibition of Fas dependent pathway by translocation of Daxx) with the response of prosurvival proteins has to be studied in order to associate them.
- To understand whether the different response of Bak protein to different 4-HNE concentration is related with the proposed unique role of Bak, the interactions of Bak with mitofusins have to be studied

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APPENDIX A

CHEMICALS

(in alphabetical order)

Name of Chemical	Supplier Company	Catalog Number
Acrylamide/Bis-acrylamide	Sigma, Germany	A3699
Ammonium persulfate	Sigma, Germany	A3678
Annexin V-FITC	Alexis Biochemicals	ALX-209-250-T100
Anti-Beta-actin Ab	Cell Signal Tech., USA	4967
Anti-rabbit IgG HRP linked	Cell Signal Tech., USA	7074
Anti-mouse IgG HRP linked	Amersham Biosciences, UK	RPN4201
Anti-Bcl-2 Ab	Cell Signal Tech., USA	2872
Anti-Bcl-xl Ab	Cell Signal Tech., USA	2762
Anti-Mcl 1 Ab	Cell Signal Tech., USA	4572
Anti-Bak Ab	Cell Signal Tech., USA	3814
Anti-Bax Ab	Cell Signal Tech., USA	2772
Anti-Bim Ab	Cell Signal Tech., USA	2819
Anti-Bid Ab	Cell Signal Tech., USA	2002
Protease inhibitor cocktail	Roche, Germany	11697498001

tablet		
ECGS	Upstate Cell Signaling, USA	02-101
EDTA	Riedel-de Haén, Germany	27248
Ethanol	Riedel-de Haén, Germany	32221
F12K Medium	ATCC, USA	30-2004
Foetal Bovine Serum	Sigma, Germany	F2442
Glycerol	Riedel-de Haén, Germany	15523
Glycine	Amnesa, USA	0167
4-HNE	Calbiochem,USA	393204
Heparin	BiochromAG, Germany	L6510
HCl	Merck, Germany	100314
Hyperfilm ECL	Amersham Biosciences, UK	RPN2103K
Hybond P-membrane (PVDF)	Amersham Biosciences, UK	RPN2020F
Isopropanol	Riedel-de Haén, Germany	24137
KCl	Fluka, Switzerland	60129
Liquid nitrogen	Karbogaz, Turkey	
2-Mercaptoethanol	Sigma, Germany	M370-1
Methanol	Riedel-de Haén, Germany	24229
MgCl2	Sigma, Germany	M9272
Milk Diluent concentrate	KPL, USA	50-82-00
NaCl	Riedel-de Haén, Germany	13423
NaOH	Merck, Germany	106462

NP-40	Sigma, Germany	I3021
Pen/Strep. Solution	Biological Industries	
Phosphate buffered saline	Sigma, Germany	P4417
PMSF	Sigma, Germany	P7626
Prestained Protein MW Marker	Fermentas,Germany	#SM0441
RPMI 1640	PAN Biotech, Germany	P04-22100
Sodium Dodecyl Sulphate	Sigma, Germany	L4390
TEMED	Sigma, Germany	T7029
Triton X-100	Applichem, Germany	A1388
Tris	Fluka, Switzerland	93349
Trypsin/EDTA 1X	Biological Industries, Israel	03-050-1
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
DC Assay	Biorad	

APPENDIX C

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

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-20^{o} C, Bosch, TURKIYE

Distilled Water: Millipore, Elix-S, FRANCE

Millipore, MilliQ Academic, FRANCE

Electrophoresis: Biogen 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, TÜRKIYE

pH meter: WTW, pH540 GLP MultiCal®, GERMANY

Power Supply: Biorad, PowerPac 300, USA

Wealtec, Elite 300, USA

Refrigerator: +4◦ C, Bosch, TÜRKiYE

Shaker: Forma Scientific, Orbital Shaker 4520, USA

GFL, Shaker 3011, USA

New Brunswick Sci., Innova™ 4330, USA

C25HC Incubator shaker New Brunswick Scientific, USA

Spectrophotometer: Schimadzu, UV-1208, JAPAN

Schimadzu, UV-3150, JAPAN

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