RESPONSE OF THP-1 CELLS TO OXIDATIVE DAMAGE INDUCED BY AAPH

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Figures

Abbreviations

$^{\bullet}NO$	nitric oxide
$^{\bullet}OH$	hydroxyl radical
AAPH	2, 2'-azobis(2-methyl-propionamidine) dihydrochloride
AIF	apoptosis-inducing factor
ANOVA	analysis of variance
APAF-1	apoptotic protease activating factor-1
ATP	adenosine triphosphate
BSA	bovine serum albumine
BSO	Buthionine Sulfoximine
CAD	caspase-activated DNase
CHAPS	(3[(3-chloramidopropyl) dimethylammonio]-1-propane-sulfonate
CNTP	3-carboxylato-4-nitrothiophenolate
CO_2	carbon dioxide
Cyt.*c*	cytochrome *c*
DEVD-AMC	Ac-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin)
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTNB	5,5 dithiobis (2-nitrobenzoic acid)
DTT	1,4-dithiothreitol
e^-_{aq}	solvated electron
EBSS	Earle's balanced salt solution
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
Fe-XO	ferric ion – xylenol orange complex
FOX	ferric-xylenol orange
GSH	reduced glutathione
GSSG	oxidised glutathione
GSSR	mixed disulfide
GuHCl	Guanidine hydrochloride
HEPES	(N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])
HI-FCS	heat inactivated fetal calf serum
H_2O_2	hydrogen peroxide

H_2SO_4	sulphuric acid
HOCl	hypochlorite
HPLC	high performance liquid chromatography
LDH	lactate dehydrogenase
LDL	low density lipoprotein
MPO	myeloperoxidase
mBBr	monobromobimane
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)
NAC	N-Acetyl-Cysteine
NaCl	sodium chloride
NADPH/$NADP^+$	nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	sodium dihydrogen orthophosphate
NaOH	sodium hydroxide
O_2	oxygen
$O_2^{\bullet-}$	superoxide
$ONOO^-$	peroxynitrite
PBS	phosphate buffered saline
PCA	Perchloric acid
PI	propidium iodide
PS	phosphatidylserine
$Pr^{\bullet}$	protein radical
PrH	protein
$PrO^{\bullet}$	protein alkoxyl radical
$PrOO^{\bullet}$	protein peroxyl radical
PrOOH	protein hydroperoxide
$R^{\bullet}$	radical
Redox	reduction-oxidation
R-N=N-R	azo compound
R-SS-R	disulphides
$ROO^{\bullet}$	carbon centred peroxyl radical
ROOH	carbon centred hydroperoxide
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
R-SH	thiol

SDS	sodium lauryl sulphate
SOD	superoxide dismutase
TCA	trichloroacetic acid
XO	xylenol orange

Abstract

Monocyte cells can be damaged when exposed to reactive oxidative species during inflammatory events within the body. This research project has examined in detail the cellular events associated with free radical damage induced death of monocyte cells, using the water soluble peroxyl radical generator 2, 2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) as a model of inflammation induced oxidative stress.

The human monocyte-derived cell line THP-1 was incubated with 10mM AAPH in Earle's Balanced Salt Solution at 37°C for up to 24 hours. Protein hydroperoxide formation was observed to occur after a six hour lag, period which corresponded to the time in which most of the intracellular glutathione was lost from the cell. Cell viability loss, measured by the MTT reduction, was also observed after six hours of incubation while no caspase-3 activation was observed. A control ethanol treatment confirmed that caspase-3 could be activated within the THP-1 cells. Flow cytometry analysis failed to show phosphatidylserine exposure due to the AAPH treatment but DNA staining by propidium iodide confirmed the loss of viability seen with the MTT cell viability assay. This data strongly suggests that THP-1 cells undergo AAPH induced necrosis as a result of cellular damage including GSH loss and protein hydroperoxide formation. Microscopic examination of the cells identified many of the morphological characteristics of necrosis in the AAPH treated cells.

Studies on the effect of AAPH on U937 cells were unsuccessful due to the possible presence of intracellular infection, resulting in an increased oxidant stress in the untreated cells.

1 INTRODUCTION

1.1 Overview

The role of free radicals in biology has been the subject of extensive studies in relation to oxidative stress and cellular signalling. It is now accepted that at high concentrations, free radicals are hazardous, whereas at moderate concentrations, they can play an important role as regulatory mediators in signalling processes (Yoshida, Itoh et al. 2004). Inflammation is a good example of the importance of free radicals. Cells of the inflammatory systems (macrophages, neutrophils, monocytic cells) produce free radicals to kill ingested microorganisms or as signalling mediators. However, sometimes inflammation can become chronic as in arthritis, cancer and heart diseases. In these diseases free radicals are generated under less controlled situations, causing damage to macromolecules. This damage occurs if there is an imbalance between free radical production and the effectiveness of the defence systems (Berg, Youdim et al. 2004). The principal macromolecules being damage has been shown to be the proteins, usually resulting in the formation of protein hydroperoxide (Gebicki 1997). If the imbalance between the production of free radicals and the removal by antioxidants persists, oxidative stress in the cell could lead to cell death. Glutathione (GSH) is an important antioxidant which provides a major intracellular defence against oxidative injury and has been show to react with protein hydroperoxides (Halliwell and Gutteridge 1999).

This study has examined the response of THP-1 cells to oxidative stress induced by a peroxyl initiator, AAPH. The formation and reactivity of protein hydroperoxide formed toward GSH has been characterised over time. The consequence of the oxidative state of the cell in correlation with the type of cell death was explored.

1.2 Free radicals

Free radical can be defined as reactive chemical species possessing at least one unpaired electrons, capable of independent existence (Halliwell and Gutteridge 1999). Free radicals can be formed in three ways: (i) by the homolytic cleavage of a covalent bond, with each fragment retaining one of the paired electrons; (ii) by the loss of a single

electron from a neutral molecule; (iii) by the addition of a single electron to a neutral molecule (Cheeseman and Slater 1993).

The presence of unpaired electrons makes free radicals highly reactive species, which also means that they do not exist for a long period of time. However during the time they do exist, some free radicals have the ability to react with biomolecule in the vicinity to their production. Free radicals can be generated within cells as intermediates of normal biochemical process, such as those involving redox enzymes and bioenergetic electron transfer (Dean, Gieseg et al. 1993).

Probably the most important oxidant in biological systems is oxygen and radical derivatives of oxygen (superoxide and hydroxyl radicals), hydrogen peroxide and transition metals. When stimulated by inflammation or tissue injury, leukocytes (including macrophage cells and neutrophils) show a marked increase in oxygen uptake, in a process known as the respiratory burst (Winrow, Winyard et al. 1993). This results in the production of reactive oxygen species (ROS) (Halliwell and Gutteridge 1999). ROS is a term used to describe not only oxygen free radicals, but also some non-oxygen free radicals, such as hypochlorite (produced by activated phagocytes). ROS are generated as a result of normal metabolism. However, the deleterious condition termed oxidative stress occurs when ROS are excessively produced or insufficiently degraded (Berg, Youdim et al. 2004).

Because of the high amount of oxygen respired within the normal living system, the superoxide free anion ($O_2^{-\bullet}$) is considered to be the most abundant ROS *in vivo*. Superoxide is formed by the reduction of oxygen by transfer of a single electron (formed in the mitochondria, endoplasmic reticulum or chloroplasts) to the oxygen.

(1) $O_2 + e^-_{aq} \rightarrow O_2^{-\bullet}$

Superoxide ($O_2^{\bullet-}$) is also produced on microsomal membranes in the process of detoxification of toxic compounds by the NADPH-cytochrome P450 reductase (Halliwell and Gutteridge 1999; Winrow, Winyard et al. 1993). Superoxide is not a particularly damaging radical by itself, unless it is present at high concentrations (Conner and Grisham 1996). The importance of $O_2^{-\bullet}$ lies in the length of its half-life and its ability to diffuse across membranes (Barber and Harris 1994). It is also a source of hydrogen peroxide (H_2O_2) through a dismutation reaction with superoxide dismutase (SOD), which converts $O_2^{-\bullet}$ to H_2O_2. Two $O_2^{-\bullet}$ molecules react together to form H_2O_2

and oxygen (reaction 2). This can occur spontaneously at acidic pH, or by the action of SOD.

(2) $2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$

Hydrogen peroxide is not a free radical due to the absence of unpaired electrons but it is an oxidising agent (Barber and Harris 1994). It is not a strong oxidising agent, but it can diffuse across cellular membranes and initiate damage at sites other than where it was produced (Halliwell and Gutteridge 1999; Benzie 1996). In the presence of transition metal ions such as copper or iron, hydrogen peroxide produces an extremely reactive and damaging oxidant, the hydroxyl radical ($^{\bullet}OH$) (Cheeseman and Slater 1993). This reaction is known as the Fenton reaction (reaction 3) and involves transitions metals acting as catalysts to transfer electrons to hydrogen peroxide (Berg, Youdim et al. 2004).

(3) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$

The hydroxyl radical is an extremely reactive oxidising agent that reacts with most biomolecules at a diffusion-controlled rate (Cheeseman and Slater 1993; Conner and Grisham 1996). With a half-life of 10^{-9} seconds at 37°C, the $^{\bullet}OH$ radical can react within 6nm from its origin of formation and therefore react with those biomolecules closest to its site of generation (Gebicki 1997; Nohl 1993).

During the inflammation response, hypochlorite (HOCl), another important ROS, is released by the activated neutrophils (Daugherty, Dunn et al. 1994). The neutrophil specific enzyme myeloperoxidase (MPO) catalyses the reaction of H_2O_2 with the chloride ion (Cl^-) forming HOCl. HOCl has an essential role in microbial killing due to its ability to oxidize most biomolecules (Kettle, Gedye et al. 1993; Weiss 1989).

Another ROS released by some cells is nitric oxide ($NO^{\bullet}$). It is a widely distributed intracellular messenger, modulating the blood flow, thrombosis and neural activity (Palmer, Ferrige et al. 1987; Beckman and Koppenol 1996). Reaction of $NO^{\bullet}$ with the superoxide ion results in the generation of peroxynitrite ($ONOO^-$), a strong and relatively long-lived oxidizing agent which can attack protein molecules causing tissue injury (Ischiropoulos and al-Mehdi 1995).

1.3 Role of Free radicals in the process of inflammation

Inflammation is a local response of bodily tissue to injury or irritation. The causes of inflammation are extremely variable. They could be infectious (bacteria, virus), physical (hot, cold, irradiation) or chemical toxicity. During inflammation, the cells of the inflammatory system, phagocytes cells (neutrophils, mononuclear phagocytes, and eosinophils) are stimulated and move towards the site of damage. These cells produce potentially toxic ROS which can provide the body with a mechanism of defence against invading organisms (Cheeseman and Slater 1993; Henson and Johnston 1987). In immune cells, large scale production of $NO^{\bullet}$ by macrophages, or superoxide by neutrophils, provides a necessary host defence function. While when produced in smaller doses in non-phagocyte cells, these same reactive molecules functions as signalling molecules (Yoshida, Itoh et al. 2004).

1.4 Cellular damage during oxidative stress

There is a lot of evidence of the involvement of ROS in the initiation and development of various forms of damage in biological systems. Effort to reduce or eliminate the effects of ROS has proved difficult to achieve in practice, due to a lack of knowledge of the pathway linking the formation of the ROS *in vivo* with the end-point damage. During attack by ROS it is likely that the first molecule attacked in cells is converted to a secondary free radical or reactive intermediate, which in turn generates further reactive species (Gebicki 1997). In this sequence of events, damage to vital molecules can induce irreversible cell dysfunction.

1.4.1 Protein damage

The formation of reactive oxygen groups upon protein is considered to be a significant contributor to cellular damage and as an indicator of the interruption to normal biological systems. It has been thought for a number of years that membrane lipids were the main targets for cellular oxidative damage due to their proximity and their tendency to undergo self-perpetuating chain reactions. However, protein is the major constituents of the cell and the membrane, excluding the non reactive water (Du and Gebicki 2004). Protein constitutes 75% of the combined masses of potential initial molecular targets in eukaryotics cells. It is therefore sensible to assume that protein would be a highly important biological target.

Many proteins can stabilise metals in forms able to redox cycle, allowing site-specific formation of very reactive products from hydrogen peroxide and superoxide (Halliwell and Gutteridge 1999). The biologically derived free radical, such as the hydroxyl and peroxyl radicals, have been shown to cause amino acid oxidation, decarboxylation, deamination and cross-linking (Dean, Gieseg et al. 1993; Dean, Fu et al. 1997). This oxidative modification of proteins *in vivo* may affect a variety of cellular functions involving proteins: receptors, signal transduction mechanisms, transport systems, and enzymes. Investigation suggests that accumulated protein damaged may not solely be a marker for pathologies of ageing, but actually a component mechanism (Dean, Gieseg et al. 1993). This accumulation is associated with many diseases such as Alzheimer's disease, atherosclerosis, Parkinson's disease, cataractogenesis, cystic fibrosis, muscular dystrophy, rheumatoid arthritis and Werner's syndrome (Berlett and Stadtman 1997; Fu, Dean et al. 1998). An important element of this hypothesis is that oxidized proteins may themselves contain reactive species which can go on to further damage normal proteins and other biomolecules. For instance, oxidation and inactivation of DNA repairs enzymes or DNA polymerases in replicating DNA, will cause the gradiant loss of cell viability (Cakatay, Telci et al. 2003).

1.4.2 Protein hydroperoxide formation

The formation of reactive protein hydroperoxides begins when an $H^{\bullet}$ is extracted from a protein by radical species ($R^{\bullet}$), forming a carbon-centred radical ($Pr^{\bullet}$) (equation 4), which in the presence of O_2 is rapidly converted into a protein peroxyl radical ($PrOO^{\bullet}$) (equation 5). The addition of a proton and an electron to the protein peroxyl is sufficient to convert it into a protein hydroperoxide (PrOOH) (equation 6). Alternatively, the proton and electron from the side chain of another protein (R'H) can also aid in the formation of PrOOHs, but with a by-product of a new carbon-centred radical ($R'^{\bullet}$) (equation 7).

(4) $PrH + R^{\bullet} \rightarrow Pr^{\bullet} + RH$

(5) $Pr^{\bullet} + O_2 \rightarrow PrOO^{\bullet}$

(6) $PrOO^{\bullet} + e^{-} + H^{+} \rightarrow PrOOH$

(7) $PrOO^{\bullet} + R'H \rightarrow PrOOH + R'^{\bullet}$

The potential biological importance of protein hydroperoxides lies in their formation, stability and reactivity. Protein hydroperoxides have a relatively long lifetime, enabling

them to diffuse a considerable distance and therefore diffuse through intracellular as well as extracellular spaces (Gebicki 1997).

Protein hydroperoxides have been shown to react with ascorbate and glutathione (GSH), two important protective agents of the cells against oxidative stress (Simpson, Narita et al. 1992).

This ability, to transfer damage to other biological molecules has given strength to the argument that protein radicals are a relevant form of oxidative stress. Protein hydroperoxides themselves should therefore be considered as ROS, capable of propagating further damage (Gebicki 1997).

1.4.3 Protein oxidation by the peroxyl-radical generator AAPH

2, 2'-azobis(2-methyl-propionamidine) dihydrochloride (AAPH) has been used in a number of studies as a peroxyl-radical generator to investigate the formation of protein hydroperoxides. AAPH induced protein hydroperoxides have been studied on isolated protein (Gebicki and Gebicki 1993), red blood cell ghosts (Firth 2001), low density lipoprotein (Pearson 2002) and the human monocyte like cell line U937 (Gieseg, Duggan et al. 2000).

AAPH is a thermally labile azo compound which above 5°C, decomposes to produce carbon centred radicals and nitrogen gas (equation 8) (Niki 1990). At 37°C and in presence of oxygen, the rate constant for peroxyl radicals formation is of 3.19×10^{-7} $M.s^{-1}$. The interaction of the carbon centred radicals with adjacent proteins will generate the protein radical, $Pr^{\bullet}$ (equation 9) and (equation 10). Protein hydroperoxides are then formed via reactions (equation 6) and (equation 7) above.

(8) $R\text{-}N{=}N\text{-}R \rightarrow 2R^{\bullet} + N_2$

(9) $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$

(10) $ROO^{\bullet} + PrH \rightarrow Pr^{\bullet} + ROOH$

1.5 Cellular defence against free radicals

The reduction-oxidation (redox) state of the cell is a consequence of the balance between the levels of oxidizing and reducing equivalents (Filomeni, Rotilio et al. 2003). ROS production is usually associated with detoxification processes, requiring defence systems to counteract the deleterious effects of the damaging molecules. Similar to detoxification systems, antioxidant defences can be upregulated by means of external

stimuli (Dafre, Medeiros et al. 2004). Antioxidants are defined as "substances that when present at low concentrations compared to those of an oxidizable substrate significantly delay or inhibit oxidation of that substrate" (Halliwell and Gutteridge 1990). Cells utilise both enzymatic and non-enzymatic defences against free radicals. The major enzymatic defences are superoxide dismutase, glutathione peroxidase and catalase, which inactivate superoxide anion and peroxides, respectively. Among the non-enzymatic antioxidants are ascorbic acid, tocopherols and glutathione.

1.5.1 Thiols

Thiol groups in protein and non-proteins are involved in the maintenance of various cellular functions, including many enzyme activities. A number of thiol (R-SH) containing molecules act as antioxidants either directly, or as co-factors for antioxidant enzymes. Thiols are readily oxidised during the onset of oxidative stress and, by scavenging ROS, will protect key cellular targets. Protein thiols are present at levels significantly higher than those reported for glutathione (GSH) (Di Simplicio, Cacace et al. 1998). Protein thiols may serve an antioxidant function by several mechanisms; protein thiols may preemptively scavenge oxidants which initiate peroxidation, thus sparing tocopherols and/or lipids from attack (Dubey, Forster et al. 1996; Cakatay, Telci et al. 2003). However, the most important thiol reported for its antioxidant action is glutathione.

1.5.2 Glutathione

Within the cellular context, the redox status depends on the relative amounts of the oxidized and reduced partners of the major redox molecules. Glutathione is the most prevalent non-protein intracellular thiol. Glutathione is considered to be the major thiol/disulfide cellular redox buffer and appears to have several fundamental functions ranging from detoxification to messenger molecule (Sies 1999; Cotgreave and Gerdes 1998; Arrigo 1999).

Glutathione exists as a reduced form (GSH), as a disulfide (GSSG), or as mixed disulfide (GSSR) with protein thiols (Meister and Anderson 1983). The ratio of GSSG/GSH reflects the redox status. Therefore the oxidation of a limited amount of GSH to GSSG can drastically change this ratio, greatly affecting the redox status within the cell (Filomeni, Rotilio et al. 2003). The level of glutathione is typically 100 folds higher than the level of GSSG (Schafer and Buettner 2001).

Glutathione and mixed disulfides are continuously reduced back through the reaction of the NADPH-dependent glutathione disulfide reductase (reaction 11) and by the catalysis of cellular enzymes such as glutaredoxin or thioredoxin (Filomeni, Rotilio et al. 2003), (Meister and Anderson 1983).

(11) $GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$

Glutathione is synthesized from its amino acid precursors in two steps. First, the enzyme γ-glutamylcysteine synthetase catalyzes the synthesis of γ-glutamylcysteine from glutamate and cysteine. Glutathione synthetase then catalyzes the formation of glutathione from glycine and γ-glutamylcysteine, and thus, its own synthesis (Luperchio, Tamir et al. 1996).

Its thiol moiety allows glutathione to reduce disulfides (R-SS-R) and other molecules, to serve as a sulfhydryl buffer in the cytoplasm and therefore, to influence and to maintain the overall reduction-oxidation (redox) potential of the cell (Hwang, Sinskey et al. 1992). Thiol groups on proteins are targets of ROS. Glutathione alone has been shown to directly reduce protein hydroperoxides to hydroxides *in vitro*, whilst concomitantly becoming oxidized (Simpson, Narita et al. 1992). Also, *in vitro*, glutathione can scavenge reactive species such as $^{\bullet}OH$, HOCl, $RO^{\bullet}$ and $ROO^{\bullet}$ (Halliwell and Gutteridge 1999). The oxidation of the thiol group on cysteine is involved in several functions of glutathione and proteins. Free radical-mediated oxidation of this thiol group results in the formation of a thiyl radical. This radical can undergo dimerisation to the corresponding disulphide or react with oxygen or H_2O_2 to produce oxygen containing products such as $R\text{-}SO_4^-$ (Mason and Rao 1990). The non-enzymatic function of glutathione in the detoxification of ROS is believed to be non-stoichiometric and may lead to the formation of further radicals.

A number of other crucial roles have been described for glutathione including involvement in signal transduction, gene expression, apoptosis, protein glutathionylation and NO metabolism (Wu, Fang et al. 2004).

1.6 Mechanism of cellular death

The mechanism of cellular death occurs principally by two distinct mechanisms: apoptosis and necrosis. Necrosis is generally associated with a severe damage to the cell

(Ueda and Shah 1994). The cell is killed by the disruption of the ion homeostasis and the release of various proteases (Borner and Monney 1999).

Apoptosis, or programmed cell death, is a more deliberate process. Apoptosis is an efficient and highly regulated process involved in physiological as well as pathological conditions (Rizzo, Regazzi et al. 1999). It is an essential process for development and maintenance of homeostasis during embryogenesis, cell growth and immune regulation as well as elimination of damaged, mutated or infected cells in eukaryotic organisms (Takano, Aramaki et al. 2001).

As an example, a number of reports clearly demonstrated that H_2O_2 can trigger apoptotic cell death and that the mode of cell death switches from apoptotis to necrosis when the concentration of the oxidant is increased (Lennon, Kilfeather et al. 1992; Ueda and Shah 1992; Hockenbery, Oltvai et al. 1993; Nosseri, Coppola et al. 1994).

1.6.1 Apoptosis

Apoptosis, a form of cell suicide, is a type of cell death that is accomplished by specialized cellular machinery. Apoptosis has been subdivided into at least three major classes, defining the chronological sequence of biochemical and morphological modifications (Leist and Nicotera 1997). During the initial or induction phase, signalling action occurs and morphological modifications are few in number although biochemical changes are proceeding. In the second phase, the execution phase, the molecular machinery of apoptosis becomes active and the cell is committed to die. This middle phase of apoptosis is characterized by the presence of condensed chromatin. The third phase of apoptosis has only been observed in *in vitro* and is characterised by visible morphological changes including condensation and margination of the nuclear chromatin, DNA fragmentation, condensation of the cells with preservation of the organelles and apoptotic body formation (Wyllie, Kerr et al. 1980; Abbro, Lanubile et al. 2004). Although from some observation, no unique morphological feature exists during apoptosis, even in the same cell type. The concept of apoptosis cannot therefore be linked to a specific morphology for all types of cells and/or systems (Abbro, Lanubile et al. 2004).

The hallmark of apoptosis ("programmed cell death"), as opposed to necrosis, is its active character, in that it requires the presence of a functional mitochondrial respiratory chain. The central component of this machinery is a proteolytic system involving a

family of cystein proteases called caspases. The caspases are activated by a signalling pathways initiated either by ligation of cell surface death receptors (the extrinsic pathway) or by mitochondria perturbation by different stressors (intrinsic pathway) (Moldovan and Moldovan 2004). Caspases play critical roles in the execution phase of apoptosis, there are responsible for many of the biochemical and morphological changes associated with apoptosis. Caspase activation is therefore a point of no return in the apoptotic program.

Perturbation of mitochondrial membrane integrity is followed by release in the cytosol of a number of proapoptotic proteins: cytochrome *c*, the flavoprotein apoptosis-inducing factor (AIF), endonuclease G, and others (Fumarola and Guidotti 2004; Moldovan and Moldovan 2004). Several studies have suggested that when cells receive apoptotic stimuli, cytochrome *c* (Cyt.*c*) released from mitochondria activates caspase-3 and plays an important role in the initiation of apoptosis (Kluck, Bossy-Wetzel et al. 1997; Liu, Kim et al. 1996; Kim, Lee et al. 2003). Caspase-3 has been shown to be either partially or totally responsible for the proteolytic cleavage of many key proteins (Kojio, Zhang et al. 2000). Active caspase-3 cleaves an inhibitor of caspase-activated DNase (CAD) to release caspase-dependent DNase, which then enters the nucleus to degrade internucleosomal DNA (Enari, Sakahira et al. 1998; Sakahira, Enari et al. 1998). Caspase-3 also cleaves a nuclear factor termed Acinus, which induces chromatin condensation (Zamzami and Kroemer 1999; Sahara, Aoto et al. 1999).

Studying the caspases shows their participation in apoptosis to be controlled. They cut off contacts with surrounding cells, reorganize the cytoskeleton, shut down DNA replication and repair, interrupt splicing, destroy DNA, disrupt the nuclear structure, induce the cell to display signals that mark it for phagocytosis, and disintegrate the cell into apoptotic bodies, which are phagocytosized by nearby cells without associated inflammation (Thornberry and Lazebnik 1998; Savill and Fadok 2000; Dini, Pagliara et al. 2002). Typical biochemical changes comprise cytochrome *c* release from mitochondria, activation of cysteine-dependent caspases and labelling of the cell for clearance by phagocytes through exposure of phosphatidylserine on the surface of the cell (Vicca, Hennequin et al. 2000).

In the absence of other cells to engulf the apoptotic cells, secondary necrosis may follow apoptosis where the condensed cell lyses releasing its contents (Fadeel and Kagan 2003).

1.6.2 Necrosis

Necrosis generally occurs after severe cellular damage, leading to acute cellular dysfunction. During necrosis there is a precipitous decline in cellular ATP levels, loss of osmotic control, swelling and cell lysis. Inflammation and further tissue damage result when the dying cell's contents spill into the extracellular space (Chandra, Samali et al. 2000). A loss in plasma integrity results in disregulation of osmotic pressure causing swelling and rupture of the cell. The spilling of the cellular content can cause further injury or death to the surrounding cells, which can ultimately lead to tissue damage (Haslett 1992; Samali, Gorman et al. 1996). Necrosis is characterized by cytoplasmic swelling, disruption of internal organelles, membrane lysis and release of cell debris into the extracellular spaces (Abbro, Lanubile et al. 2004; Pagliara, Chionna et al. 2003).

Necrosis appears to be a no-return path which cannot be modulated. This view stems from the general opinion that necrotic cell death occurs as a result of massive damage which overwhelms the defence and repair capacities of the cell (Palomba, Sestili et al. 1996).

1.7 Objectives of the study

Previous studies in our laboratory have shown that human histiocytic lymphoma cell line U937 cells exposed to AAPH lead to protein hydroperoxide formation (Duggan 2000; Cassidy 2003). Additionally when U937 cells were treated with AAPH, the loss of cellular thiols and the loss of cell viability was observed (Duggan 2000). AAPH have been used in other studies to enhance apoptosis by hyperthermia in U937 cells (Li, Kondo et al. 2001). These studies were conducted with a range of AAPH concentration to observe a suitable level of cellular damage.

In our studies we used a fixed AAPH concentration. We investigate the cellular events during AAPH induced cellular death at 37°C in the human monocyte-derived cell lines THP-1 and U937. The timing of these events and the importance of protein hydroperoxide formation were investigated.

Protein hydroperoxide formation, a major product of the reaction of peroxyl and hydroperoxyl groups with proteins (Fu, Gebicki et al. 1995) was measured to follow the oxidation state of the cell, as proteins are now widely recognised as the main targets during oxidative stress. The role of the thiol and intracellular glutathione antioxidant

will be assessed for their ability to protect the cell against peroxyl radical-mediated protein hydroperoxide formation. Finally, the measure of cell viability and the manner of the cell death will be investigated to follow any correlation with the oxidative state of the cell.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All reagents used in this research were analytical grade. Water used was de-ionised and ultrafiltered by a Milli-Q ultrafiltration system.

1,4-dithiothreitol (DTT)	Boehringer Mannheim GmbH, Germany
2, 2'-azobis(2-methyl-propionamidine) dihydrochloride (AAPH)	Aldrich., Chemical Co., WI, USA
5 5'-dithiobis(2-nitrobenzoic acid) (DTNB)	Sigma Chemical Co., St louis, MO, USA.
Acetic acid (glacial)	BDH Lab Supplies, Poole, England
Acetonitril	Merck
DEVD-AMC	Sigma Chemical Co.
Ammonium ferrous sulphate	Hopkin and Williams Ltd, Essex
Apoptotest™ FITC	DakoCytomation
Bovine serum albumine (BSA)	Sigma Chemical Co.
CHAPS	Sigma Chemical Co.
Chelex 20 resin	Bio-Rad laboratories.
DEVD-AMC	Sigma Chemical Co
Dimethyl sulphoxide (DMSO)	Sigma Chemical Co.
Earle's balanced salt solution (EBSS)	Sigma-Aldrich
Ethanol, 96%	BDH chemicals Ltd
Glutathione, reduced from	Sigma Chemical Co.
Guanidinium hydrochloride (GuHCl)	Sigma Chemical Co.
Heat inactivated foetal calf serum (HIFCS)	Gibco BRL; Grand Island, NY, USA
HEPES	Sigma Chemical Co.
Hydrochloric acid (HCl)	BDH chemicals Ltd
Monobromobimane (mBBr)	Sigma-Aldrich
MTT	Sigma Chemical Co.
Penicillin/Streptomycin	Gibco BRL; Grand Island, NY, USA
(1000 units/mL penicillin G and 1000µg/mL streptomycin)	
Perchloric acid (PCA)	BDH chemicals Ltd
Roswell Park Memorial Institute 1640 (RPMI)	Sigma-Aldrich
RPMI 1640, without phenol red	Sigma-Aldrich
Sodium chloride (NaCl)	BDH chemicals Ltd
Sodium dihydrogen orthophosphate (NaH_2PO_4)	Merck
Sodium dodecyl sulphate (SDS)	Sigma Chemical Co.
Sodium hydroxide (NaOH)	BDH chemicals Ltd
Sulphuric acid (H_2SO_4)	BDH chemicals Ltd

Tergitol, type NP-40	Sigma Chemical Co.
Trichloroacetic acid (TCA)	Merck
Trypan blue solution (0.4%)	Sigma Chemical Co.
Xylenol orange	Sigma Chemical Co.

2.1.2 Solutions, buffers and media

A) Cell culture media

All solutions used in cell culture were autoclaved or filter sterilised with a 0.20 μm filter before use under sterile conditions in a class 2 cabinet.

RPMI

A 500 ml bottle of Roswell Park Memorial Institute (RPMI) 1640 medium with phenol red and glutamine was supplemented with 5% heat inactivated fetal calf serum (HI-FCS), 1000 units/ml penicillin G and 1000 μg/ml streptomycin. Media was prepared under aseptic conditions and stored at 4°C. Media was warmed in a water bath to 37°C before use.

Earle's balance salt solution

250 ml bottles of Earle's balance salt solution (EBSS) were prepared and filter sterilised under aseptic conditions and stored at 4°C. Media was warmed in a water bath to 37°C before use.

Phosphate buffered saline (PBS) general use

Phosphate buffered saline, composed of 150mM sodium chloride (NaCl) and 10mM sodium dihydrogen orthophosphate (NaH_2PO_4) pH 7.4, was prepared from stock solutions. An overnight treatment with Chelex 20 resin followed to remove any contaminating transition metals. The chelex resin was removed by filtration through a 0.45 μm filter. The solution was stored at 4°C.

Phosphate buffered saline (PBS) aseptic use

PBS solution was sterilised by autoclaving (15 minutes, 121°C, 15 psi) and stored at 4°C. PBS was warmed in a water bath to 37°C before use for the washing of cells.

AAPH solutions

A 100 mM stock solution of 2,2'-azobis (2-methyl-propionamidine) dihydrochloride (AAPH) was prepared fresh on each day of experimentation in EBSS and kept on ice in the dark until required, and then passed trough a sterile filter (0.2 μm) before being added to the cells.

B) FOX assay solutions

Sulphuric acid solution

A 1 M sulphuric acid stock solution was made up by diluting concentrated H_2SO_4 (18.44 M) in milli-Q water. The solution was stored at room temperature and used for several months. A diluted 25 mM H_2SO_4 solution was prepared using the stock solution once a month.

Trichloroacetic acid solution (TCA)

TCA 72% $^{w}/_{v}$ solution was made up by weighing 28.8 g of TCA and adding milli-Q water to a final volume of 40 ml. A 5% TCA solution was made up by dilution of the 72% TCA solution.

Xylenol orange solution

5 mM Xylenol orange in 25 mM H_2SO_4 solution was stored at room temperature and used within a month.

Ferrous ammonium sulphate

A 5 mM ferrous ammonium sulphate solution was made up in 25 mM H_2SO_4. The solution was stored at room temperature and used within three days.

C) G-PCA FOX assay solutions

Guanidine hydrochloride (GuHCl)

A 6 M solution of guanidine hydrochloride was prepared in water. The solution was sonicated for 5 min and the volume adjusted to 100 ml with water.

Xylenol orange solution

Xylenol orange was prepared to a concentration of 5 mM in 110 mM PCA. 190 mg of xylenol orange were dissolved in 50 ml of 110 mM PCA. The solution was stored at room temperature and used within a month.

Ferrous ammonium sulphate

A 5 mM ferrous ammonium sulphate solution was prepared using 110 mM PCA solution. 98 mg of ferrous ammonium sulphate were dissolved in 50 ml of 110 mM PCA. The solution was stored at room temperature and used within a week.

D) MTT solutions

MTT reagent

5 mg/ml of MTT was prepared in RPMI 1640 containing no phenol red (no HI-FCS and pen/strep). The solution was sterilised via aseptic filtration through a 0.22 μm

filter in the C2 cabinet and stored at 4°C or -20°C protected from light. The solution was kept for a month.

SDS

A 10% SDS solution $^{w}/_{v}$ in 0.01 M HCl was prepared using Milli-Q water. The solution was stored at room temperature.

E) HPLC Monobromobimane (mBBr) GSH solutions

Monobromobimane (mBBr)

The stock solution consisted of a 40 mM mBBr solution in acetonitrile. The solution was stored at 4°C in a dark coloured bottle. The solution was kept for up to 2 weeks.

Trichloroacetic acid

100% TCA solution $^{w}/_{v}$ was prepared in water and stored at 4°C. 10 g of TCA solid were weight out and dissolved in enough water to make it to 10 ml.

Glutathione (GSH) standard

The 10 mM GSH solution stock was made up by dissolving 30.7 mg of GSH in 10 ml of PBS. A fresh solution was made prior to experiment. Standard solutions were made up from the stock solution at 2, 5 and 10 μM in PBS. These were stored at 4°C until analysis.

NaOH

A solution of 0.1 M NaOH was made from a 10 M stock solution.

F) DTNB assay

NaH_2PO_4 solution

A stock solution of 10 mM NaH_2PO_4 was made by adding 400 μL of 250 mM NaH_2PO_4 to 10 ml of water.

5,5'-dithiobis (2-nitrobenzioc acid) solution (DTNB)

A 300 mM DTNB stock solution was made in NaH_2PO_4 solution. The working solution was diluted 100 times in NaH_2PO_4 solution. The solution was stored at 4°C protect from the light for a month.

G) Activity of Caspase-3 assay

DTT solution

A 1 M DTT solution was prepared prior to each experiment by adding 154 mg of DTT to 1 ml of water. This solution was kept on ice in the dark.

HEPES buffer

100 mM HEPES, at pH 7.25 with 10% sucrose, 0.1% CHAPS and 10^{-4} % NP-40 in a final volume of 100 ml. The pH was adjusted using 10 M NaOH. 5 ml of the solution was aliquoted and stored at -20°C.

Caspase substrate: Acetyl-Asp-Glu-Val-Asp-7-amido-4-methyl-coumarin (DEVD-AMC)

A stock solution of 50 mM DEVD-AMC $^{w}/_{v}$ was made up in 100% DMSO.

The stock solution was aliquoted by 5 µl and stored at -20°C.

Caspase-3 working solution

The solution was made prior to each experiment by mixing 1ml of the HEPES buffer, 5 µL of the DTT solution and 1µL of the caspase substrate. The solution was kept at 37°C in the dark prior to use in experimentation.

H) Apoptest™ FITC

Apoptotest™ FITC was supplied by DakoCytomation.

The kit contained: 100 µl Annexin V-FITC solution

3x 1.7 ml 10x concentrated buffer

250 µg red solid Propidium Iodide

2.2 Methods

2.2.1 U937 and THP-1 cell culture

a) U937 cell

U937 cells are a human histiocytic lymphoma cell line with monocyte-like characteristics (Sundstrom and Nilsson 1976) and were obtained from Dr B Hock, Haematology Research Group, School of Medecine.

b) THP-1 cell

THP-1 cells are a human monocyte-derived cell line established in 1980 (Tsuchiya, Yamabe et al. 1980) and were obtained from Dr B Hock, Haematology Research Group, School of Medecine.

Cells were maintained in a suspension culture in RPMI 1640 media supplemented with 5% HI-FCS and Pen/Strep as described previously (Section 2.1.2), at 37°C in a

humidified atmosphere containing 5% carbon dioxide (CO_2). Cells were maintained at a density between 5×10^4 and 1×10^6 cells per ml.

The cells were observed under an inverted microscope (Nikon TMS) in order to check their condition, absence of infection and abundance.

The density of the cells was determined using a Double-chambered haemocytometer. Viable cell counts were performed by taking a small sample (20 µl) of cell suspension and mixing with 0.4% trypan blue dye (80 µl). The cell dilution was left for 30 seconds and then applied to two fields of the haemocytometer. The equation used to establish the cells count was:

Cell/ml of medium = No. of cells in one square (or mean of 5 squares) × dilution factor (5 in this case) × 10 (volume of the chamber) × 10^3 per ml.

2.2.2 Cell culture experiments

All cell manipulations were performed under aseptic conditions in a class 2 biological safety cabinet (Clyde –Apex BH 2000). All instruments and equipment used were either sterilised by autoclave (15 minutes, 121°C, 15 psi), sterile plasticware (Nunc products, Nalge Nunc International, or Falcon products, Becton Dickinson and Co.) or sprayed with 70% ethanol.

Setting up plates for experiments

The required amount of cell suspension for the experiment was pelleted in a 50 ml tube after centrifugation at 400 g at 25°C for 5 minutes (Eppendorf high-speed benchtop centrifuge 5403). Supernatant was removed and the resulting pellet of cells was washed twice with warm sterilised PBS and re-suspended in the required amount of warm Earle's Balanced Salt Solution (EBSS).

For each experiment a calculated amount of cell suspension containing 0.5×10^6 cells/ml were plated in 6 or 12-well tissue culture plates. Three replicates for each treatment were plated.

The cells were incubated for a period of time of 0 to 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. After incubation, samples were removed and processed according to the individual experimental procedure.

2.2.3 Cellular protein hydroperoxide determination using the FOX assay

The Ferric-Xylenol Orange (FOX) assay as modified by Gebecki (Gay, Collins et al. 1999) was used in this study to examine the kinetics of protein hydroperoxide formation within cells (U937 or THP1) exposed to a peroxyl radical generator (AAPH) solution. The assay relies on the protein hydroperoxide's ability to oxidise ferrous iron (Fe^{2+}) to ferric ion (Fe^{3+}) (equation 1). Ferric ions then bind to xylenol orange dye (equation 2). The absorbance of the resulting coloured complex iron-xylenol can be measured spectrophotometrically at 560 nm (Shimadzu, UV-1601PC).

1. $Fe^{2+} + ROOH \rightarrow Fe^{3+} + OH^{-} + RO^{\bullet}$
2. $Fe^{3+} + XO \rightarrow Fe\text{–}XO$

The peroxyl radical generator AAPH interferes with the FOX assay, therefore it was removed by precipitating and washing the resulting protein pellet with TCA, prior to analysis.

3 ml of cell suspension were removed, after having been gently mixed to resuspend cells which had settled on the bottom of the plate. The cells were washed twice in glass centrifuge tubes with PBS, and resuspended in 1.7 ml centrifuges tubes in 1 ml of PBS. To the remaining solution, 140 µl of 72% $^{w}/_{v}$ cold TCA was added and the samples left on ice for 5 min to allow the protein to precipitate. The precipitate was centrifuged for 5 min at full speed (23100g) at 4°C. The supernatant was removed and drained on tissue paper, and the pellet resuspended in 1 ml of 5% $^{w}/_{v}$ cold TCA. The pellet was dissolved, and the sample was centrifuged as above. This washing step was repeated once more. To the resulting protein pellet, 900 µl of 25 mM H_2SO_4 was added and dissolved, prior to the addition of 50 µl of xylenol orange. The remaining solution was vortexed and 50 µl of ferrous ammonium sulphate was added. This solution was vortexed and incubated in the dark at room temperature for 30 minutes.

At the end of the incubation time, samples were centrifuged (23100g, 4°C, 5 minutes) to remove any cell debris. The supernatants were transferred to plastic semi-micro cuvettes and the absorbance read at 560 nm against a water blank using a spectrophotometer. The concentration of protein hydroperoxide was calculated using the extinction coefficient, of $0.035 \times 10^{6}\ M^{-1}\ cm^{-1}$ (Gay, Collins et al. 1999).

2.2.4 Protein hydroperoxide determination using the G-PCA FOX assay

The principle of the G-PCA-FOX assay is the same as for the FOX assay describe above, in that the peroxide react with ferrous iron to give ferric ion which then binds to a dye xylenol orange. The standard FOX assay suffers from a high sensitivity to small changes in pH. Gay & Gebicki (Gay and Gebicki 2002) modified the use of the solvent, and substituted the perchloric acid to sulphuric acid. This substitution produced a much broader pH optimum profile, allowing greater tolerance of pH shifts.

The G-PCA-FOX assay involved taking 3 ml of cells sample, placed into a glass tube and washed twice with PBS by centrifugation at 400 g for 5 minutes. The cell pellets were suspended in 745 µl of 6 M GuHCl and the remaining solution transferred in a 1.7 ml centrifuge tube. After addition of 40 µl of 0.5 M PCA, the samples were vortexed and the FOX reagents were added, 25 µl of each 5 mM XO and Fe^{2+} in 110 mM PCA. This was followed by the addition of 15 µl of water, to adjust the final volume to 850 µl. The mixture was vortexed and incubated at room temperature in the dark for 30 min. The absorbance at 560 nm was measured against water blank. The concentration of protein hydroperoxide was calculated with the extinction coefficient of 0.036×10^6 M^{-1} cm^{-1} (Gay and Gebicki 2002).

2.2.5 HPLC analysis of intracellular GSH

Intracellular GSH in the cells was measured by the Monobromobimane (mBBr) derivatistion method on reverse phase high performance liquid chromatography (HPLC).

Monobromobimane (mBBr) is a cell-permeable dye that binds thiol groups. This assay utilises these properties to measure GSH by reverse phase HPLC after remove of the protein by precipitation (Cotgreave and Moldeus 1986).

A standard solution was prepared in order to be treated at the same time as the samples (method described in section 2.1.2 E).

1 ml of the cell suspension was placed in a 1.7 ml centrifuge tube and washed twice in PBS by centrifugation (5min, 400g, 25°C). The cells were suspended in 400 µl PBS.

To the remaining solution, 9 µl of 0.1 M NaOH was added followed by 10 µl of mBBr. Samples were incubated in the dark at room temperature for 20min. After the incubation, 20 µl of 100% $^{w}/_{v}$ TCA was added and the tubes were centrifuged at full

speed (23100g) for 5 min at 4°C. The remaining supernatant was transferred in another 1.7 ml centrifuge tube for the mBBr analysis on HPLC.

2.2.6 Determination of total cellular thiols

Total cellular thiols was measured by the reaction with the 5,5'-dithiols (2-nitrobenzoic acid) (DTNB). Thiol groups react with DTNB and forms the brightly coloured 3-carboxylato-4-nitrothiophenolate (CNTP) anion, which can be detected spectrophotometrically at 412 nm (equation 3). This assay is based on a general method described by Boyne & Ellman (Boyne and Ellman 1972).

3. $R\text{-}SH + DTNB \rightarrow CNTP$

Peroxyl radicals produced by AAPH destroy the CNTP anion, therefore a minimum of three washes of the cells with PBS were performed before analysis.
3 ml of cell suspension were removed from the six well plates. The cells were placed in glass tubes and were centrifuged at 400g for 5 minutes at 4°C and washed twice in cold PBS. Cells were resuspended in 1ml cold PBS and then lyses by sonication for 2 minutes (Soniclean 160T, Transtek systems, Australia). 500 µl of 10% SDS were added and the samples vortexed. To the remaining solution 15 µl of DTNB (3 mM) was added, and the samples were incubated for 30 minutes at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 412 nm. The concentration of total cellular thiols was calculated using the extinction coefficient, of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$ (Riddles, Blakeley et al. 1979).

2.2.7 Measure of the cellular metabolic activity by MTT reduction

The MTT assay measures the cellular metabolic activity of NADH/NADPH dehydrogenases to gauge the overall health of the cell. Living cells can metabolise MTT, a yellow water soluble triazolium salt, into MTT-formazan, an insoluble purple compound. Cell lysis releases the MTT-formazan which is solubilised by an SDS buffer and measured via its absorbance at 570 nm. The MTT assay used in this study is based on the original method described by Mosmann (Mosmann 1983).

Cell experiments using MTT analysis were performed in 12 well suspension plates, under aseptic conditions. 1 ml of cell suspension was removed and centrifuged (400g, 5

minutes, 25°C). The supernatant was discarded and 500 µl warm PBS was added followed by another centrifugation (as above) to wash the cells. Supernatant was removed and 100 µl warm RPMI (without phenol red) was added into the eppendorf tubes and then plated into 12 well suspension plates that contained 900 µl warm RPMI (without phenol red). 100 µl of the MTT reagent, filtered sterilised, was added to each well, and the plate incubated at 37°C for 2 hours. After the incubation time, under aseptic conditions, 1 ml 10% sodium dodecyl sulphate (SDS) was added to each well and a pipette tip was used to dissolve all the purple MTT formazan crystals. The cells were incubated at 37°C for 20 minutes to help dissolve all the crystals, and the absorbance of the resulting solution was measured spectrophotometrically at 570 nm against a no cell blank. The percentage of living cells was measured compared to the percentage of the control value at time zero.

2.2.8 Determination of Caspase-3 activity

Caspase-3 is a part of a cysteine protease family that are key mediators of programmed cell death or apoptosis (Thornberry and Lazebnik 1998). Caspase-3 has been found to cleave the artificial substrate Ac-DEVD-AMC by releasing the AMC fluorophore, which is detected by excitation at 370 nm and emission 440 nm on the fluorometer (Varian Cary Eclipse fluorescent spectrophotometer). 100 µl of working reagent are required per million of cells.

2 ml of cell suspension was removed from the six well plates and centrifuged at 400 g for 5 minutes at 25°C. The remaining pellet was washed in 1 ml PBS. The 1.7 ml centrifuge tubes were centrifuged at 2600g, for 5 minutes. The cells were washed once more in 1 ml PBS, before a final spin at 23100g at 4°C for 5 min. The supernatant was discarded and the pellet was immediately frozen at -80°C for up to two weeks until analysis.

Prior to analysis the caspase working reagent was prepared by mixing 1µl caspase substrate and 5 µl DTT for every 1 ml caspase buffer (as described in section 2.1.2 F). The working solution was kept in the dark at 37°C. To the cell sample, 200 µl of working solution at 37°C was added and homogenised in the 1.7 ml centrifuge tube and placed in a quartz micro-cuvette. The release of AMC was measured over two minutes. Fluorescence measurements (excitation 370 nm, emission 440 nm) were converted to pmoles of AMC/min by the use of a calibration curve produced with free AMC.

2.2.9 Fluorescence Activated Cell Sorter (FACS)

During apoptosis, cells expose phosphatidylserine at the cell surface. Annexin V is a phospholipid-bonding protein, which in the presence of calcium ions binds selectively and with high affinity to phosphatidylserine. This binding profile makes annexin V a powerful and selective tool for the detection of apoptotic cells.

After treatment with AAPH and washing with cold PBS, 10^5 cells were pelleted and suspended in binding buffer containing Annexin V-FITC and propidium iodide (PI), as per the manufacturer's instructions (Apoptotest™ FITC, DakoCytomation). Following ten minutes in the dark, 10,000 cells were analysed with Vantage fluorescence-activated cell sorter from Becton Dickinson (San Jose, CA). The cells were classified as viable, apoptotic or necrotic based on regions drawn on the dot plots. Cells with low Annexin V-FITC and PI fluorescence were classified as viable, cells binding Annexin V-FITC but excluding PI were classified as apoptotic and double labelled cells were classified as necrotic (Vermes, Haanen et al. 1995).

2.3 Statistical analysis

Statistical analysis of the data was performed using PRISM software (version 4, GraphPad Software, Inc.). The comparison among treatments within an experiment was analysed using one way analysis of variance (ANOVA). When significance was observed ($p \leq 0.05$) Tukey's post test was performed to determine significance from the appropriate control values. Significant levels are indicated in the text. The statistical significance, indicated in the graphs uses the following notation, (* $p \leq 0.05$), (** $p \leq 0.01$), (*** $p \leq 0.001$) and represents variation from the relevant time zero value. The data points are shown as the means and standard errors of triplicate treatments. Standard errors were calculated and are presented on the graphs as symmetrical error bars. Results shown were obtained from single experiments, representing a minimum of three repetitions of the same experiment.

3 RESULTS

3.1 AAPH-induced oxidative damage to U937 cells

In order to measure and time the effects of free radical attack on cells, we used a model cell line, and a well known peroxyl radical generator. The monocytic cell line U937, will be incubated over a period of time with the water-soluble azo compound 2,2'-azobis (2-methyl-propionamidine) dihydrochloride (AAPH). 10 mM of AAPH appeared to be the optimal concentration to induce a suitable level of cellular, based on previous studies in our laboratory (Duggan 2000; Cassidy 2003). The following parameters were analysed; protein hydroperoxide formation, total thiols, intracellular glutathione, cell viability and, as a marker of apoptosis, caspase-3 activity and the level of phosphatidylserine exposure.

3.1.1 Protein hydroperoxide formation measured by the standard FOX assay

Initially, AAPH-induced protein hydroperoxide formation in U937 cells was measured. Three wells of 4 ml of U937 cells at 4×10^6 cells/ml were incubated at 37°C in EBSS media exposed to 10 mM AAPH for a period of time up to 24 hours. Control cells in three wells were incubated at the same time without AAPH.

Samples were taken from the wells after 0, 3, 14, 18 and 24 hr and analysed for protein hydroperoxides using the standard FOX assay (Section 2.2.3).

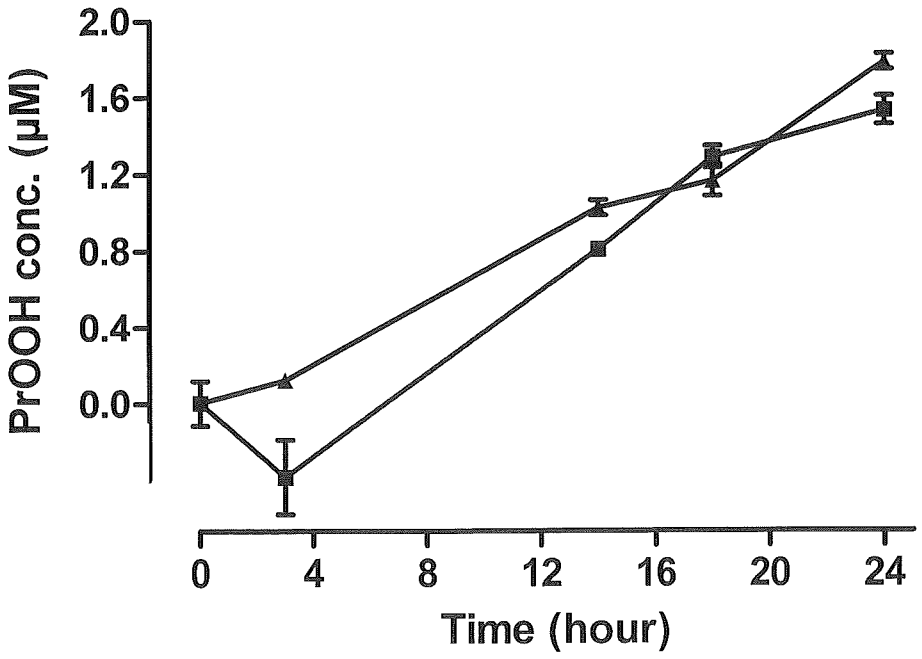


Figure 3.1.1 Protein hydroperoxide formation on U937 cells measured by standard FOX analysis
U937 cells (4×10^6 cells/ml) were incubated in the presence (▲) and absence (■) of 10 mM AAPH in EBSS at 37°C, 5% CO_2. At indicated time points, samples were taken for standard FOX analysis of protein hydroperoxides. Each value shown is the mean ± SEM of three replicates.

Surprisingly, results clearly showed that there was formation of protein hydroperoxides over time in both AAPH treated cells and in control cells (Fig 3.1.1).

Peroxidation of protein in both samples seemed to follow the same kinetics. After 3 hours of treatment it increased in a linear fashion, to reach a concentration of 1.6 µM after 24 hours of incubation in both cases. The presence of AAPH did not seem to significantly affect the formation of protein hydroperoxide on the cells. The experiment was repeated several times and the same trend was obtained each time. This differs from the previous work, where U937 cells incubated with AAPH showed an increase of protein hydroperoxide formation, and the control did not present any formation of protein hydroperoxide (Duggan 2000; Cassidy 2003).

It was possible that the FOX values obtained for the control samples were the product of the assay system used. We therefore examined protein hydroperoxide formation on the U937 cells using a different FOX assay.

3.1.2 Protein hydroperoxide formation measured by the G-PCA-FOX assay

The FOX assay has been modified to produce a number of different versions. The FOX assay used previously was shown to be stable but sensitive to a change of pH for the detection of the FeXO complex by spectrophotometer (Gay, Collins et al. 1999). As the results did not agree with previous work, the idea of measuring the protein hydroperoxide formation with a different FOX assay was investigated.

G-PCA-FOX assay (Gay and Gebicki 2003) used a different delipidation system from the standard FOX assay, and had a less pH sensitive molar absorption coefficient for the measurement of the FeXO complex, due to the use of perchloric acid instead of sulphuric acid for pH control.

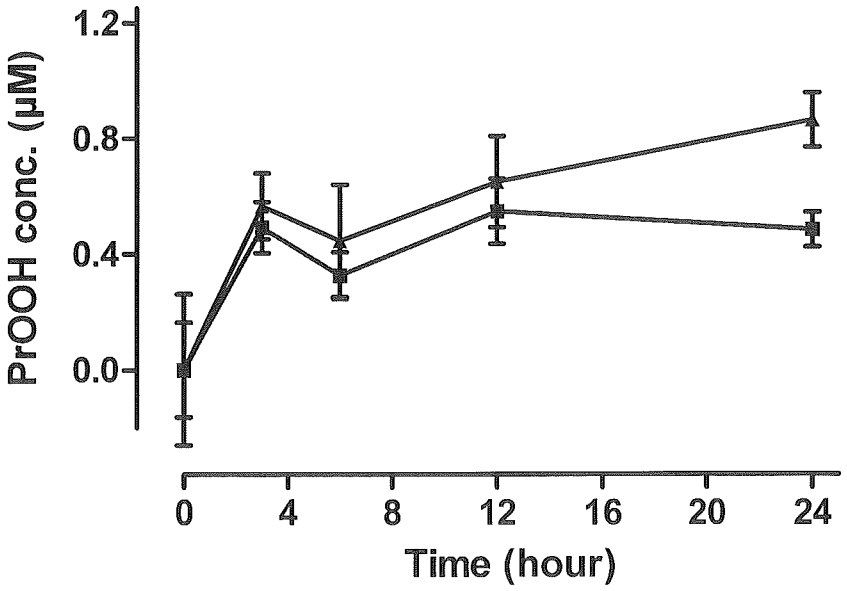


Figure 3.1.2 Protein hydroperoxide formation on U937 cells measured by G-PCA-FOX assay

U937 cells (4×10^6 cells/ml) were incubated in the presence (▲) and absence (■) of 10 mM AAPH in EBSS at 37°C, 5% CO_2. At indicated time points, samples were taken for G-PCA-FOX analysis of protein hydroperoxides. Each value shown is the mean ± SEM of three replicates.

Again an increase in protein hydroperoxide formation was observed after 3 hours in both the AAPH treated cells and in the control cells (Fig 3.1.2). The formation of protein hydroperoxide increased to a concentration of 0.8 μM for the AAPH treated cell and 0.5 μM for the controls cells after 24 hours.

The lack of difference between the two treatments seen with the G-PCA-FOX assay suggests that pH alteration and lipid contamination are not the cause of the observed kinetics.

3.1.3 Protein hydroperoxide formation on BSA incubated in PBS

Incubating bovine serum albumine (BSA) with AAPH has been widely used by past students in this laboratory to measure the formation of protein hydroperoxide (Duggan 2000; Ling 2003). A significant increase in protein hydroperoxide formation had always been observed. This experiment is used here as control for the FOX solutions and in view to check the effect of sample manipulation during the FOX assay. BSA possesses the advantage of not being a cellular complex and is therefore easier to manage than the U937 cells.

The same protocol used for the experiment with U937 cells, standard FOX assay, was followed. BSA (2mg/ml) was incubated at 37°C in PBS (pH 7.4) in the presence of 10 mM of AAPH for a period of time up to 5 hours. The control BSA was incubated without AAPH. At 0, 1, 3 and 5 hr time point, three replicates of the samples were removed for analysis.

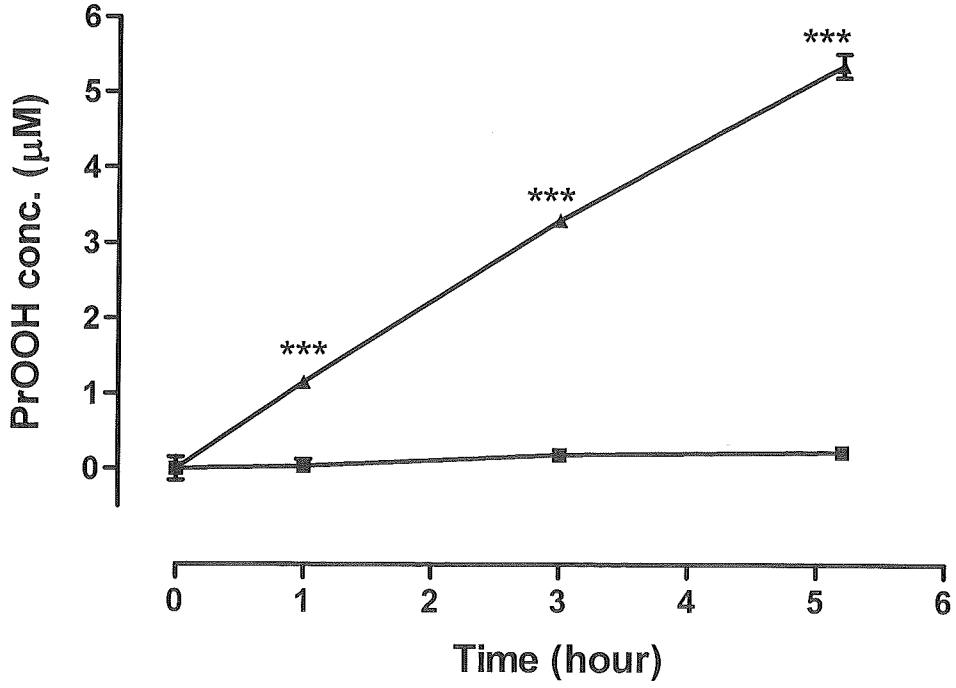


Figure 3.1.3 AAPH-induced formation of protein hydroperoxides on BSA
BSA (2 mg/ml) in PBS pH7.4 was incubated at 37°C in the presence (▲) and absence (■) of 10 mM AAPH for 5 hours. At indicated time points, samples were taken for standard FOX analysis of protein hydroperoxides. Each value graphed is the mean ± SE of three replicates.

The formation of protein hydroperoxide on BSA was measured by the standard FOX assay (Fig 3.1.3). Steady linear increase (r^2= 0.9981) was observed over a period of 5 hours when the BSA was incubated with AAPH, whereas the BSA control did not show any state of oxidation over that time. A highly significant difference ($p \leq 0.001$) was observed in the formation of protein hydroperoxides in response to AAPH compared to the control.

These results show that neither the technique nor the FOX assay solutions were inducing formation of protein hydroperoxides on the control BSA.

3.1.4 Protein hydroperoxide formation on BSA incubated in EBSS

Manipulation of the sample during the FOX assay appears to not be problematic. The following step was therefore taken to create the same controlled conditions as for the cell experiment, but with BSA. Oxidation of the control cells was observed even though they were only incubated in the EBSS minimal media, so the previous experiment with BSA was repeated but this time BSA was incubated in EBSS instead of PBS. The difference between these two solutions is that EBSS is a physiological salt solution supplemented with glucose whereas PBS is a simple phosphate buffer solution with no supplements. The experiment described previously was repeated with 10 mM AAPH incubated in 2 mg/ml of BSA for 5 hours.

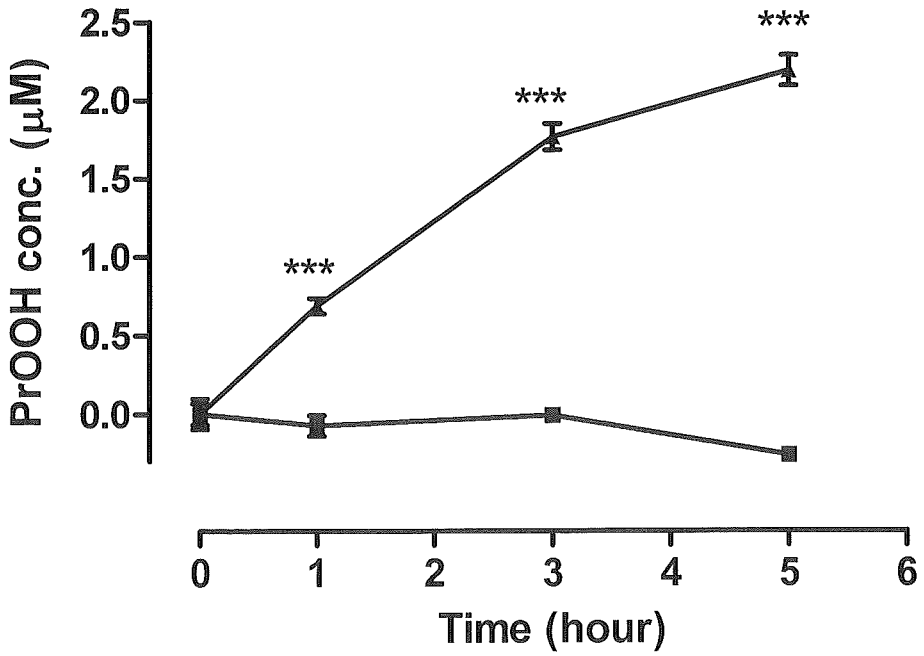


Figure 3.1.4 Protein hydroperoxide formation on BSA incubated in EBSS
BSA (2 mg/ml) in EBSS was incubated at 37°C in the presence (▲) and absence (■) of 10mM AAPH for 5 hours. At indicated time points, samples were taken for standard FOX analysis for protein hydroperoxides. Each value shown is the mean ± SEM of three replicates.

Though AAPH incubation with EBSS appears to induce less protein hydroperoxide formation in BSA than with PBS, it did show there was no protein hydroperoxide formation in the absence of AAPH (Fig 3.1.4). This result indicates that the minimal media, EBSS, did not cause the oxidation of the control BSA.

3.1.5 Effect of cell concentration on protein hydroperoxide formation

High concentrations of U937 cells were used in the above experiments to measure the formation of protein hydroperoxide. As this raised the concern that the high concentration of cells in the reaction vial could induce cellular stress, the concentration of cells per well was reduced to the same cell concentration used by previous students. Cell concentration was decreased from 4×10^6 cells/ml to 0.5×10^6 cells/ml. Three wells containing 3 ml of 0.5×10^6 cells/ml were treated with 10 mM AAPH over a period of 24h at 37°C. Three wells of control cells were incubated without AAPH.

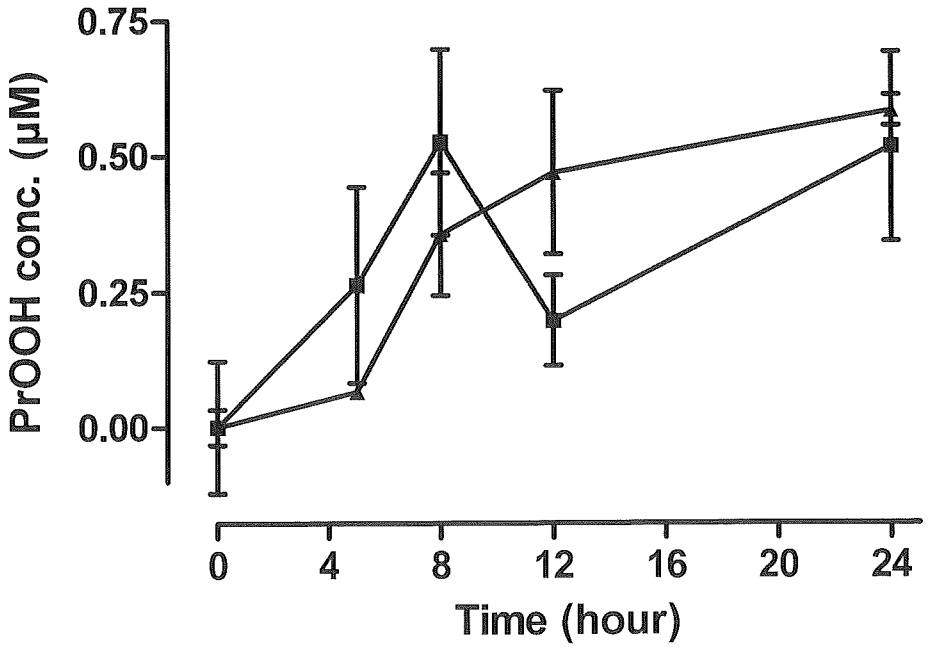


Figure 3.1.5 Protein oxidation on U937 cells with lower cell concentration
U937 cells (0.5×10^6 cells/ml) in EBSS were incubated in the presence (▲) and absence (■) of 10mM AAPH at 37°C, 5% CO_2. At indicated time points, samples were taken for standard FOX analysis of protein hydroperoxides. Each value shown is the mean ± SEM of three replicates.

Less protein hydroperoxides were formed than in the experiment using U937 cells at a higher concentration (Fig 3.1.5). The total level of protein hydroperoxide was 0.6 μM in this experiment compared with 1.6 μM in Fig 3.1.1. The formation of protein hydroperoxides in the control cells was observed to be similar to the AAPH-treated cells with the same concentration observed (0.6 μM) after 24 hours. Cell concentration therefore did not account for the formation of protein hydroperoxides on the control cells.

3.1.6 A comparison of protein hydroperoxide formation in different media

We demonstrated that neither the FOX assay, the experimental solutions or the cell concentration were responsible for presence of protein hydroperoxide formation in the control. While it had been shown that EBSS was not producing any protein hydroperoxides on the control BSA, it was still unclear whether this media was adversely affecting the health of the cells.

Protein hydroperoxide formation on cells incubated in EBSS was therefore compared with protein hydroperoxide formation on cells incubated in the growth media, RPMI

1640. No AAPH was added and the cells were incubated at 0.5×10^6 cells per ml for up to 24 hours at 37°C.

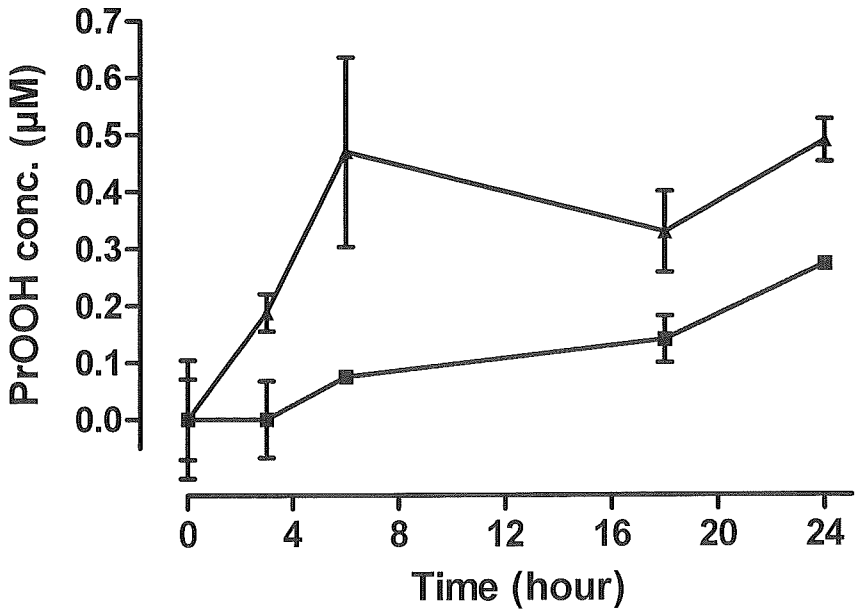


Figure 3.1.6 Effect of different media on protein hydroperoxide formation on U937 cells
U937 cells (0.5×10^6 cells/ml) were incubated in EBSS (■) or RPMI 1640 (▲) at 37°C, 5% CO_2. At indicated time points, samples were taken of FOX analysis for protein hydroperoxides. Each value shown is the mean ± SEM of three samples.

Protein oxidation on cells was observed, by the increase in protein hydroperoxides measured with the FOX assay, in both media (Fig 3.1.6). A significant increase ($p \leq 0.001$) in protein hydroperoxide formation was observed for the cells incubated in EBSS and RPMI 1640 after 24 hours.

These results show that formation of protein hydroperoxides occurred in the U937 cells whether they were incubated in EBSS or RPMI 1640 media. It had been previously shown that EBSS did not induce any oxidation in protein, measured by the BSA experiment. It appears from these findings that the cells are undergoing an oxidative stress.

3.1.7 Loss of cell viability

In order to observe cell viability over time, the level of cell metabolism as determined by the MTT assay, has been measured.

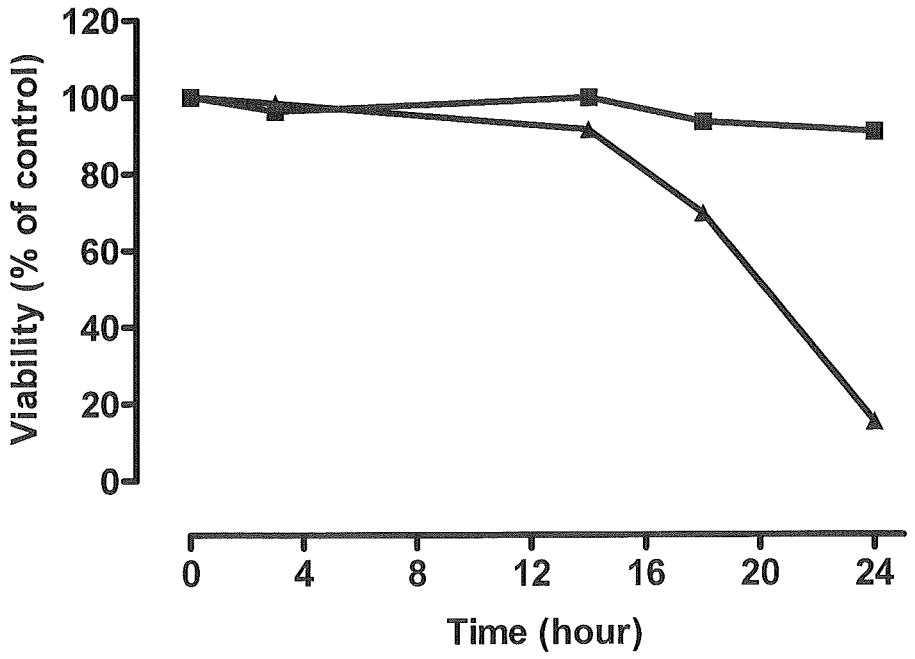


Figure 3.1.7 Effect of AAPH on U937 cell viability
U937 cells (4×10^6 cells/ml; 1ml/well) in EBSS were incubated in the presence (▲) and absence (■) of 10mM AAPH at 37°C, 5% CO_2. At indicated time points, samples were taken for MTT analysis as a measure of cell viability. The data were expressed as a percentage of viability of the control cells at time 0. Each value shown is the mean ± SEM of the triplicate samples.

The loss of cell viability in U937 cells incubated with AAPH did not occur before 14 hours of incubation, and this was followed by a linear decrease of cell death until very few viable cells remained after 24 hours (Fig 3.1.7).

The level of viability in the control cells stay constant, with no decrease in cell viability observed after 24 hours. This is interesting because the level of protein hydroperoxides formed in these control cells was as high as the AAPH-treated cells. It is important to notice that the MTT assay and the standard FOX assay from Fig 3.1.1. were measured from the same experiment.

3.2 AAPH-induced oxidative damage to THP-1 cells

The problems encountered with U937 cells led to the use of THP-1 cells for the remainder of the research project. THP-1 cells are also a human monocyte-like cell line, and have also been used in apoptosis studies (Vicca, Hennequin et al. 2000 ; Li, Dalen et al. 2001). It was initially important to measure protein hydroperoxide formation on this cell line in order to make a comparison with the results obtained with U937 cells. The kinetics of other parameters such as thiol loss and cell death mechanisms were then investigated to elucidate the timing of events occurring when THP-1 cells were exposed to oxidative stress induced by the peroxyl radical generator AAPH.

For each experiment an identical treatment was applied to the THP-1 cells, and different parameters were then measured. THP-1 cells at 0.5×10^6 cells per ml were incubated at 37°C in Earle's Balanced Salt Solution (EBSS) with 10 mM AAPH for a period of time up to 24 hours. The control cell samples were incubated without AAPH. After the time points indicated in the figure legends for each set of data, samples were removed from the wells and washed prior to analysis.

3.2.1 Protein hydroperoxide formation in THP-1 cells

To measure the action of 10 mM of AAPH on the protein oxidation of THP-1 cells, the production of protein hydroperoxides was analysed using the standard FOX assay (Gay, Collins et al. 1999).

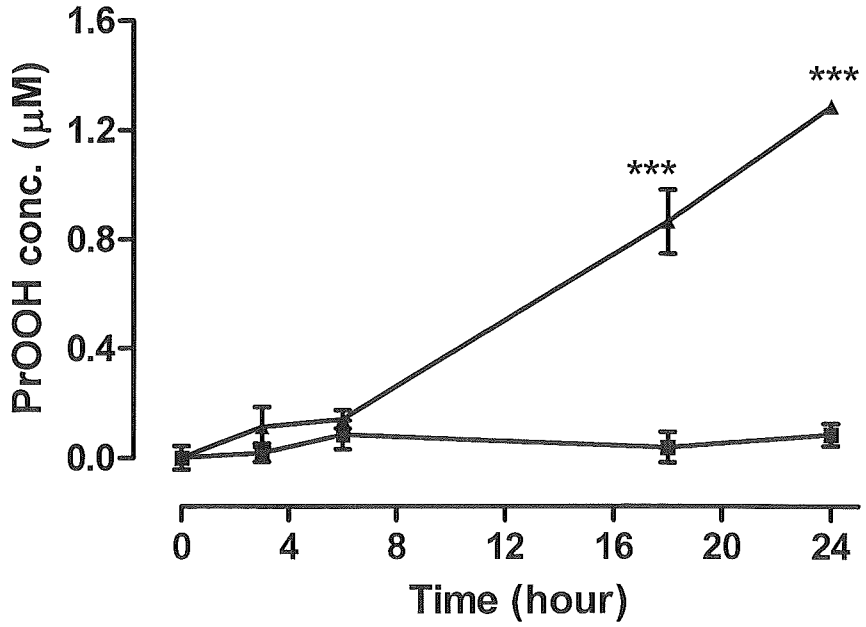


Figure 3.2.1 AAPH induced protein hydroperoxide formation on THP-1 cells
THP-1 cells (0.5×10^6 cells/ml; 3 ml/well) in EBSS were incubated in the presence (▲) and absence (■) of 10 mM AAPH at 37°C, 5% CO_2. At indicated time points, samples were taken for standard FOX analysis of protein hydroperoxides. Each value graphed is the mean ± SEM of the triplicates.

When the cells were not exposed to AAPH, no production of protein hydroperoxide was observed at any time whereas the cells incubated with AAPH showed protein hydroperoxide formation after 6 hours of incubation (Fig 3.2.1). This 6 hour lag phase was followed by an increase in hydroperoxide formation over 24 hours. The concentration of protein hydroperoxides at this time was 1.3 μM.

The level of protein hydroperoxide formation in the cells treated with AAPH was significantly different from the non-treated control at all time points after 6 hours of treatment ($p\leq0.001$). The protocol was repeated and the same results obtained at least three times, the 6 hour lag phase occurring each time.

These results are in agreement with the previous findings of the lab in that AAPH induced significant protein hydroperoxide formation in the cells compared to control cells, and validated the use of THP-1 as a model cell to conduct the study upon.

3.2.2 Loss of total cellular thiols in THP-1

The levels of intracellular thiols regulate the intracellular redox homeostasis, and have an important role in antioxidant defence systems.

To elucidate the effect of AAPH on the level of thiols in the THP-1 cells, total cellular thiols were measured over time. The thiol concentration was quantified with Ellman's reagent (DTNB).

Samples were removed at certain times of incubation as indicated on Fig 3.2.2. The thiol concentration per mg of protein could not be determined because oxidised protein interferes with the protein concentration assay. Therefore presentation of the results was expressed as μM thiol concentration.

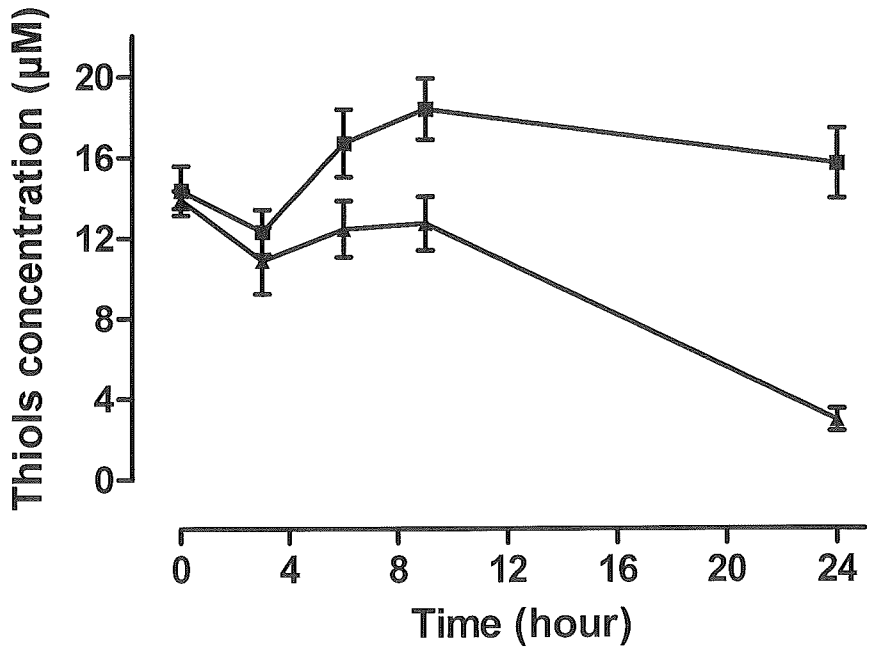


Figure 3.2.2 Total thiol loss from THP-1 cells during incubation with AAPH
THP-1 cells (0.5×10^6 cells/ml; 2 ml/well) in EBSS were incubated in the presence (▲) and absence (■) of 10mM AAPH at 37°C, 5% CO_2. At indicated time points, samples were taken for DTNB analysis of total thiols. Each value shown is the mean ± SEM of triplicate samples.

The control and AAPH treated cellular thiol concentrations were 16 μM at time zero (Fig 3.2.2). The total cellular thiols decreased 9 hours after AAPH exposure. Initial thiol loss began after 3 hours of incubation, follow by a lag period of 6 hours during which the concentration of thiol (12 μM) remained constant until 9 hours. The total thiol concentration then dropped to 2.9 μM after 24 hours of incubation. The amount of thiol remaining (2.9 μM) could be considered a minimum intracellular concentration and we could conclude that a complete decrease of total available cellular thiol occurred after 24 hours of AAPH exposure.

Thiol concentration in the control cells rose from 12 μM to 16 μM at 6 hours and to 18μM at 9 hours and slightly decreased to 15 μM after 24 hours of incubation.

Level of protein and non-protein thiol in THP-1

The previously described DTNB assay was used to determine the level of non-protein thiols (mainly glutathione) and of protein thiols contained in the total thiols of the THP-1 cells. This experiment was performed on cells which had had no treatment applied to them and were taken directly from growth media containing RPMI and foetal calf serum.

For the determination of non-protein thiols, proteins were precipitated with 100% TCA. The remaining solution was neutralized with 10 M NaOH to adjust the pH to the optimum pH for the DTNB assay, pH 7.2. The DTNB reagent was then added to the sample to determine the non-protein thiols.

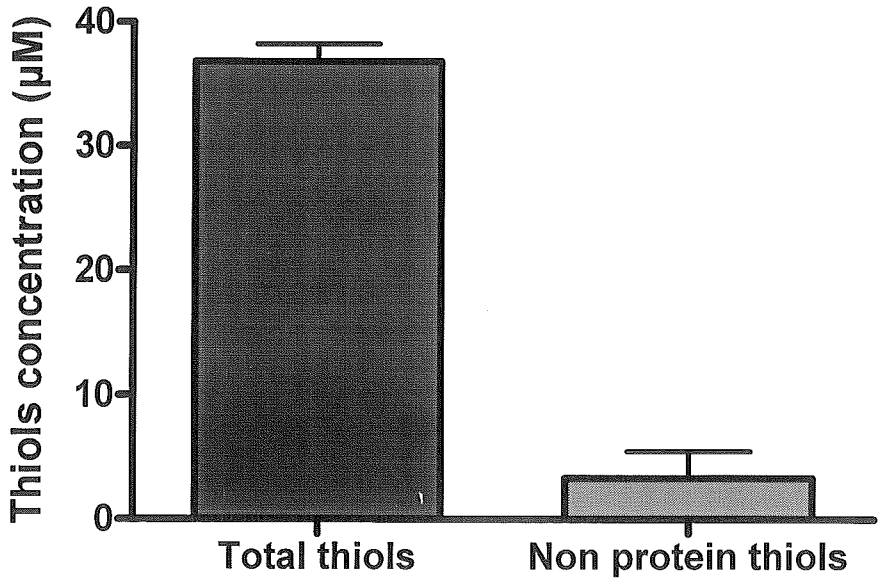


Figure 3.2.3 Concentration of total thiols and non protein thiols in THP-1 cells
THP-1 cells in RPMI (1.5×10^6 cells/ml; 3 ml/well) were analysed for total thiols and non-protein thiols with the DTNB assay. Proteins were precipitated with 100% TCA prior analysis for the non-protein thiol samples. Each value shown is the mean ± SEM of three replicates.

Total thiols in THP-1 cells were detected at a concentration of 36.8 μM and the non-protein thiols were measured at 3.3 μM (Fig 3.2.3). Therefore the non-protein thiol represents 8.9 % of the total thiol.

3.2.3 Intracellular glutathione loss

To gain a better understanding of the use of thiols by the cells when in an oxidatively stressed state, the amount of intracellular glutathione was measured by monobromobimane derivatisation and detection by reverse phase HPLC.

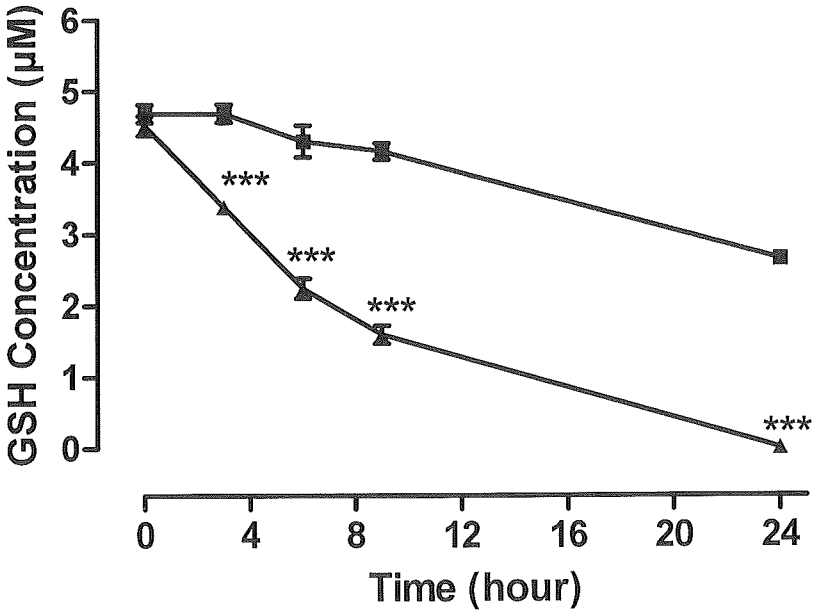


Figure 3.2.4 Intracellular GSH loss in THP-1 cells treated with AAPH
THP-1 cells (0.5×10^6 cells/ml; 2 ml/well) in EBSS were incubated in the presence (▲) and absence (■) of 10 mM AAPH at 37°C, 5% CO_2. At indicated time points, samples were taken for GSH analysis by HPLC. Each value shown is the mean ± SEM of the three samples.

The general level of intracellular glutathione in normal THP-1 cells was measured at time 0, and found to be present at a concentration of 4.7 μM (Fig 3.2.4). The intracellular glutathione content of THP-1 cells was observed to decrease immediately following incubation with AAPH. After 24 hours of incubation, a complete loss of glutathione was observed. A significant loss of glutathione was observed for the control cells after 9 hours, which decreased from 4 μM to 2.6 μM at 24 hours. Incubation of the cells in a minimal media for 24 hours could be the reason for the intracellular GSH decrease even within the cells not treated with AAPH. A highly significant difference was observed between the control cells and the cells incubated with AAPH ($p\leq0.001$).

3.2.4 Markers of apoptosis measured on THP-1 cells treated with AAPH

A major question when studying the effect of oxidative stress on cells is the measurement of cell death. It is important to identify how oxidative agents are acting on cell viability, as every cell type responds differently to each oxidative agent.

Therefore, after measuring the cell viability, the next step was to look at the type of cell death occurring. As is well known, cell death may occur by one of two mechanisms, apoptosis or necrosis.

A. Loss of cell viability

The ability of AAPH to cause cell death was assessed by the use of the MTT reduction assay, as previously described (section 2.2.8). This assay measures the cellular metabolic activity of NADH/NADPH dehydrogenases to gauge the overall viability of the cell.

The cell viability of THP-1 cells treated with AAPH was measured over a period of 24 hours.

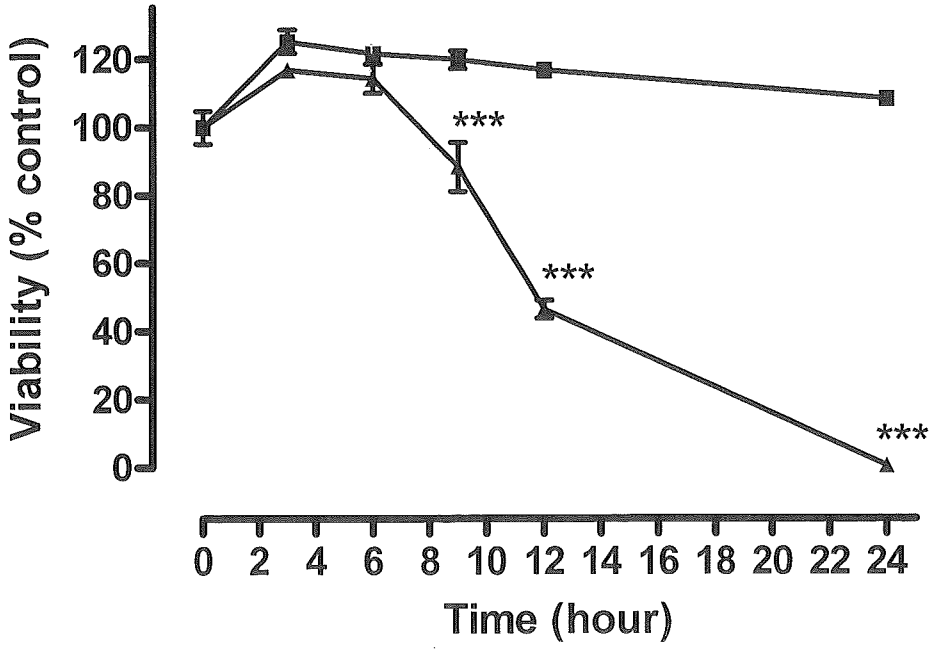


Figure 3.2.5 Effect of AAPH on THP-1 cell viability
THP-1 cells (0.5×10^6 cells/ml; 2 ml/well) in EBSS were incubated in the presence (▲) and absence (■) of 10 mM AAPH at 37°C, 5% CO_2. At indicated time points, samples were taken for MTT analysis as a measure of cell viability. The data were expressed as a percentage of viability of the control cells at time 0. Each value shown is the mean ± SEM of the triplicate samples.

An increase in cell viability was observed in the control cells after the first time point (Fig 3.2.5). The viability changed from 100% at the start, to 125% after 3 hours and then slightly decreased to 24 hours. These results were thought to be an artefact of the reaction or, alternatively, an increase in cell metabolism in response to changes in their environment.

Cells treated with AAPH followed a similar increase in cell MTT reduction from 100% to 116% of the control after 6 hours of incubation, and then the cell viability decreased significantly from 9 hours onwards ($p \leq 0.001$). An almost complete loss of cell viability was observed after 24 hours of incubation with AAPH.

B. Caspase-3 activation

Cell death in THP-1 cells treated with AAPH has been observed after 6 hours of incubation. In our aim to determine the type of cell death induced by AAPH, the cells were assayed for caspase-3 activation, a biochemical marker of apoptosis.

Fluorescence measurements (excitation 370 nm, emission 440 nm) were converted to pmoles of AMC/min by the use of a calibration curve produced by the excitation of free AMC (Fig 3.2.6).

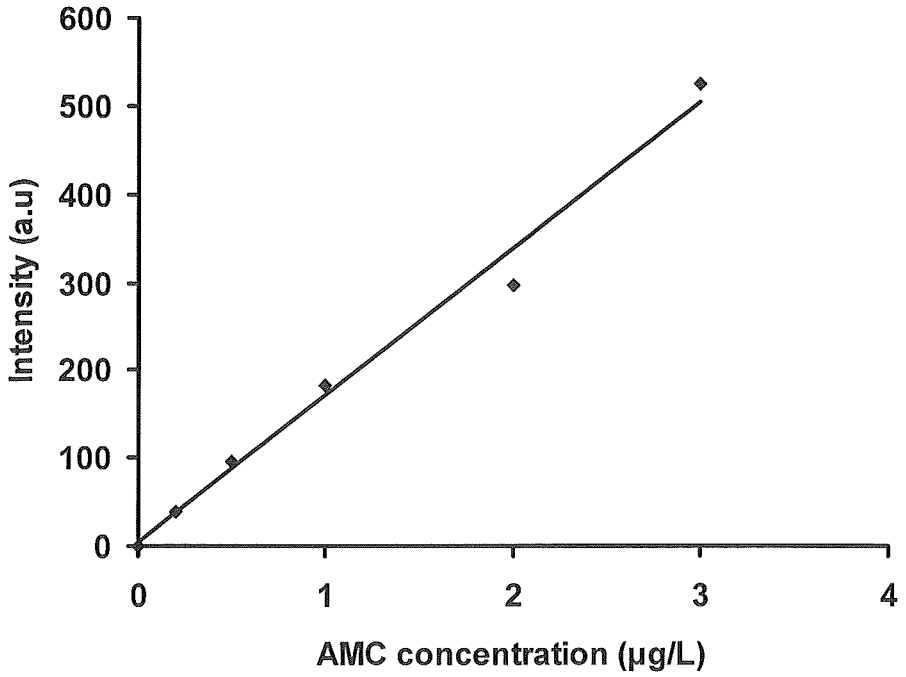


Figure 3.2.6 AMC calibration curve
Different concentrations of free AMC were prepared in 100% DMSO. The fluorescence (excitation 370 nm, emission 440 nm) of each concentration was measured and plotted on a graph. The calibration equation obtained was: Intensity= 168.42*Concentration.

Four millilitres of cells were incubated at various periods of time up to 24 hours with AAPH, and assayed for caspase-3 activation (DEVD-AMC cleavage), by fluorescence as described in section 2.2.8.

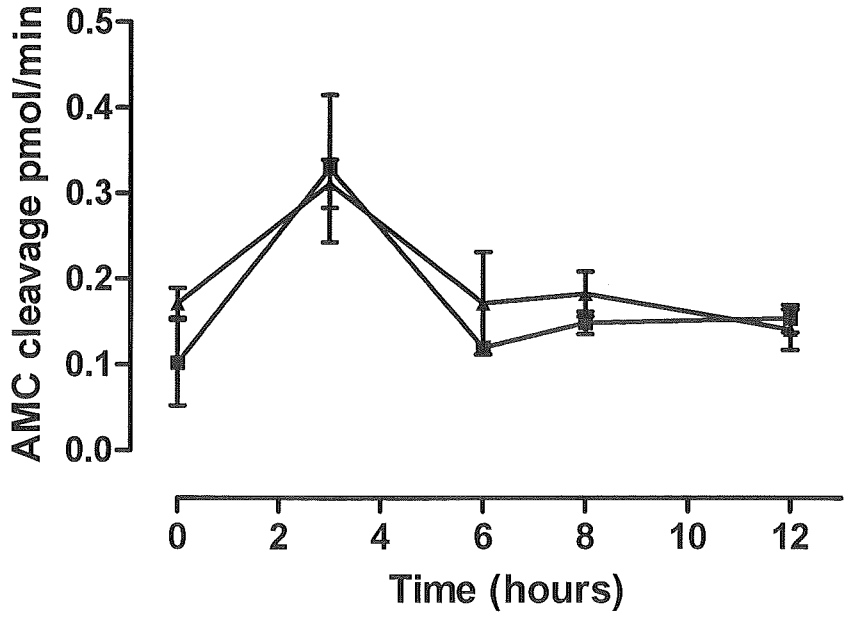


Figure 3.2.7 Effect of AAPH on caspase-3 activation in THP-1 cells
THP-1 cells (1×10^6 cells/ml; 2 ml/well) in EBSS were incubated in the presence (▲) and absence (■) of 10 mM AAPH at 37°C, 5% CO_2. At indicated time points, samples were taken for analysis by caspase assay. Each value shown is the mean ± SEM of the triplicate samples.

The addition of AAPH to the THP-1 cells did not significantly affect the level of caspase activity (Fig 3.2.7). The level of caspase activity found in the THP-1 was similar for the AAPH treated cell and the control cells. This level was low compared to the alcohol positive control (Fig 3.2.8).

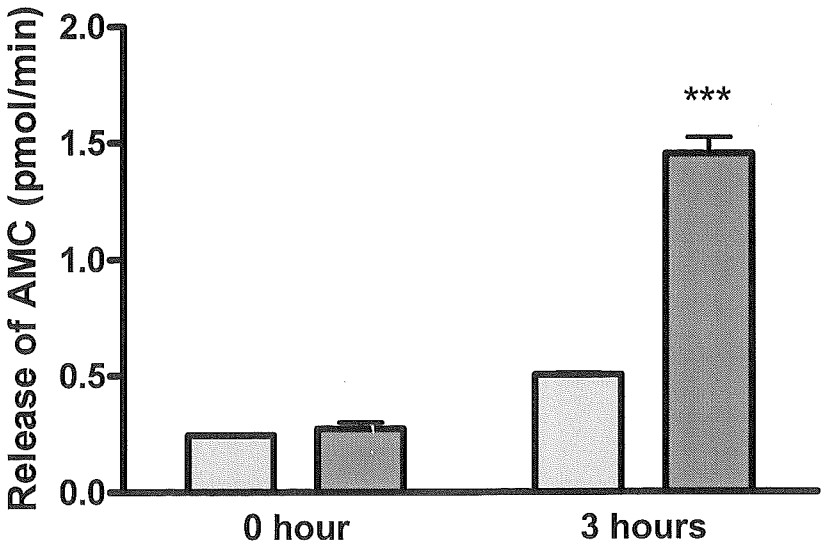


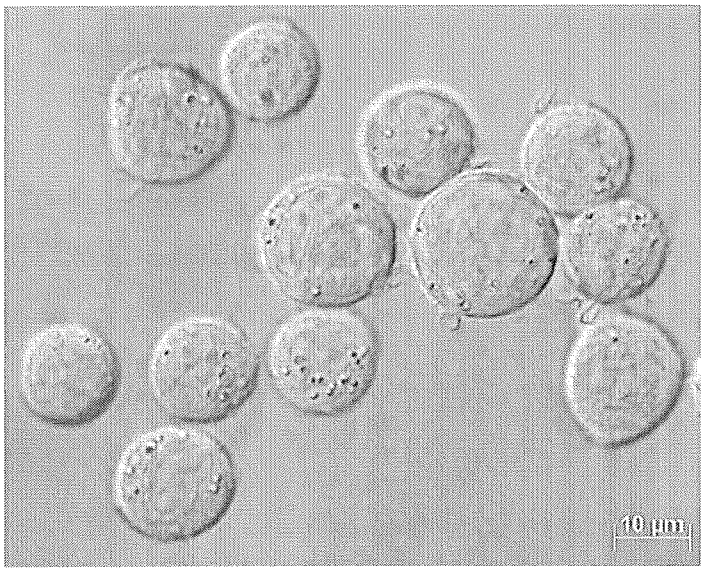
Figure 3.2.8 Caspase-3 activation on THP-1 cells incubated with 6% methanol
THP-1 cells (1×10^6 cells/ml; 2 ml/well) in EBSS were incubated in the presence (dark) and absence (clear) of 6% ethanol at 37°C, 5% CO_2 at 0 and 3 hours for a positive control. Each value shown is the mean ± SEM of the triplicate samples.

The positive control was made by incubating the THP-1 cells with 6% of alcohol. A highly significant increase ($p\leq0.001$) in caspase-3 activity was measured after 3 hours of incubation.

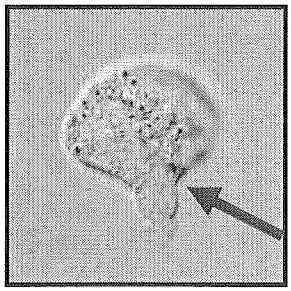
C. Cell morphological changes

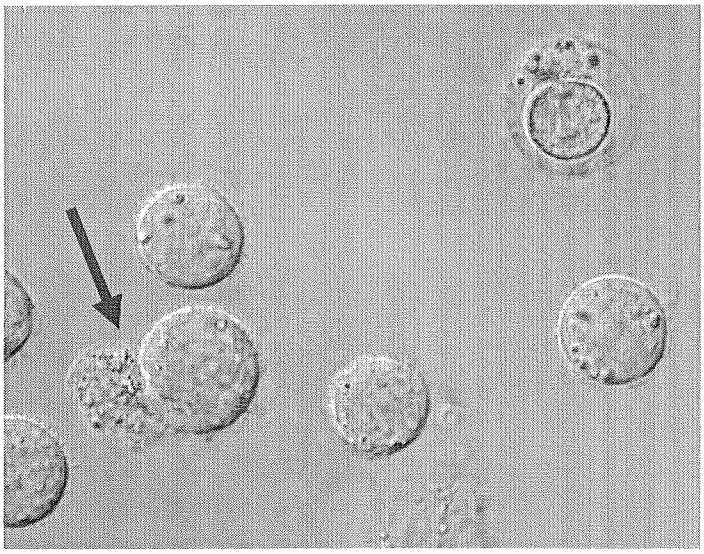
Some authors attempt to categorise the type of cell death by studying the ultrastructure of the cell by microscopy. During apoptosis some changes in the morphology of the cell have been described. Moreover, morphological description cannot always be linked to a specific morphology for all types of cells and/or systems (Abbro, Lanubile et al. 2004). However it was interesting to observe the morphology of the cells during AAPH treatment to see whether they appeared more apoptotic or necrotic. Photographs of the cells during the incubation with AAPH were taken.

❖ **Control cells at time 0**
Objective x100



❖ **Cells incubated with AAPH after 6h**
Objective x100





❖ **Cells incubated with AAPH after 24h**
Objective x100

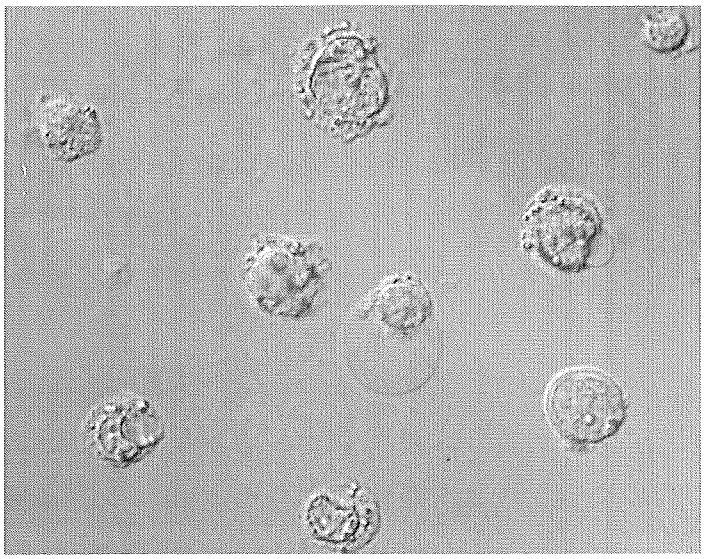


Figure 3.2.9 Morphological changes in THP-1 cells treated with AAPH
A drop of suspension cells in EBSS was transferred onto a slide and a coverslip was applied above the cells. The slides were analysed under Axioskop 2 microscope from Zeiss. Photos of representative cells were taken, using x100 magnification lenses.

The morphology of the cells at different times of treatment showed interesting features (Fig 3.2.9). After 6 hours, we observed several cells with cytoplasmic swelling, indicated by the arrow. These morphologies observed will be characterized as necrotic cells (Abbro, Lanubile et al. 2004). After 24 hours the cells had lost their normal morphology.

D. Phosphatidylserine exposure

The caspase assay data suggests that the cell death induced by the treatment of AAPH was either caspase independent or necrosis. Another feature of the apoptotic process is the phosphatidylserine exposure. During apoptosis phosphatidylserine in the cell membrane is flipped over so that it is exposed to the outside of the cell. There it functions as part of the recognition process for phagocytes (Yu, Byers et al. 2000). Flow cytometry measurements were used in this study to show percentage of cells in necrosis or apoptosis by the use of double staining. Annexin V is a Ca^{2+}-dependent protein that preferentially binds to phosphatidylserine, and propidim iodide (PI) is a dye (like trypan blue) that enters cells with damaged cell membranes and incorporates itself into the DNA.

Two millilitres of the cells at $1x10^6$ per ml were incubated with AAPH for various periods of time, 3, 6, 7.5 and 16 hours. $5x10^5$ cells/ml were used for the analysis, the cells were washed prior to fluorescent staining, and assayed by flow cytometry.

The results were analysed by looking at whether the cells were stained by Annexin V or propidium iodide (PI). The results are presented on a two-dimensional dot plot, with PI on the y axis and Annexin V on the x axis. On the lower left of the plot, the cells are described as viable. Cells in the lower right quadrant, Annexin V positive but PI negative, can be said to be apoptotic. Cell that are PI positive and Annexin V positive are necrotic and may be undergoing secondary necrosis (Vermes, Haanen et al. 1995). Graphs of the percentage obtained have been plotted.

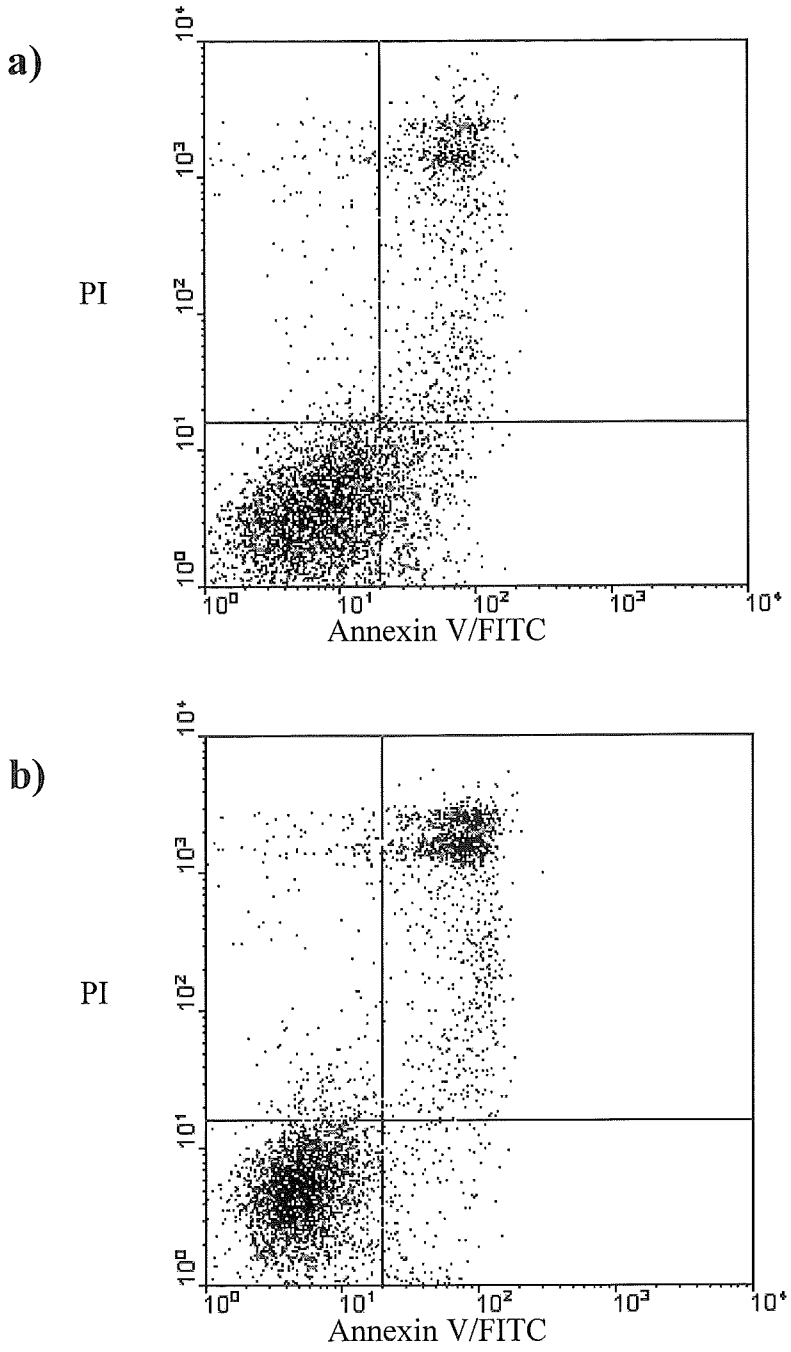
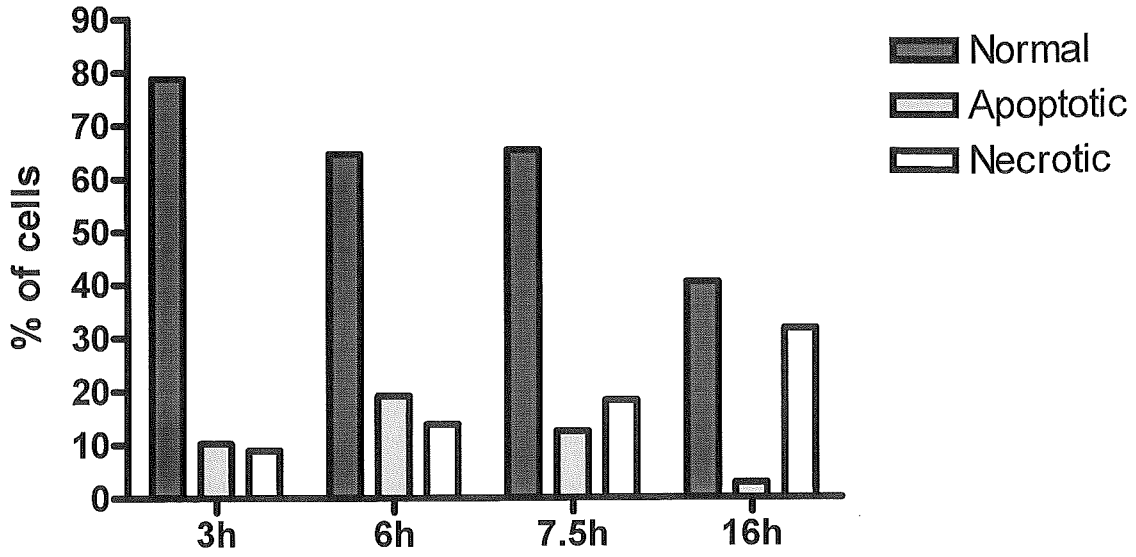


Figure 3.2.10 Flow cytometry dot plots showing the effects of AAPH on THP-1 cells

THP-1 cells (1×10^6 cells/ml; 2 ml/well) in EBSS were incubated in the absence a) and presence b) of 10 mM AAPH at 37°C, 5% CO_2. The cells were washed and incubated with propidium iodide and annexin V-FITC for 10 minutes before analysis by flow cytometry. Regions indicate approximate locations of cells that are viable (Lower Left), undergoing apoptosis (Lower Right), and in the process of (secondary) necrosis (Upper Right).

a)



b)

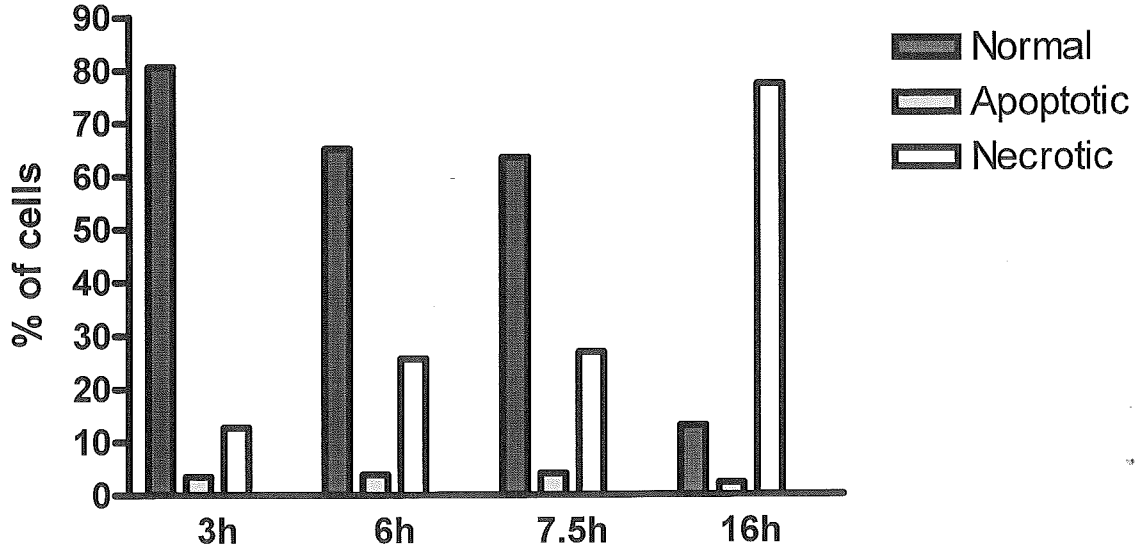


Figure 3.2.11 Effect of AAPH on THP-1's phosphatidylserine exposure and PI uptake

THP-1 cells (1×10^6 cells/ml; 2 ml/well) in EBSS were incubated in the absence a) and presence b) of 10 mM AAPH at 37°C, 5% CO_2. At indicated time points, samples were taken for analysis of APOPTEST™-FITC by flow cytometry to measure phosphatidylserine exposure and PI uptake. Regions were drawn on the dot plots to indicate approximate locations of cells that are viable, undergoing apoptosis and in the process of (secondary) necrosis. The number of cells in each area is expressed in the diagram.

A large decrease in cell viability is observed at 16 hours of AAPH incubation, with smaller decreases is measured at 6 and 7.5 hours (Fig 3.2.11). THP-1 cells incubated

with AAPH did not show any sign of apoptosis at any time during the incubation. Necrotic cells began to be detected at 6 and 7.5 hours of AAPH incubation but the number had dramatically increased by 16 hours.

The presence of apoptotic cells in the control is thought to be an artefact of the analysis, as the same apoptosis level, around 10%, is measured at each time point. It appears that the type of cell death induced by AAPH in THP-1 cells is necrosis.

4 DISCUSSION

4.1 Discussion on the U937 cells

It was expected that no protein hydroperoxide would form on controls cells incubated in EBSS but, while this was observed twice, all other experiments showed protein hydroperoxides formation in control cells (Fig 3.1.1). These results were in total contrast with the work of previous researchers in the laboratory who used the same methods as in this study but did not observed protein hydroperoxide formation in the control cell (Duggan 2000; Cassidy 2003). In comparison to control, AAPH did not appear to have any effect on protein hydroperoxide formation when added to the U937 cells. Therefore it was impossible to follow the effect of AAPH on protein hydroperoxide formation in U937 cells.

Surprisingly the MTT reduction viability assay (Fig 3.1.7) show no loss of cell viability with the control cells, even though they had the same amount of protein hydroperoxide as the AAPH treated cells (Fig 3.1.1) which did show a loss in cell viability with time. It seems like the protein hydroperoxide in the control cells had no affect on the cell viability. These results made it impossible to correlate protein oxidation with other parameters of oxidative stress in the U937 cells.

The use of other stocks of U937 cells from our laboratory and other laboratories within Christchurch also failed to correct the problem. Due to time constraints for this project, we were unable to investigate this problem but did suspect that the U937 cells line was contained or being infected with mycoplasma. Mycoplasma could have been present in the cell culture without being observed, as mycoplasma can not be directly observed under the microscope. Another possibility is the presence of chemical contaminants in the cell culture media but as this problem was not observed with the other cell lines this was very unlikely.

As the U937 cell line was unusable an alternative monocytic cell line, THP-1, was investigated for the rest of the study.

4.2 AAPH induced oxidative damage to THP-1 cells

In an attempt to clarify the results obtained in this study, a chronological presentation of the events is shown below.

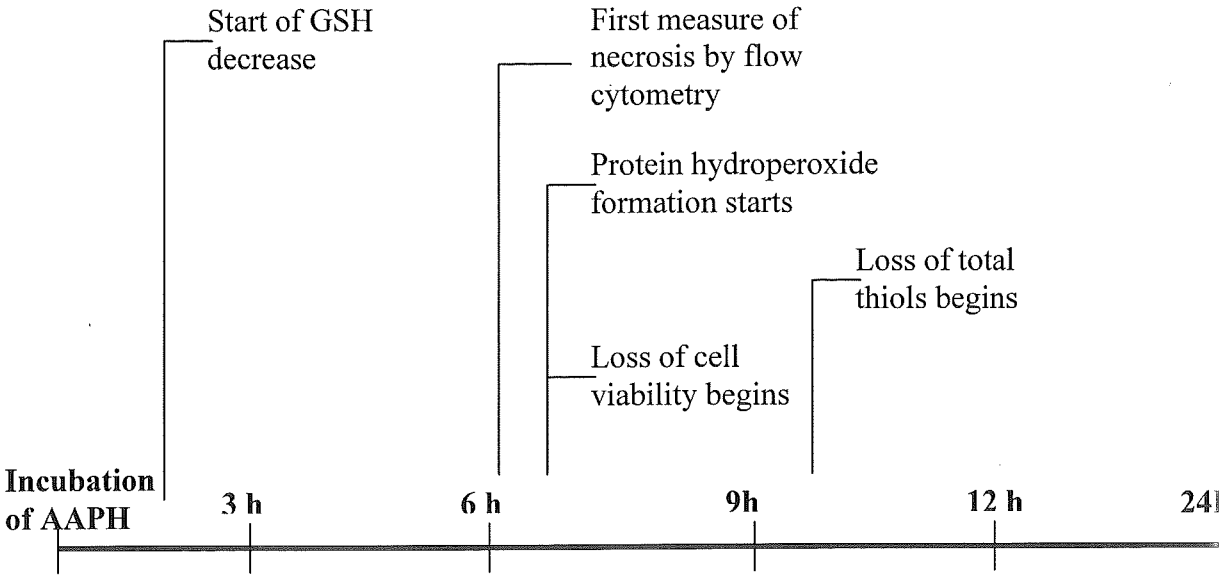


Figure 4.2.1 Sequence of events in THP-1 cells incubated with AAPH at 37°C

The effect of AAPH damage to THP-1 cells was the induction of cell death by necrosis. The cause of the necrosis was investigated by observing individual biochemical sites for indications of damage. Measurements of protein damage, loss in total cellular thiol and loss in GSH were made while also monitoring loss of cell viability over time.
AAPH's cellular toxicity was reported in several publication to be due to the reactive nature of the peroxyl radical ($ROO^{\bullet}$), (Gebicki and Gebicki 1993; Gieseg, Duggan et al. 2000; Li, Kondo et al. 2001). It is important to remember that AAPH is a labile azo compound, which is soluble in water and it is supposed to not cross the lipid membrane of the cell. Protein hydroperoxide formation on U937 cells appears to be only associated with the cellular membranes (Cassidy 2003).

The exposure of THP-1 cells to 10 mM AAPH caused the formation of protein hydroperoxides after 6 hours of incubation (Fig 3.2.1). This 6 hours lag phase was quite surprising as the kinetics of protein hydroperoxide formation found in previous AAPH studies using the U937 human histocytic lymphoma cell line, present an increase over time without showing of a lag phase (Duggan 2000; Cassidy 2003). This absence in lag phase from past studies could be due to the large interval between measurements used and the lag phase could have been missed. Interestingly Du & Gebicki demonstrate that cultured cells exposed to biologically significant amounts of hydroxyl radicals acquire

reactive protein peroxide groups immediately on exposure of the radical (Du and Gebicki 2004). This is unlikely to be the case in our study as the first protein hydroperoxide formed were not measure before 6 hour of treatment. The possibility of the formation of lipid hydroperoxides during this lag phase has been ruled out because AAPH was shown to induce protein and not lipid peroxidation in U937 cells. With Sp2/0 mouse myeloma cells exposed to gamma irradiation generated $HO^{\bullet}$ radicals, no lipid peroxides were detected (Du and Gebicki 2004).

Differences in response to oxidative stress between U937 and THP-1 cell lines have been previously shown (Baird, Hampton et al. 2004). U937 cells exhibit a lower level of glutathione expression compared to the THP-1 cells (Ferret, Soum et al. 2000), and this difference could explain why a lag phase in protein hydroperoxide formation is present for the THP-1 cells, but supposedly to be absent for the U937 cells. This statement support our finding that we present in section 4.2.2 where the direct and rapid decrease of glutathione measure is thought to be the cause of the protein hydroperoxide formation lag phase. Glutathione is one of the main intracellular antioxidant and may have initially protected THP-1 cells against the formation of protein hydroperoxides. Other factors, including radical acceptors and redox enzymes, could also participate in this lag phase. For instance the thioredoxin system has been thought to play a major role as a buffering mechanism which maintains the redox status of the cell (Holmgren 1979; Iwata, Hori et al. 1997). The importance of thioredoxin and other antioxidants in THP-1 cells may provide further insight to the development of the lag phase. Alteration of GSH levels may also provide further insight (see section 4.2.2).

After the lag phase, a linear increase in protein hydroperoxide was observed, reaching a final concentration of 1.3 μM after 24 hours. Cells incubated in the absence of AAPH showed no significant increase in protein hydroperoxide, indicating that an oxidative stress is normally needed in order to generate protein hydroperoxides in THP-1 cells.

4.2.1 AAPH induced decrease in intracellular GSH

The initial concentration of intracellular glutathione in the THP-1 cells was measured at 4.7 μM (Fig 3.2.4). After only 6 hours of incubation with AAPH, glutathione levels dropped to half, with no glutathione remaining after 24 hours. The cellular thiol status is of great importance among antioxidant defence systems. Under oxidative challenge, peptide thiols like glutathione, can participate as reducing buffers, leading to a decrease in protein thiols (protein-SH) or an increase in the levels of protein-mixed disulfide

(PSSG). Another general response elicited by ROS is the activation of glutathione synthesis, which enhances the cellular antioxidant capacity (Dafre, Medeiros et al. 2004; Dickinson and Forman 2002; Dickinson and Forman 2002). Reduced glutathione (GSH) is known to provide a major intracellular defence against oxidative injury, by serving as a cofactor for glutathione peroxidases, which detoxify hydrogen peroxide and lipid peroxides (Meister and Anderson 1983; Meister 1994). Depletion of intracellular glutathione has been shown to increase susceptibility in rodent and human cells to the damaging effects of oxidizing species like nitric oxide ($NO^{\bullet}$) (Luperchio, Tamir et al. 1996). Furthermore inhibition of glutathione synthesis in human monocytes has been shown to increase the toxicity of oxidised low density lipoprotein (LDL) towards these cells (Gotoh, Graham et al. 1993). The loss of glutathione can even be the cause of oxidative stress by altering the reducing potential of cells (Ghibelli, Coppola et al. 1995).

Upon oxidative stress, GSSG may either recycle to GSH or exit from the cells, leading to overall glutathione depletion (Reed 1990). The significant glutathione depletion in cells treated with AAPH suggests that glutathione is not being synthesized *de novo* and/or recycled to compensate for its shift to the oxidized form. Some studies suggest that glutathione can only be resupplied to the cells at a limited rate (Luperchio, Tamir et al. 1996). In our experiments, the cells were constantly incubated with AAPH for a period of 24 hours therefore the damage to the cell became permanent and became stronger over time as more protein hydroperoxides form. The continued presence of AAPH appear to make it impossible for the cells to recycle its glutathione, leading to a loss in cellular viability (Pascoe and Reed 1987; Salgo, Squadrito et al. 1995) as measured in the present study by the MTT assay (Section 3.2.4A).

In the future it would be interesting to measure the glutathione oxidation product. Measuring the GSH/GSSG ratio and GSSR would allow us to understand the mechanism of intracellular GSH loss when the cell is attacked by AAPH. It would also be interesting to see if the cells could recover from the glutathione loss by removing the AAPH after few hours of incubation.

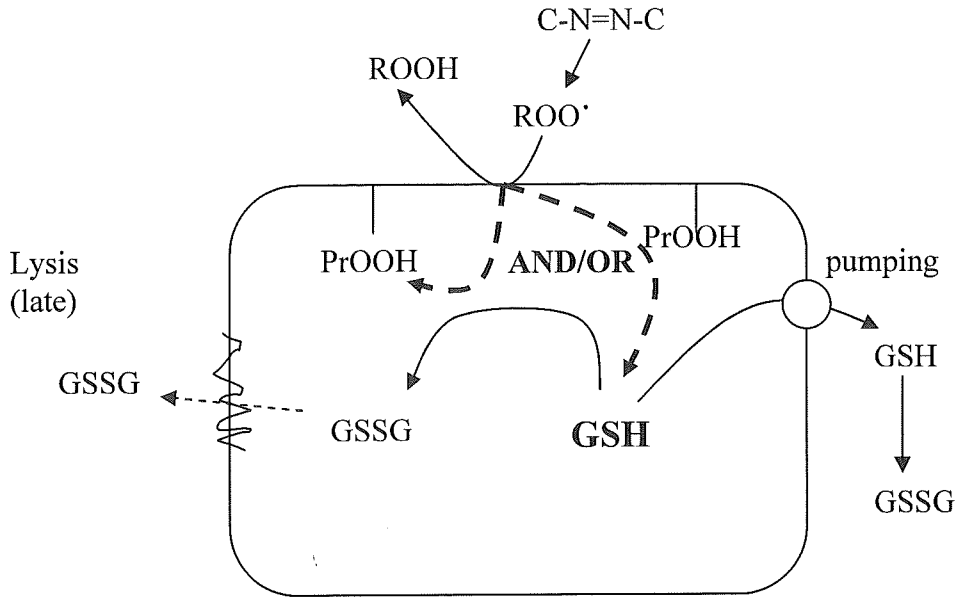


Figure 4.2.2 Possible mechanism of GSH depletion in THP-1 incubated with AAPH

In the future, taking samples of glutathione between 9 and 24 hours would allowed to make a more complete analysis of the correlation between protein hydroperoxide formation and the loss of glutathione. Furthermore the glutathione levels could also be artificially altered with N-Acetyl-Cysteine (NAC) or Buthionine Sulfoximine (BSO) during AAPH treatment. Different concentrations of AAPH could be applied to the cells to observe correlation between the lag phase of protein hydroperoxide formation and the loss of glutathione.

A decrease in intracellular glutathione was also observed with cells in the absence of AAPH (Fig 3.2.4), where half of the initial concentration (4.7 μM) was observed after 24 hours of incubation. Given that the cells are incubated in only a minimal media for the course of the experiment, it is to be expected that they would be under a certain amount of stress, which would explain the decrease in the main antioxidant of the cell.

4.2.2 Difference between total thiol and GSH loss in THP-1 cells

A number of studies have shown that ROS can cause depletion in total cellular thiol levels (Kennedy, Matsumoto et al. 1999; Baird, Hampton et al. 2004) and more specifically, depletion of glutathione levels (Luperchio, Tamir et al. 1996 ; Carr and

Winterbourn 1997). In our study we were interested in examining the effects of ROS on both total thiol and intracellular glutathione.

In the study of Carr & Winterbourn the oxidative stress induced by HOCl on neutrophils provoked a proportional decrease in both glutathione and protein thiols (Carr and Winterbourn 1997). This contrast with our work, as THP-1 cells treated with AAPH resulted in a sudden decrease in glutathione level (Fig 3.2.4) although the depletion in total thiols did not begin until after 9 hours of AAPH treatment (Fig 3.2.2). Total thiols in THP-1 cells therefore do not seem to have a similar protector effect here which could be due to the oxidants being different and involving a different cell defence mechanism. They also found that level of protein thiols initially present in the neutrophils is approximately 5-fold the glutathione content (Carr and Winterbourn 1997). However, we measured the level of total thiol (mainly protein thiol, Fig 3.2.3) initially present in the THP-1 cells to be 10-fold the glutathione content (Fig 3.2.3). The level of total thiols and non protein thiols (GSH) has also been measured in muscle tissue of the rat and the level of non protein thiol was less than 10% of the total thiol content (Cakatay, Telci et al. 2003). In support of this variability between studies, other literature indicates that the levels of reduced and oxidized thiols, particularly GSH, GSSG and GSSR, in cells grown in culture are highly cell-line specific and also dependent upon culture condition and the cell cycle (Luperchio, Tamir et al. 1996 ; Atzori, Dypbukt et al. 1994; Allalunis-Turner, Lee et al. 1988). It is therefore not completely possible to compare our results and the level of thiols with studies using different cells.

4.2.3 AAPH induced total thiol decrease in THP-1 cells

The presence of protein thiol groups has been demonstrated to protect BSA against peroxyl radical-mediated peroxidation, and the reason of this was attributed to the protein thiols ability to scavenge peroxyl radicals (Platt and Gieseg 2003). As it has been shown above (Fig 3.2.3), protein thiols represent more than 90% of the total thiols, we could talk about protein thiols when presenting the total thiols results. Total thiols in the cells both with and without AAPH decreased during the first 3 hours but subsequently increased by the next time point, however still lower than the time 0 for the cells with AAPH. The noticeable increase in the control cells between 3 and 6 hours could be due to the cell's new environment in a minimal media with no amino acids or nutrients, where they tried to compensate for the change from RPMI 1640 to EBSS by

producing more thiols. This increase was consistently observed in repeats of this experiment (n=3).

The possible protective mechanisms of thiols toward ROS include scavenging and/or detoxifing ROS, blocking their production, or sequestering the transition metals that are the source of free electrons (Halliwell and Gutteridge 1999; Halliwell and Gutteridge 1990). In our study, the total thiols of the THP-1 cells treated with AAPH did not seem to scavenge the radicals formed. The kinetics for total thiols shows a decrease after 9 hours, which is more than 3 hours after the formation of protein hydroperoxide starts (Fig 3.2.2). The decrease in glutathione in addition to cause the lag phase in protein hydroperoxide formation, could also maintain the redox balance of the cell. Then, once a small concentration of glutathione is left, the protein thiols start to be oxidised, and the redox balance of the cell is lost (Luperchio, Tamir et al. 1996). Nevertheless, GSH seems to play the major role in protecting the cell from radical attack because the formation of protein hydroperoxides starts before the decrease in protein thiols begins.

A difference of initial total thiol concentration is observed between Fig 3.2.2 and Fig 3.2.3. The explanation could be that the cells used for Fig 3.2.3 were taken straight from the growth media (RPMI 1640) whereas the other cells (Fig 3.2.2) were taken from the experimental media (EBSS). It is possible that some thiols from the growth media, which contains free amino acids, may have remained attached to the cells after the washing steps and been measured by the DTNB assay. However more washing step were made for the experiments in EBSS, and even if the same amounts of cells was counted, the washing process could have induced a lost of cells which is seen by this lower value.

4.2.4 AAPH induced loss in cell viability

Since the aim of this study was to understand the mechanism and kinetics of cell death when attacked by ROS, measuring cell viability and determining the mode of cell death was the key part of this research.

Cell viability was monitored by the MTT assay which has been extensively used in our laboratory. The assay gauges the overall viability of the cell by measuring the cellular metabolic activity of NADH/NADPH dehydrogenases (section 2.2.8). The MTT assay was chosen for this work as previous studies in our laboratory have shown it to be more sensitive than the lactate dehydrogenase (LDH) assay or the trypan blue exclusion assay (Duggan 2000).

Incubation of the THP-1 cells with AAPH induced a complete lost of cell viability after 24 hours. The cells are unable to repair the oxidative damage due to the decrease in GSH and total thiols. This suggests that cell death resulted from loss of the redox balance in the THP-1 cells. The concentration of AAPH used (10 mM) is very high compared to *in vivo* measurements of H_2O_2, which are kept at low micromolar concentrations in human blood plasma (Halliwell and Gutteridge 1990). However, when killing of bacteria and at sites of inflammation there is an increase in the consumption of oxygen, which is used in the production of H_2O_2. This increase in ROS would occur in the immediate vicinity of the cell and would likely be of a high concentration. At 37°C, AAPH decompose to form peroxyl radicals at a rate of 3.19×10^{-7} $M.s^{-1}$ as a result of the carbon-centred radicals reacting with oxygen (reaction 9) (Niki 1990). In our study, 10mM AAPH will generate 11.48 $\mu M.h^{-1}$. Therefore the high concentration in AAPH may be physiologically relevant in this study.

The thiol redox status in the cell is known to be an important determinant for the state of the cell viability. Some studies have shown that the loss of cell viability is closely correlated with the ROS-induced depletion of cellular thiol levels (Salgo, Squadrito et al. 1995). In our study the THP-1 cell metabolism decreases before the level of total thiols starts to decrease. The viability of THP-1 cells seems more related to glutathione depletion than to loss of total thiols. A strong correlation is also observed between the start of the decrease in cell viability and the start of protein hydroperoxide formation. It suggests that protein hydroperoxides may be toxic to the cell by reducing intracellular thiols level resulting in the inhibition of the Na/K ATPase (Kurella, Tyulina et al. 1999; Chen, Zhang et al. 1992).

4.2.5 AAPH induced necrosis to THP-1 cells

Studies by Li, Kondo *et al.* have shown that AAPH and hyperthermia could induce apoptosis measure by PS externalization in U937 cells (Li, Kondo et al. 2001). In this study we were interested in observing the effect of AAPH on cell death at a normal physiological temperature of 37°C, and with the use of THP-1 cells.

To observe the type of cell death induced by AAPH, caspase-3 activity was initially monitored. Caspase-3 is an inducer of apoptosis and, as such, is a suitable marker for apoptosis. When THP-1 cells were treated with AAPH, a very low caspase-3 activity baseline was observed over the time. This baseline was similar for both the cells treated with AAPH and the control cells. Caspase-3 was therefore not present in THP-1 cells

during AAPH-induced oxidative stress. This lack of caspase-3 activity was not due to a problem with the assay because activation was observed with the positive control. Six percent of alcohol incubated with THP-1 cells for 3 hours induces caspase-3 activity. These results suggest that the THP-1 cell death induced by AAPH was either caspase independent apoptosis or necrosis.

In this study, we hypothesize that the oxidative environment created by glutathione loss may produce changes in enzymatic activities such as protease activation. Since protease activation is crucial for triggering apoptosis, these changes will result in the loss of all background caspase activity in the THP-1 cells. Furthermore, the decrease in caspase activity has been observed in cells placed under oxidative stress, consistent with the observation that the active site cysteine of the caspase has to be reduced to be active (Hampton, Fadeel et al. 1998; Hampton and Orrenius 1997). The loss of thiols could have therefore caused the rapid loss of caspase activity. The presence of caspase activation alone is often considered to be definitive evidence of apoptosis, since their cleavage of structural and regulatory proteins is responsible for the irreversible dismantling of the cell. However there is a growing awareness that apoptosis can proceed in the absence of caspase activation, and there can be differences in the quantification of apoptosis depending on the parameter being assessed (Asmis and Begley 2003). This often makes it difficult to compare different studies when only a limited number of apoptosis markers have been measured (Baird, Hampton et al. 2004).

Morphological observations suggested that the cells could be dying by necrosis. However, making conclusion about the type of cell death based solely on morphology is inadvisable. Despite the use of typical morphological features that characterize apoptosis, a "universal morphology of apoptosis" does not exist (Bonanno, Ruzittu et al. 2000).

Phosphatidylserine (PS) exposure and propidium iodide (PI) uptake were monitored to further determine the type of cell death induced by AAPH. Phosphatidylserine is present in the cell membrane but during apoptosis it is flipped over and exposed to the outside of the cell, while PI is a dye (like trypan blue) that enters cells with damaged cell membranes and incorporates itself into the DNA. The fact that PI uptake was increased but no PS exposure was observed indicates that necrosis is the type of cell death induced by AAPH.

Often necrosis is reported to follow apoptosis if the incubation is carried out for long enough, or if there are insufficient quantities of other cell to engulf the apoptotic cells

and clear debris (Fadeel and Kagan 2003). However, no sign of apoptosis was detected at any time up to 24 hours in this study. Necrosis is therefore the main form of cell death induced in THP-1 cells incubated with AAPH at 37°C.

4.3 Summary

The focus of this work was to identify the sequence of events at various intracellular sites for indications of damage which will ultimately lead to cell death when the monocytic THP-1 cell line was incubated with the water-soluble radical generator AAPH.

With the results obtained in our study, glutathione seem to be the major antioxidant able to block the production of protein hydroperoxides in the THP-1 cells. Interestingly, protein thiols did not appear to be efficient in preventing the formation of protein hydroperoxides. Glutathione could have protected the protein thiols from oxidation by keeping the thiols in a reduced state. However once most of the glutathione is depleted, the loss of the total thiols appears to start. More research on glutathione depletion need to be carried out in order to make a firm conclusion.

The THP-1 cell death appears to be due to the loss of redox status in the cell. The morphological examination and the flow cytometry analysis shown that necrosis in THP-1 cells is the main form of cell death induced by AAPH. Caspase activity may have been lost due to AAPH oxidation of the caspases resulting in a necrotic form of cell death.

High oxidative stress induced by the decomposition of AAPH to peroxyl radicals, shows that the THP-1 cells cannot maintain a redox status after 6 hours. This stress leads to the induction of necrosis in THP-1 cells.

The study of the effects of AAPH on the U937 cells remains unsuccessfully due to a possible presence of intracellular contamination, resulting in an increase of protein hydroperoxide formation in the control cells.

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