Modulation of intracellular GSH in THP-1 cells during oxidative stress induced by AAPH.

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Abbreviations

$^{\cdot}OH$	hydroxyl radical
AAPH	2,2'-azobis(2-methyl-propionamidine) dihydrochloride
Apaf-1	apoptotic protease activating factor
ATO	adenosine triphosphate
BSA	bovine serum albumin
BSO	Buthionine Sulfoximine
CO_2	cabon dioxide
Cu^+	cuprous ion
Cu^{2+}	cupric ion
DNA	deoxyribonucleic acid
e-	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
GuHCl	Guanidine hydrochloride
HI-FCS	heat inactivated foetal calf serum
H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
HOCl	hypochlorite
HPLC	high performance liquid chromatography
MPO	myeloperoxidase
MBB	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
$O_2^{\cdot -}$	superoxide
PBS	phosphate buffered saline
PCA	perchloric acid
PI	propidium iodide
PS	phosphatidyl serine
$Pr^{\cdot}$	protein radical
PrH	protein
$PrO^{\cdot}$	protein alkoxyl radical
$PrOO^{\cdot}$	protein peroxyl radical
PrOOH	protein hydroperoxide
$R^{\cdot}$	radical
Redox	reduction-oxidation
R-N=N-R	azo compound
R-SS-R	disulphides
$ROO^{\cdot}$	carbon centered peroxyl radical
ROOH	carbon centered hydroperoxide
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SDS	sodium lauryl sulphate
SOD	superoxide dismutase
TCA	trichloroacetic acid
XO	Xylenol Orange

Abstract

The human monocyte-derived THP-1 cell line was incubated with 10mM AAPH in Earle's Balanced Salt Solution at 37°C for up to 24 hours. Protein hydroperoxide formation occurred after an 8 hour lag phase which corresponded to glutathione loss observed in the cells. SDS-Page analysis confirmed protein degradation occurred after 6 hours. Cell viability measured by the MTT reduction assay also dropped after 8 hours. Reduction of intracellular glutathione levels using BSO caused reduction of the lag phase seen in protein hydroperoxide formation. Cell viability of BSO-treated cells was lower than control cells, indicating the initiation of apoptotic events. Flow cytometry analysis showed no difference between BSO-treated and control cells, indicating that GSH levels do not have an effect on the type of cell death observed in AAPH-induced oxidative damage on THP-1 cells. These results confirmed previous data in the lab suggesting THP-1 cells undergo AAPH-induced necrosis as a result of cellular damage, including the loss of GSH and the formation of protein hydroperoxides.

1 Introduction

1.1 Overview

Free radicals have been widely studied in biology, with a heavy focus on the role they play in oxidative stress. In animal cells, free radical production is a result of either accidental or deliberate generation. How the body's defence systems then control these molecules plays a significant factor in the outcome of the initial reaction (Berg, Youdim et al. 2004). Many age-related diseases such as Alzheimer's, arthritis, heart disease and cancer are the result of poorly controlled free radical reactions at a specific site. Due to the increasing frequency of these diseases throughout society, a lot of funding worldwide is being directed into research that focuses on the initial phases of disease progression.

Of all the major classes of biomolecules that may be attacked by free radicals, research has shown that proteins are the most susceptible (Gebicki 1997). This is due to the high ratio of proteins on the surface of the cell membrane- they comprise around 50% total mass of the cell membrane (Alberts, B. et al. 2002; Gebicki, J. 1997). This reaction results in the formation of protein hydroperoxides (PrOOH), which can upset the maintenance of redox balance in cells controlled by antioxidants. When this occurs, the oxidative stress becomes too intense and cell death occurs. One of the most important antioxidants involved in the neutralisation of free radicals is glutathione (GSH), and it has been shown to react with effective efficiency against PrOOH molecules (Halliwell & Gutteridge 1999).

This study examined the effect of AAPH on the monocyte-like cell line THP-1. The formation of PrOOH and progression of cell death was monitored when GSH levels are altered from normal basal levels.

1.2 Free radicals

A free radical is a chemical species possessing an unpaired electron(s), which is capable of existing on its own (Halliwell & Gutteridge 1999). There are three ways free radicals can be formed: (i) by the homolytic cleavage of a covalent bond- a process generally incurred by high energy input, eg: high temperature, UV light or ionising radiation; (ii) by the loss of a single electron from a normal molecule; (iii) by the addition of a single electron to a normal molecule (Cheeseman & Slater 1993).

Electron transfer (iii) is the most common mode of free radical formation in biological systems.

Oxygen-derived free radical products are considered highly important in free radical biochemistry. The two unpaired electrons in the molecule enable it to react quickly and easily with free radicals, either accepting or donating these electrons when in close proximity. These reactions are found in many biological pathways, including the process known as respiratory burst, which results in free radical products termed Reactive Oxygen Species (ROS) (Winrow, Winyard et al. 1993; Halliwell & Gutteridge 1999). Examples of ROS include hydrogen peroxide and the non-oxygen free radical hypochlorite, which is produced by activated phagocytes (Berg, Youdim et al. 2004). The most abundant ROS is the superoxide anion. The major source of these free radicals is from modest leakage originating at the electron transport chains of mitochondria, chloroplasts and the endoplasmic reticulum (Dean, Fu et al. 1997). Superoxide is formed by the single electron reduction of oxygen:

$$(1)\ O_2 + e^- \rightarrow O_2^{-\cdot}$$
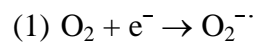
A two-electron reduction forms hydrogen peroxide:

$$(2)\ O_2 + 2e^-\ 2H^+ \rightarrow H_2O_2$$
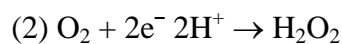
Under acidic pH, or with the help of the enzyme Superoxide Dismutase (SOD), hydrogen peroxide can also be formed by reaction of two superoxide molecules:

$$(3)\ 2O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$$

McCord and Friovich in the 1960's recognised SOD as the first example of an enzyme that uses a free radical as a substrate, catalysing the dismutation of $O_2^{-\cdot}$ into H_2O_2 (Shinobu & Flint Beal 1998). The H_2O_2 formed is a non-radical product, and its formation in the method above is known as a dismutation reaction (Cheeseman & Slater, 1993). Although superoxide is reactive, it has only a limited damaging effect in itself on biological systems. Rather, it is its ability to form H_2O_2 by the dismutation reaction (reaction 3) that is important in biochemistry, for it is this product that acts a damaging oxidising agent (Barber & Harris 1994) due to its ability to diffuse across cell membranes. In this way, damage can be caused at sites other than the initial area that the superoxide was produced (Halliwell & Gutteridge 1999).

Hydrogen Peroxide is also important in biochemistry because it is able to react with transition metals ions to produce the most reactive of the oxygen free radicals, the hydroxyl radical ($\cdot OH$):

$$(4)\ H_2O_2 + Fe^{2+} \rightarrow \ ^\cdot OH + OH^- + Fe^{3+}$$

$$(5)\ H_2O_2 + Cu^+ \rightarrow \ ^\cdot OH + OH^- + Cu^{2+}$$
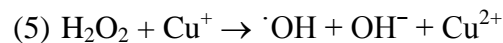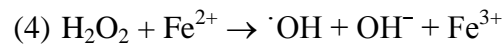
This reaction is referred to as the Fenton equation. It uses the reactivity of the unoxidised transition metals ferrous (Fe^{2+}) iron and cuprous (Cu^+) copper to form a $^\cdot OH$ radical. The non-catalysed formation of $^\cdot OH$, i.e. without the oxidation of transition metals, is less likely in biology due to the low steady-state concentrations of the reactants (Cheeseman & Slater, 1993). The reactivity of the $^\cdot OH$ molecule is reflective of its very short half-life of 10^{-9} seconds at body temperature, 37°C (Gebicki 1997; Nohl 1993). As it is able to react with any biomolecule within a 6nm vicinity, the need to control its formation is clearly evident, especially within a biological context.

When free radicals are produced deliberately in biological systems, it occurs in a constrained environment, at a targeted site in order to reduce the risk of electrons leaking. Activated phagocytes produce $O_2^{\cdot -}$ in order to destroy bacterial cells. The reaction is targeted to the interface of the phagocyte plasma membrane and the bacterium (Cheeseman & Slater, 1993). H_2O_2 plays an important role in the immune response, where white blood cells use the catalytic properties of NADPH oxidase to form the ROS:

$$(6)\ O_2 + NADPH \rightarrow O_2^{\cdot -} + NADP^+ + 2H^+ \rightarrow H_2O_2$$
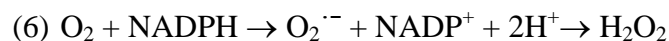
This product can then be used by neutrophils to form the very reactive HOCl species (Daugherty, Dunn et al. 1994), which is used to combat microbial invasion. It requires the catalytic activity of myeloperoxidase (MPO):

$$(7)\ H_2O_2 + Cl^- \rightarrow HOCL + OH^-$$
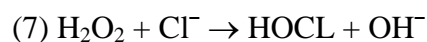
Some cells release Nitric Oxide ($NO^\cdot$), which is recognised as an endothelial-derived relaxing factor. Used throughout biological systems as an intracellular messenger, its role is to control blood flow by regulating smooth muscle contraction (Alberts 2002), thrombosis and neural activity (Palmer, Ferrige et al. 1987; Beckman and Koppenol 1996). It is a remarkable example of a signalling molecule because it can diffuse through most cells and tissues with little consumption or direct reaction (Beckman and Koppenol 1996). It is seen in both plants and animals, and is a gas so it has the ability to cross the plasma membrane, unlike many other signalling molecules that are too large to do so (Alberts 2002). $NO^\cdot$ can react with superoxide to form peroxynitrite

(ONOO˙), a strong oxidising agent with a comparatively long half-life that can attack protein molecules and cause tissue injury (Ischiropoulos & al-Mehdi 1995).

1.3 Effect of oxidative stress on cellular components

From the perspective of understanding free radical reaction mechanisms, one of the most important events to identify is the initial reaction targeting the primary cellular components by Reactive Oxygen Species (ROS). The most damaging ROS is the ˙OH radical, as it does not discriminate between organic targets; it reacts with the most abundant cell constituent (Gebicki 1997). Data in previous research has shown that according to this argument, the most likely cell target would be the proteins (Davies & Dean 1997). Due to the importance of proteins in a biological context, this project focuses on this area.

1.3.1 Protein damage

For many years, the belief was held that the lipid bilayer of cell membranes was the main target for oxidative attack. Publications exhibiting the ability of lipids to undergo self-perpetuating chain reactions added further research interest into the area, overshadowing the study of protein oxidation. The more difficult processes involved in order to prove proteins were oxidising gave little prospect for providing publishable results. However, Gieseg et al. published the first report of the formation of protein hydroperoxides in living cells attacked by reactive oxygen species in 2000. Using the FOX assay on the monocyte-like cell line U937, the group showed that reactive hydroperoxide groups can and do form in high yields on a wide range of proteins exposed to a wide range of ROS. Gebicki also showed that protein hydroperoxides readily form in lipoproteins, blood serum and mouse myeloma cells (Gebicki 2000). The theory that proteins undergo oxidation before other cell components has come about by the statistical analysis of cell components. Excluding water; which is non-reactive, proteins constitute 75% of the combined masses of potential initial molecular targets in eukaryotic cells (Du & Gebicki 2004).

The mechanism of ROS formation generated by protein oxidation has been studied with great interest, and has resulted in the general understanding; that the reaction is driven in a non-random fashion using transition metals complexed in a redox active form (Gebicki 1997). The protein-metal complex acts as an effective chelating agent,

enabling site-specific production of ROS from hydrogen peroxide and superoxide (Halliwell & Gutteridge 1999; Gebicki 1997). These damaging products, such as $\cdot$OH and $-OO\cdot$ radicals, have been proven to cause amino acid oxidation, decarboxylation, deamination and cross-linking (Dean, Gieseg et al. 1993; Dean, Fu et al. 1997). Downstream effects of these reactions can affect many cell processes that proteins are involved in, including receptor function, enzymatic properties, signal transduction, and transport systems. Damaged proteins can also clump together, causing an effect not only on the aforementioned cell functions, but also providing a component mechanism for age-related pathological diseases. These include Alzheimer's, atherosclerosis, Parkinson's, cataractogenesis, cystic fibrosis, muscular dystrophy, rheumatoid arthritis and Werner's syndrome (Berlett & Stadtman 1997; Fu, Dean et al. 1998).

1.3.2 Mechanism of Protein hydroperoxide formation

Oxidative attack of the polypeptide backbone is initiated by the subtraction of an $H\cdot$ of a protein by a radical species ($R\cdot$) to form a carbon-centred radical ($Pr\cdot$) (equation 8). This carbon-centred radical formed can then react with O_2 to form a protein peroxyl radical intermediate ($PrOO\cdot$) (equation 9), giving rise to the protein peroxide ($PrOOH$) (equation 10). Protein-protein cross-linking may occur if the $PrOO\cdot$ reacts with another carbon-centred radical (equation 11) (Berlett & Stadtman 1997).

$$(8)\ PrH + R\cdot \rightarrow Pr\cdot + RH$$
$$(9)\ Pr\cdot + O2 \rightarrow PrOO\cdot$$
$$(10)\ PrOO\cdot + e^- + H^+ \rightarrow PrOOH$$
$$(11)\ PrOO\cdot + R'H \rightarrow PrOOH + R'\cdot$$
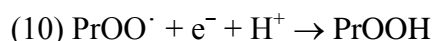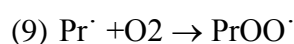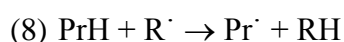
The common fate of most organic hydroperoxides is decomposition, usually involving redox reactive transition metals (equations 12 and 13). These transition metals are often bound at specific sites on macromolecular structures, so hydroxyl radical generation is essentially causing site-specific damage on the molecules (van Dyke & Saltman 1996).

$$(12)\ PrOOH + Fe^{2+} \rightarrow RO\cdot + Fe^{3+} + HO^-$$
$$(13)\ PrOOH + Fe^{3+} \rightarrow PrOO\cdot + Fe^{2+} + H^+$$
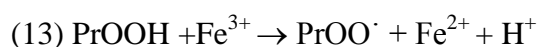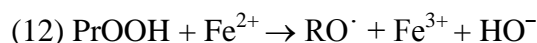
These alkoxyl and peroxyl products are usually unstable and react with other molecules, initiating new chain reactions. In effect, the protein hydroperoxides are

acting as a ROS, giving evidence to the argument that protein oxidation is an important and highly significant factor of oxidative stress (Gebicki 1997).

1.3.3 Peroxyl radical generation using AAPH

The water-soluble azo compound 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) has been widely used as a temperature-dependant free radical generator (Li, Kondo et al. 2003). Its effects have been studied on isolated protein (Gebicki & Gebicki 1993), red blood cell ghosts (Firth 2001), low density lipoprotein (Pearson 2002), and in human monocytic leukaemia U937 cells (Gieseg, Duggan et al. 2000; Li & Kondo et al. 2001).

When in the presence of oxygen and at a temperature of 37°C, AAPH forms peroxyl radicals at a rate of 3.19×10^{-7} M.s^{-1} (Niki 1990). When the carbon-centred radical reacts with adjacent proteins, a protein radical (Pr$^{\cdot}$) is generated; this in turn forms protein hydroperoxides seen previously in equations 10 and 11 (Ma, Chao et al. 1999).

$$(14)\ R\text{-}N\text{=}N\text{-}R \rightarrow 2R^{\cdot} + N_2$$
$$(15)\ R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$$
$$(16)\ ROO^{\cdot} + PrH \rightarrow Pr^{\cdot} + ROOH$$
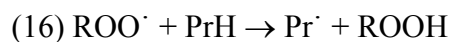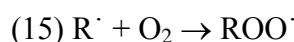
1.4 Cellular defence against free radicals

When biomolecules are damaged within a cell, it is important that the cells detoxifying and antioxidant defence systems work without interference from damaged molecules in order to maintain overall redox balance (Filomeni, Rotilio et al. 2003). ROS production is associated with detoxification, and antioxidants can be upregulated by external stimuli in order to increase the amount of defence molecules within the cell, reducing as much damage as possible (Dafre, Medeiros et al. 2004). Antioxidants are compounds that provide protection against the harmful effects of damaging free radicals and other ROS. They are able to react with free radicals at low concentration to form harmless, non-reactive molecules (Halliwell & Gutteridge 1990). There are two antioxidant defence systems; enzymatic and non-enzymatic components, which contribute to the homeostasis of the intracellular redox state (Kim et al. 2004). Antioxidants are either reactive chemicals such as α-tocopherol, ascorbic acid and glutathione; or specialised enzymes such as superoxide dismutase (SOD), glutathione

peroxidase and catalase. These scavenging molecules are chemicals that at low concentrations can protect biomolecules from oxidative damage by reacting with and neutralising free radicals. The neutralising effect on free radicals is caused by: the possession of a ring structure (α-tocopherol, ascorbic acid) or stabilisation of the unpaired electron using a metal-based active site (SOD, catalase, glutathione peroxidase) that is able to delocalise electrons. Even though the antioxidant chemical will then possess an unpaired electron, it lacks enough energy to form further radicals. As we age, the effectiveness of our biological protective systems appears to decrease, resulting in the onset of many age-related diseases. It is widely believed that a dietary increase of antioxidant chemicals will show down the effects of oxidative attack (Gebicki 2000). Proving this theory is difficult due to the extensive length of time involved in developing pathological symptoms associated with these diseases.

1.4.1 Glutathione

Glutathione is a ubiquitous tripeptide, γ-glutamylcysteinylglycine (Kim et al. 2004). It plays several important roles in biological systems, including catabolism of H_2O_2 and other peroxides through an enzymatic coupling reaction, detoxifying electrophiles, and protecting the thiol groups of proteins from oxidation (Wang & Ballatori 1998; Lu 1999; Dobashi, Aihara et al. 2001). Glutathione is the most abundant antioxidant in the cell, with a high intracellular concentration in the mM range (James, Slikker et al. 2005). It is found predominantly in its two redox forms: reduced (GSH) and oxidised (GSSG) depending on the redox status of the cell (Ghibelli, Fanelli et al. 1998; Filomeni, Rotilio et al. 2003). Its protective effect against free radical attack is based on the ability to oxidise its thiol group within the cysteine residue, forming GSSG. This can be catalytically changed back into its reduced form (GSH) by glutathione reductase (Figure 1.4.1) (Meister & Anderson 1983).

NADPH + H$^+$ **NADP$^+$**

Glutathione Reductase

GSSG   **2GSH**
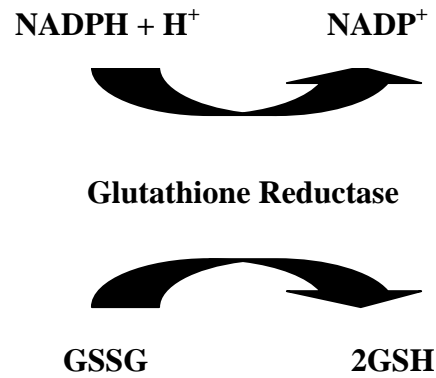
Figure 1.4.1 The catalytic reduction of glutathione (Garrett & Grisham 1999).

Glutathione is synthesised from amino acids in two phases. Glutamate and cysteine form γ-glutamylcysteine by the catalytic reaction involving γ-glutamylcysteine synthetase. This product is then combined with glycine by an enzymatic reaction with glutathione synthetase to form the tripeptide γ-glutamylcysteinylglycine, better known as GSH (Figure 1.4.2).

γ-glutamylcysteine synthetase

Glutamate

Cysteine

γ-glutamyl cysteine

Glycine

glutathione synthetase

GSH
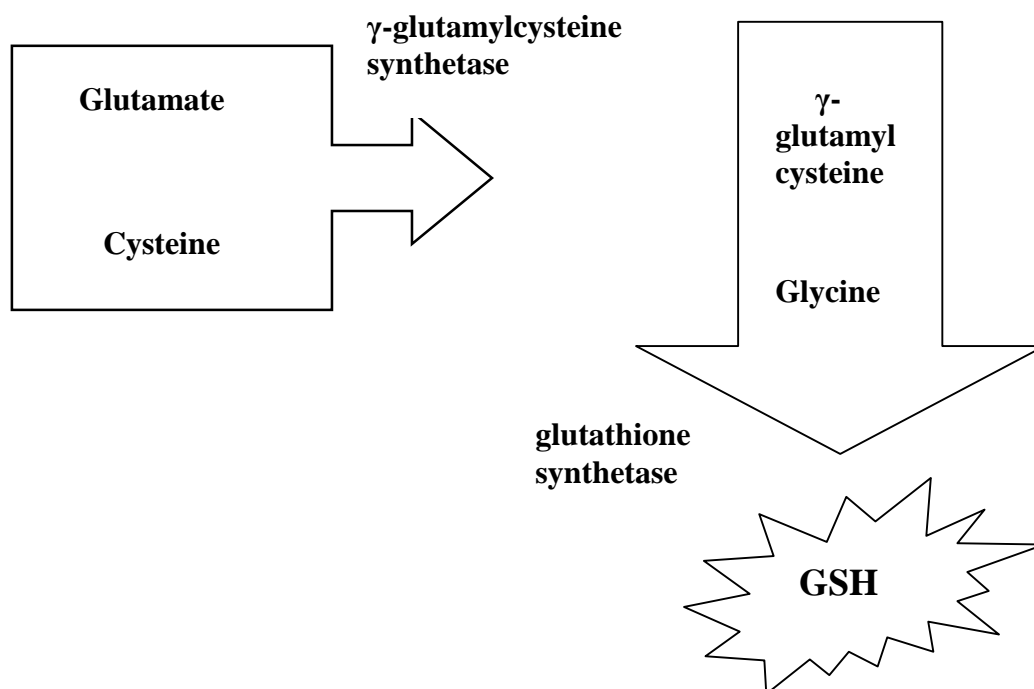
Figure 1.4.2 Glutathione synthesis from its amino acid building blocks (Luperchio, Tamir et al. 1996).

The role of GSH is to maintain the overall redox potential of the cell, essentially acting as a redox buffer system by virtue of its thiol group (Hwang, Sinskey et al. 1992). It acts as a detoxification molecule by reducing disulfides (R-SS-R), and as a

messenger molecule through chain reactions (Sies 1999; Cotgreave & Gerdes 1998; Arrigo 1999). As sulphur-containing amino acid residues are particularly susceptible to oxidation, it is important that these residues be converted back to their unoxidised form in order to prevent major disruption to protein function. GSH is essentially acting as a "built-in" ROS scavenger system to protect proteins from more extensive, irreversible damage (Berlett & Stadtman 1997; Simpson, Narita et al. 1992).

The behaviour GSH/GSSG cycling in apoptosis is a widely debated topic. The major question focused on in this area is whether GSH loss occurs prior or concomitantly to apoptotic signalling. Upon oxidative stress, GSSG may recycle back into GSH (figure1.4.1) or it may exit from the cell via specific efflux transporters, leading to overall glutathione depletion (Reed 1990). Ghibelli et al. have shown that in U937 cells, active extrusion of GSH could favour the onset of apoptosis by passively allowing oxidative stress to take place, i.e. GSH loss can trigger the apoptotic signalling cascade (Ghibelli, Fanelli et al. 1998). On the other hand, van den Dobblesteen et al. using Human Jurkat T lymphocyctes observed that cells already undergoing the apoptotic signalling cascade became dramatically depleted of GSH 30 minutes after exposure to anti-FAS/APO-1 antibody, signifying that GSH loss occurs concomitantly with apoptosis (van den Dobblesteen, Stefan et al. 1996).

1.5 Mechanisms of cell death

1.5.1 Apoptosis

Apoptosis, or programmed cell death is a normal cell response to oxidative stress, associated with the generation of ROS (Cai & Jones 1998; Alberts, Johnson et al. 2002). The process is required for both normal physiological development of biological systems, and in a wide range of diseases including cancer and Human Immunodeficiency Virus (HIV). Apoptosis is a highly regulated process which is accomplished by specialised complex cellular machinery including several proteases, such as caspases. It is characterised by proteolysis, chromatin condensation, nuclear and DNA fragmentation (Plantin-Carrenard, Bernard et al. 2005). The process differs from necrosis as it may occur in a single cell surrounded by a group of viable cells, whereas necrosis involves death of a group of cells simultaneously.

There are three major sub-classes of apoptosis, used to describe the chronological order of both biochemical and morphological modifications the apoptotic cell (Leist & Nicotera 1997). The initial "induction" phase involves signalling actions. Biochemical changes are proceeding but morphological alterations are minimal. In the second "execution" phase, apoptotic molecular machinery is activated and the cell is committed to death. This phase is visually characterised by the condensation of chromatin within the cell. Finally, visible signs of apoptosis appear, including condensation and margination of nuclear chromatin, DNA fragmentation, and the cell condenses while preserving its organelles (Wylie, Kerr et al. 1980, Abbro, Lanubile et al. 2004).

1.5.2 Caspase-dependent apoptosis

One of the major important factors of apoptosis is that it requires a functional mitochondrial respiratory chain. The caspase family of proteases are thiol-dependent enzymes that are activated either by signalling cascades i.e. extrinsic pathways, or via mitochondrial stress i.e. intrinsic pathways (Moldovan & Moldovan 2004; Alberts, Jonson et al. 2002). Caspases are cysteine proteases that cleave after aspartate residues in their substrate proteins (Garrett & Grisham 1999). There are two types of caspases involved in apoptogenesis. Effector caspases (caspase-3, -6, and -7) are responsible for the cleavage that disassembles the cell, and initiator caspases (caspase -8 and -9) influence the primary proteolytic cascade (Friesen, Kiess et al. 2004). The caspase cascade can be activated by several factors including Granzyme B, which is released by cytotoxic T lymphocytes; death receptors such as the FAS ligand, TRAIL and tumour necrosis factor; and the apoptosome, which is regulated by Cytochrome c and the bcl-2 family (Wang, Song et al. 2006; Garrett & Grisham 1999; Alberts, Johnson et al. 2002). Adaptor proteins that bring multiple copies of specific procaspases into an aggregated complex in order to initiate cleavage of each other, triggering their mutual activation, activate the pathway. Once activated, the initiator caspases can cleave downstream effector procaspases to amplify the death signal and spread it throughout the cell (Alberts, Johnson et al. 2002). This pathway is simplified in the schematic diagram in figure 1.5.1.
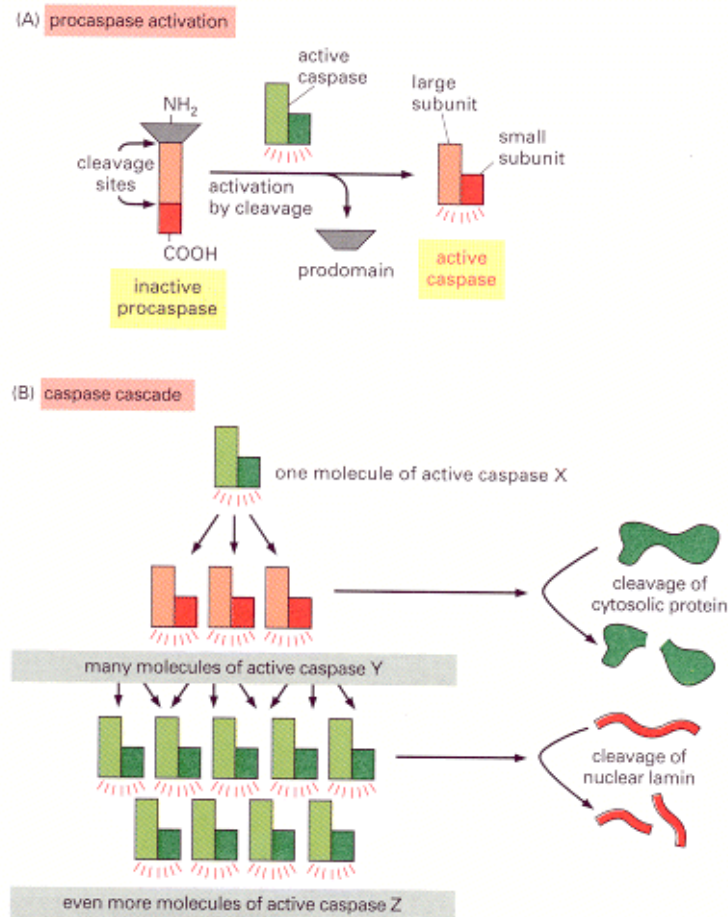
Figure 1.5.1 The caspase cascade involved in apoptosis (Alberts, Johnson et al. 2002).

Caspase activation is considered strong evidence of apoptosis due to its irreversible, all-or-nothing role in dismantling the cell, by cleaving and regulating proteins essential for normal cell function (Li, Kondo et al. 2003). Caspase cascades are controlled by positive feedback. Caspase-3 cleaves the target proteins to induce apoptosis, and it also cleaves Caspase-9 molecules, enabling more Caspase-3 to become activated (Alberts, Johnson et al. 2002).

The main intracellular regulators of the cell death program are the Bcl-2 family of proteins. Most members of this family inhibit apoptosis by blocking cytochrome c release from the inner mitochondrial membrane (figure 1.5.1). It is essential that these proteins are maintained at consistent concentrations in order to prevent uncontrolled initiation of apoptosis. On the other hand, the over expression of Bcl-2 may prove an

important and useful method of controlling apoptosis in some diseases such as cancer (Friesen, Kiess et al. 2004, Garrett & Grisham 1999).

1.6 Objectives of this study

Previous research in the free radical biochemistry laboratory have shown that the human monocyte THP-1 cell line exhibits signs of oxidative damage after exposure to AAPH (Kappler 2005; Baird 2003).This project used AAPH at a fixed concentration in order to determine the cellular events that occur in THP-1 cells during AAPH-mediated death at 37°C. A time course of the events occurring in cell death and the role of GSH in the apoptotic process were investigated. Protein hydroperoxide formation was measured in order to follow the oxidation state of the cell. As it is a major product of the reaction of peroxyl and hydroperoxyl groups with proteins, it is a reliable and widely recognised way of showing the effects of oxidative stress (Fu, Gebicki et al. 1995). The major intracellular antioxidant glutathione was assessed for its role in protecting THP-1 cells against peroxyl radical-mediated protein hydroperoxide damage. Cell viability was measured in conjunction with other experiment in order to provide a link to correlate the oxidative state of cell with apoptotic events.

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.

2,2'-azobis(2-methyl-propionamidine) dihydrochloride (AAPH)	Aldrich Chemical Co., WI, USA
2,4-DNPH	Sigma Chemical Co., St Louis, USA
Acetic acid (glacial)	Scharlau Chemie S.A., La Jota, Barcelona
Acetonitril	Merck, Darmstadt, Germany
Ammonium Ferrous Sulphate	Hopkin & Williams Ltd., Essex, England
ApoAlert Annexin V-FITC Apoptosis Kit	BD Biosciences Clontech, Palo Alto, Ca., USA
AR chromatograph Glycine	Bio-Rad laboratories, Hercules, Ca., USA
BCA protein Assay Reagent Kit	Pierce, Rockford, Il, USA
Blueranger Prestained Protein Molecular Weight Marker Mix	Pierce, Rockford, Il, USA
B-mercaptoethanol	Sigma Chemical Co., St Louis, USA
Bromophenol Blue	Sigma Chemical Co., St Louis, USA
Bovine Serum Albumin (BSA)	Sigma Chemical Co., Missouri, USA
Chelex 100 resin	Bio-Rad laboratories, Hercules, Ca., USA
Coomassie Brilliant blue R-250	Bio-Rad laboratories, Hercules, Ca., USA
DL-Buthionine-[S,R]-Sulfoximine (BSO)	Sigma-Aldrich Chemical Co., St Louis, MO, USA
Earle's Balanced Salt Solution (EBSS)	Gibco (by Invitrogen) Grand Island, NY, USA
Earle's Balanced Salt Solution (EBSS)	Sigma Chemical Co., Missouri, USA
Ethanol, 96%	Scharlau Chemie S.A., La Jota, Barcelona
Ethylacetate	BDH lab supplies Ltd., Poole, England
Ferric Ammonium Sulphate	Sigma Chemical Co., St louis, USA
Glutathione, reduced form	Sigma-Aldrich Chemical Co., St Louis, MO, USA
Glycerol	Sigma-Aldrich Chemical Co., St Louis, MO, USA
Guanidinium hydrochloride (GuHCl)	Sigma Chemical Co., Missouri, USA
Heat inactivated foetal calf serum (HI-FCS)	Gibco (by Invitrogen) Grand Island, NY, USA
Hydrochloric acid (HCl)	BDH lab supplies Ltd., Poole, England
Hydrogen Peroxide Solution	BDH lab supplies Ltd., Poole, England
Isopropanol alcohol (2-propanol)	J.T. Baker, Phillipsburg, N.J., USA
Lauryl Sulphate/Sodium dodecyl Sulphate (SDS)	Sigma-Aldrich Chemical Co., St Louis, MO, USA
Methanol	Scharlau Chemie S.A., La Jota, Barcelona

Monobromobimane (mBBr)	Fluka (by Sigma-Aldrich), Stenheim, Switzerland
(MTT) Thiazolyl Blue Tetrazolium Bromide	Sigma-Aldrich Chemical Co., St Louis, MO, USA
N-Acetyl-L-Cysteine (NAC)	Sigma-Aldrich Chemical Co., St Louis, MO, USA
NuPAGE 4-12% Bis-Tris Gel, 1.0mm×12well	Invitrogen, Carlsbad, Ca., USA
NuPAGE MOPS SDS Running Buffer (20×)	Invitrogen, Carlsbad, Ca., USA
Page Ruler Protein Ladder	Progen Biosciences, QLD, Australia
Penicillin/streptomycin	Gibco (by Invitrogen) Grand Island, NY, USA
(1000 units.mL penicillin G and 1000μg/mL streptomycin)	
Roswell Park Memorial Institute 1640 (RPMI)	Sigma-Aldrich Chemical Co., St Louis, MO, USA
RPMI 1640, without phenol red	Sigma-Aldrich Chemical Co., St Louis, MO, USA
Sodium Chloride (NaCl)	BDH lab supplies Ltd., Poole, England
Sodium dihydrogen orthophosphate (NaH_2PO_4)	Scharlau Chemie S.A., La Jota, Barcelona
Sodium Hydroxide (NaOH)	Merck, Darmstadt, Germany
Sulphuric acid (H_2SO_4)	BDH lab supplies Ltd., Poole, England
t-Butyl Hydroperoxide	Sigma Chemical Co., St Louis, USA
Trichloroacteic acid (TCA)	Merck, Darmstadt, Germany
Tris 2-Amino-2-(hydroxymethyl)-1,3-propandiol	Boehringer Mannheim, GMBH, Germany
Trypan Blue solution (0.4%)	Sigma-Aldrich Chemical Co., St Louis, MO, USA
Xylenol Orange	Sigma-Aldrich Chemical Co., St Louis, MO, USA
Xylenol Orange	Sigma Chemical Co., St Louis, USA
Xylenol Orange	Riedel-de-Haën (by Sigma-Aldrich) GMBH, Austria

2.1.2 Solutions, buffers and media

A) Cell Culture Media

All solutions used in cell culture were autoclaved or filter sterilised with a 0.2 μm filter before use, under sterile conditions, in a class 2 cabinet.

RPMI

A 500mL bottle of Roswell Park Memorial Institute (RPMI) 1640 medium with phenol red and glutamine was supplemented with 5% heat inactivated foetal calf serum (HI-FCS), 1000units/mL penicillin G and 1000μg/mL streptomycin. The media was prepared under aseptic conditions and stored at 4°C, and warmed in a water bath at 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. Chelex 100 resin was mixed into solution overnight to remove

any contaminating transition metals. The chelex resin was removed by filtration through a 0.45μm filter.

Phosphate buffered saline (PBS)-sterile use

PBS was sterilised by autoclaving (15 minutes, 121°C, 15psi) and stored at room temperature. PBS was warmed in a water bath at 37°C before using to wash cells.

AAPH solutions

A 250mM stock solution of 2,2'-azobis(2-methyl-propionamidine) (AAPH) was prepared fresh in EBSS on the day of an experiment immediately before use, and then passed through a sterile filter (0.2μm) before addition to cell suspension.

Bovine Serum albumin (BSA) for culture plate treatment

A 5% solution of BSA in EBSS was made up fresh on the day of an experiment, and then passed through a sterile filter (0.2μm). 8μL of the solution was then spread evenly over the well bases using a sterile pipette tip, and allowed to dry before cells were added.

DL-Buthionine-[S, R]-Sulfoximine (BSO)

A 5mM stock solution of BSO was made up in straight RPMI (no additives) on the day before an experiment, and then passed through a sterile filter (0.2μm). Enough of the solution was added to a flask of cells to get a final BSO concentration of 200μM.

N-Acetyl-L-Cysteine (NAC)

A 50mM stock solution of NAC was made up in straight RPMI (no additives) on the day of an experiment, and then passed through a sterile filter (0.2μm). Enough of the solution was added to a flask of cells to get a final NAC concentration of 2.5 or 5mM.

B) FOX assay solutions

Ferrous ammonium sulphate

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

Sulphuric acid solution

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

Trichloroacetic acid solution (TCA)

A 72% $^w/_v$ solution of TCA was made up by weighing 28.8g of TCA and adding Milli-Q water to make a final volume of 40mL. A 5% TCA solution was made up by dilution of the 72% TCA stock.

Xylenol orange

A 5mM Xylenol orange solution in 25mM H_2SO_4 was made up monthly, and stored at room temperature.

C) MTT solutions

MTT reagent

A 5mg/mL solution of MTT was prepared in RPMI 1640 (without phenol red, or additives). The solution was filtered through a 0.2μm filter under sterile conditions in the dark. The solution was stored for one month at -20°C, protected from light.

SDS

A 10% $^w/_v$ solution of SDS was made up by weighing 5g of SDS, making up to a final volume of 50mL by adding 0.01M HCl. The 0.01M HCl solution was made up by dilution of stock HCL (11.4M).

D) HPLC Monobromobimane (mBBr) GSH solutions

Glutathione (GSH) standard

A 10mM GSH stock standard was prepared in PBS immediately before HPLC
analysis. Two, five, and 10μM dilutions of the stock were prepared in PBS.

Monobromobimane (mBBr)

A 40mM stock solution was made up in acetonitrile on the day of an experiment,
according to the volume required for experiment. The solution was kept at 4°C in
the dark until required.

Trichloroacetic acid (TCA)

10g solid TCA was mixed with Milli-Q water to get a final volume of 10mL. The
solution was stored in the dark at 4°C.

E) BCA protein determination assay solutions

BCA protein Assay Reagent Kit was supplied by Pierce, and used as according to
the manufacturers instructions.

F) SDS-Page solutions (for use with Gadipore gels)

Cracker buffer

A 0.5M Tris-HCl solution was made in 150mL Milli-Q water. The pH was
adjusted to 6.8 using concentrated HCl drop wise.
The main solution was made up containing the following chemicals:
0.125M Tris HCl, pH 6.8
10% SDS
20% Glycerol
0.1% Bromophenol blue

This mixture was made up to a final volume of 50mL. To use, 20μL of β-mercaptoethanol was added to 1mL of the above solution, to create a lysis, or cracker buffer for each sample.

Running buffer

This was provided by Invitrogen as NuPAGE MOPS SDS Running Buffer (20×). The solution was diluted to 1× concentration with Milli-Q water.

Coomassie Blue stain

A solution containing 25% isopropanol alcohol, 10% acetic acid, and 0.2% Coomassie Blue made up to the required volume with Milli-Q water.

Coomassie De-stain

A solution containing 10% Acetic acid and 5% Methanol, made up to the required volume with Milli-Q water.

G) Carbonyl assay solutions

2,4-DNPH

A 10mM solution was prepared in 2M HCL.

Ethanol: ethyl acetate

A 1:1 ratio of this solution was prepared by adding equal volumes of ethanol and ethyl actetate.

Guanidinium hydrochloride (GuHCl)

A 6M solution was made up in 2M HCl.

Hydrochloric acid

A 2M solution was made up by diluting concentrated stock HCl (11.4M) with Milli-Q water.

Trichloroactetic acid (TCA)

Both the 72% and 28% $^w/_v$ solutions of TCA were prepared by weighing out the required amounts, and making up to volume in Milli-Q water. 5% TCA was made by diluting the 72% stock with Milli-Q water.

H) Aerobic Hydroperoxide assay solutions

Ferric Ammonium Sulphate

A 1mM stock solution was made up in 25mM H_2SO_4. This solution was stored at room temperature, and used within the week.

Hydrogen Peroxide (H_2O_2)

A dilution series was used to get a 100μM stock solution from concentrated H_2O_2 (9.27M).

t-Butyl Hydroperoxide

A dilution series was used to get a 100μM stock solution from concentrated t-Butyl Hydroperoxide (70%)

Xylenol orange

A 5mM Xylenol orange solution in 25mM H_2SO_4 was made up monthly, and stored at room temperature.

I) ApoAlert Annexin V-FITC Apoptosis Kit

ApoAlert Annexin V-FITC Apoptosis Kit was supplied by BD Biosciences Clontech, and used according to the manufacturers instructions.

2.2 Methods

2.2.1 THP-1 cell culture

THP-1 cells are a human monocytic leukaemia cell line, established in 1980 (Tsuchiya, Yamabe et al. 1980). These were obtained from the Haemotology Research lab at the Christchurch School of Medicine, University of Otago.

THP-1 cells were maintained at a density between 0.3×10^6 and 1×10^6 cells per mL in RPMI 1640 media as a suspension culture. The media was supplemented with 5% HI-FCS and Pen/Strep as previously described (section 2.1.2). Cells were kept in a humidified atmosphere containing 5% carbon dioxide (CO_2), and were regularly observed under an inverted microscope (Nikon TMS) to monitor their condition and population size.

Cell density was established using a double-chambered haemocytometer. A 20μL sample was taken from the cell suspension, mixed with 180μL of 0.4% Trypan Blue dye, and left for 30 seconds. A small amount of the diluted sample was placed onto the two fields of the haemocytometer and cells within these fields were counted. The equation used to determine the cell population within the cell suspension was:

Cells/mL of medium= No. of cells in one square (or mean of 5 squares) $\times$ dilution factor (10 in this case) $\times$ 10 (volume of the chamber) $\times$ 10^3 per mL.

2.2.2 Cell culture experiments

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

The amount of cell suspension required for an experiment was calculated using the Trypan Blue method, and this volume was then pelleted at 500g, for 5 minutes at 37°C (DuPont, Sorvall RT6000, or Kendro, Heraeus multifuge). Two washes in PBS were carried out before re-suspension of the cells in the required volume of EBSS to get a final cell concentration of 0.5×10^6 cells/mL. This was plated into 6 or 12-well non-tissue culture, low-cell-binding plates (Nunc products from Nalge Nunc International, or Falcon products from Beckton Dickinson and Co.). Each treatment was done in triplicate.

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

2.2.3 Cellular protein hydroperoxide determination using the FOX assay

The Ferric-Xylenol orange, or FOX assay, is based on the ability of hydroperoxides to react with an excess of Fe^{2+} at low pH, when xylenol orange (XO) dye is present. The amount of Fe^{3+} generated by this reaction is measured as a Fe-XO complex at 560nm (*Gay, Collins, Gebicki 1999). This can be shown in the following equation:

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

AAPH was the peroxyl radical generator used in all experimental set ups. As it interferes with the solutions used in the analytical phase of this assay, it had to be removed by washing the protein pellet with TCA prior to subsequent phases of analysis.

3mL of cell suspension was removed from each well after gentle pipetting up and down in order to resuspend any cells that had settled at the base of the plate. The cells were washed and centrifuged (DuPont, Sorvall RT6000) twice with PBS in plastic centrifuge tubes, and then transferred into 1.5mL centrifuge tubes after being resuspended in 1mL PBS. 140µL of 72% $^w/_v$ cold TCA was then added to each sample then vortexed and left on ice in the dark for 5 minutes, allowing time for the protein to precipitate. The samples were then centrifuged for 5 minutes at 1300G, 4°C (Pierce, Eppendorf centrifuge 5403). The supernatant was then removed by aspirating, the protein pellet resuspended in 1mL 5% $^w/_v$ TCA, and samples centrifuged as above. This process was repeated, and the resulting protein pellet was dissolved in 900µL 25mM H_2SO_4 before subsequent addition and vortex of 50µL 5mM XO followed by 50µL 5mM ferrous ammonium sulphate. After vortexing to mix the samples, they were incubated in the dark at room temperature for 30 minutes.

After the incubation period, the samples were centrifuged as above to remove cell debris, and the supernatant transferred to plastic semi-micro cuvettes. The absorbance was measured at 560nm against a water blank using a spectrophotometer (Shimadzu, UV-1601PC). The concentration of protein hydroperoxide in each sample was ten calculated by dividing the absorbance value by the extinction co-efficient of $30,250 \text{ M}^{-1} \text{ cm}^{-1}$.

2.2.4   Measurement of cell metabolic activity by MTT reduction assay

The MTT assay measures the activity of NADH and NADPH dehydrogenases within living cells in order to determine the viability or healthy state of the cells. Living cells are capable of forming MTT-formazan from the water-soluble MTT solution. This is seen as purple, crystal-like structures which can be dissolved in SDS buffer. The method used in this assay is based on the original method used by Mosmann (Mosmann 1983).

All experiments were performed in 12 well suspension plates under sterile conditions. 1mL of cell suspension was removed from each well after gentle pipetting up and down to resuspend any cells that had settled at the base of the plate. The samples were centrifuged (Pierce, Eppendorf centrifuge 5403) for 5 minutes at 150G, 25°C, the supernatant was aspirated off and the cell pellet was resuspended in 500μL warm PBS. This washing phase was repeated, and the cell pellet resuspended in 100μL warm RPMI (without any additives or phenol red). This suspension was added to a new well plate already containing 900μL RPMI (without any additives or phenol red), and then 100μL of the MTT reagent was added in the dark. The sampled were incubated for 2 hours at 37°C in a humidified atmosphere containing 5% CO_2. After this period, 1mL of 10% $^w/_v$ SDS was added to each well to dissolve MTT formazan crystals. 1mL of this solution was then measured spectrophotometrically (Shimadzu, UV-1601PC) at 570nm against a blank containing no cells. The percentage of living cells in each sample was measured and compared to the percentage of the control value at time zero.

2.2.5 Monobromobimane GSH measurement using HPLC

Monobromobimane (MBB) is a fluorescent dye capable of permeating the cell membrane in order to bind to thiol groups, specifically glutathione (GSH). This method was derived by Cotgreave and Moldeus (1986) from the HPLC method described by Reed, Babson et al. (1980). It utilises the above characteristics to determine GSH levels when used in conjunction with reverse phase high performance liquid chromatography (HPLC).

A standard GSH solution was made fresh immediately before sample analysis in order to provide a comparative measure for the samples (method described in section 2.1.2 D).

1mL of cell suspension was transferred to a 1.5mL centrifuge tube after gentle pipetting up and down to resuspend any cells that had settled at the base of the plate. The samples were washed twice in 1mL PBS after centrifuging (Pierce, Eppendorf centrifuge 5403) for 5 minutes at 150G, 25°C. The cell pellet was resuspended in 400μL warm PBS, with GSH standard solutions treated at the same time for consistency. 9μL of 0.1M NaOH was added to each tube to bring the pH to approximately 8, followed by 10μL MBB. The samples were left in the dark at room temperature for 20 minutes, allowing the MBB to permeate the cell membrane and bind to intracellular GSH. After this, 20μL of 100% $^{w}/_{v}$ TCA was added to each sample, followed by centrifuging at 1300G for 7 minutes at 4°C) and the supernatant transferred into plastic sample vials for measurement in the HPLC.

2.2.6 BCA protein determination assay

This assay is used to detect and quantify protein concentrations within a solution. It is based on a proteins ability to reduce Cu^{2+} to Cu^{+} in an alkaline medium, with each Cu+ ion subsequently chelating two BCA molecules to form a purple coloured complex which can be measured spectrophotometrically. The method used for this assay was as prescribed in the Pierce BCA protein Assay Reagent Kit, and samples were measured in a spectrophotometer (Shimadzu, UV-1601PC) at 562nm.

2.2.7 SDS-PAGE with Gadipore gels

SDS-PAGE measures the molecular mass of proteins, based on their mobility. It provides an index of protein purity and yields an estimate of the protein subunits molecular weights. The modern technique currently used was originally developed by Laemmli in 1970, originally used to study structural proteins in the head of bacteriophage T4. SDS is the detergent used to denature proteins, and the number of SDS molecules bound is proportional to the polypeptide chain length (and the protein's molecular weight). Mercaptoethanol further denatures protein by breaking down disulfide bridges before undergoing size separation. The gel used is porous and acts like a molecular sieve, allowing small polypeptides (with less SDS bound) to move freely, whilst restricting the movement of larger molecules (with more SDS bound). This causes the separation of polypeptide units according to their individual molecular weight.

All experiments were performed in 12 well suspension plates under aseptic conditions. After incubation in AAPH for up to 24 hours, or 5% ethanol for 12 hours, 1mL of sample was removed from each well and cracker buffer added to it. Samples could then be frozen at -80°C until required.

The samples were then thawed and placed into a boiling water bath for 2 minutes to re-dissolve the SDS within solution. A pre-made gel (pre-cast polyacrimide electrophoresis gels, Life Sciences, NSW, Australia) was placed into the plate holder and running buffer poured into container both inside and outside the gel. 6μL of magic marker was loaded into the 1st lane, followed by 20μL of each sample into the next lanes (one per sample).

The set up was run at constant voltage of 120V, 40mA until the dye runs to the end of the gel, taking approximately 1 hour.

After the gel is run, power is switched off, leads are disconnected, and the gel is opened up. The gel is then stained in 200mL Coomassie blue stain for 1 hour, followed by repeated de-staining washes until the background gel is clear. The gel can then be photographed under yellow light. Gels are stored for future reference at 4°C.

2.2.8 Carbonyl assay

Carbonyl groups may be introduced into proteins by reactions with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) generated as a

consequence of the reaction of reducing sugars, or their oxidation products, with lysine residues of proteins (Kristal & Yu 1992; Baynes, 1996; Monnier, Gerhardinger et al. 1995). The presence of carbonyl groups in proteins has therefore been used as a marker of ROS-mediated protein oxidation (De Zwart, Meerman et al. 1999; Levine, Williams et al. 1995). This assay is commonly used when studying protein oxidation of tissues *in vivo*, as an increase in protein carbonyl content is associated with a number of pathological disorders.

After each experiment was completed, two 1mL samples were removed from each well, creating two sets of triplicates for each treatment. 140μL of 72% TCA was added to each sample then vortexed and cooled on ice for 5 minutes to allow protein precipitation. Samples were centrifuged for 5 minutes at 260G, 4°C. The supernatant was aspirated off and the samples washed in 1mL 5% TCA followed by centrifugation (as above). This step was performed twice, but in the second repetition samples were resuspended one set of triplicates in 1mL of 10mM 2,4-DNPH in 2M HCL, and the second set (blanks) in 1mL of 2M HCL. If necessary, samples were frozen at -80°C at this point and analysed later.

Samples were thawed in the dark at room temperature before placing into the shaking block with gentle rotation (80-120 rpm) at 37°C for 90 minutes. Samples were then centrifuged for 5 minutes at 260G, 4°C. Supernatant was removed by aspiration, and the samples resuspended in 1mL 28% $^w/_v$ TCA, vortexed, cooled on ice for 5 minutes and centrifuged as above. The supernatant was removed by aspirating carefully, and samples washed twice in 1mL of 1:1 ethanol:ethyl acetate, with vortex and centrifuge occurring between each wash. Samples were resuspended in 1mL of 6MGuHCL and left for 1 hour in the dark, and then absorbance was read in the spectrophotometer (Shimadzu, UV-1601PC) at 360nm. Blanks were subtracted from each corresponding sample.

2.2.9 FOX assay standardisation with Hydrogen Peroxide

This assay is used to determine the accuracy of spectrophotometer readings for the FOX assay. It works on the principle that Xylenol Orange will bind to Fe^{3+} but not Fe^{2+} at acidic pH to form a coloured complex that can be measured spectrophotometrically to determine the concentration of hydroperoxides in solution (*Gay, Collins, Gebicki, 1999).

Fresh diluted H_2O_2 or t-butyl hydroperoxide were made up and diluted down in Milli-Q water immediately before use. Samples were made up in triplicate and treated at the same time. 100μL of 5mM Xylenol Orange was added, and samples were mixed by shaking. 40μL of 5mM Ferrous Ammonium Sulphate was then added to each sample and vortexed to mix. The samples were left in the dark at room temperature for 5 minutes, and the absorbance was read in a spectrophotometer (Shimadzu, UV-1601PC) at 560nm.

2.2.10 Staining of cells for PS exposure for Apoptosis analysis by Flow cytometry

This assay is based on the principle that cells express phosphatidylserine at their surface when undergoing apoptosis. Calcium ions will selectively bind to the phosphatidylserine with high affinity, allowing Annexin V to bind to apoptotic cells. The assay also uses Propidium Iodide (PI) to detect necrotic cells, as PI binds to DNA and RNA but cannot pass through an intact membrane.

After an experiment was completed, cells were washed twice in PBS, then resuspended in 1mL binding buffer, then pelleted by centrifuging for 5 minutes at 260G, 4°C. Cells were re-suspended in 100μL of binding buffer containing 2μL Annexin V and 5μL PI. They were then incubated in the dark for 15 minutes before finally adding 400μL Binding buffer immediately prior to FACS analysis. The samples were stable for up to 24 hours.

2.3 Statistical analysis

Statistical analysis of the data was performed using PRISM software (version 4, GraphPad software, Inc.). 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 Protein hydroperoxide formation on BSA

Protein hydroperoxide formation in Bovine Serum Albumin (BSA) has been measured by several students in the free radical laboratory, using the temperature-dependant Azo-initiator AAPH (Duggan 2000, Ling 2003, Kappler 2005). The experiment is used as a control for the FOX assay, providing a simplified example of protein hydroperoxide formation. The same protocol for both the BSA and cell samples were followed as described in methods (Section 2.2.1). BSA (2mg/mL) was incubated at 37°C in PBS (pH 7.4) for up to 5 hours in the presence or absence of 10mM AAPH. Three replicates of the samples were analysed at 0, 1, 3 and 5 hours after incubation began.
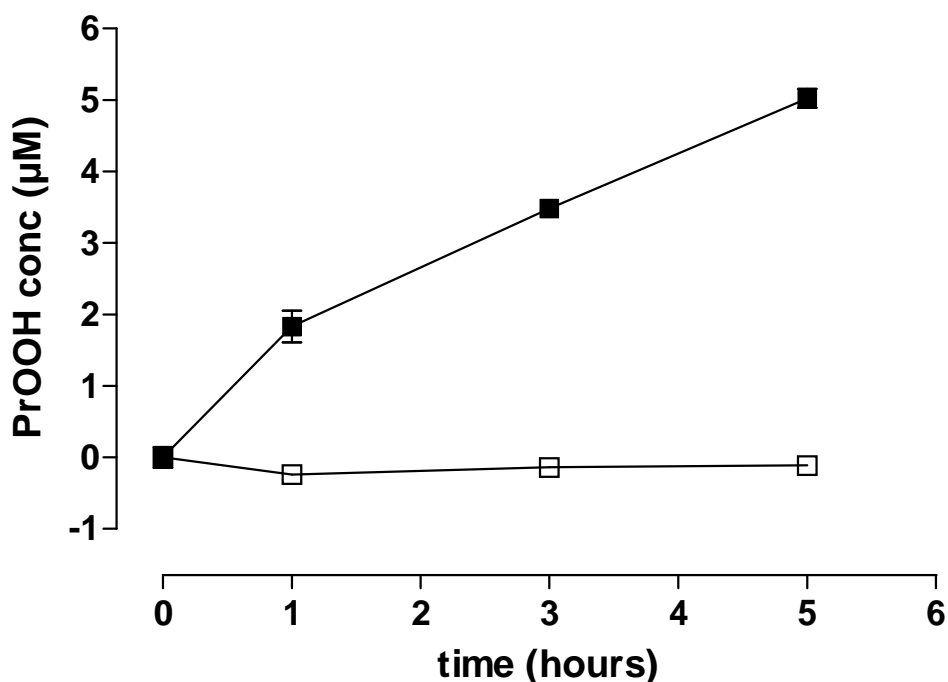

Figure 3.1.1 AAPH-induced formation of protein hydroperoxides on BSA

BSA (2mg/mL) in PBS pH 7.4 was incubated at 27°C in the presence (∎) and absence (□) of 10mM AAPH for 5 hours. At indicated time points samples were taken for standard FOX analysis of protein hydroperoxides. Each value graphed is the mean ± SEM of three replicates.

FOX assay analysis shows that incubation with AAPH causes a linear increase in the formation of protein hydroperoxides with a final concentration of 5μM after 5 hours of incubation. The control samples (no AAPH added) showed little to no protein hydroperoxide formation

3.2 AAPH induced oxidative damage to THP-1 cells

3.2.1 Protein hydroperoxide formation in THP-1 cells

The action of 10mM AAPH on the protein oxidation of THP-1 cells was measured and analysed using the standard FOX assay, using Xylenol Orange obtained from Sigma. Samples were removed from the appropriate wells after 0, 3, 6, 18 and 24 hours, and analysed for protein hydroperoxides using the standard FOX assay (Section 2.2.1).

Figure 3.2.1 Protein hydroperoxide formation on THP-1 cells measured by standard FOX analysis. THP-1 cells (0.5×10^6 cells/mL) were incubated in the presence (■) and absence (□) of 10mM 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.

Cells not exposed to AAPH showed no sign of protein hydroperoxide production, whereas the cells incubated with AAPH showed protein hydroperoxide formation after 6 hours (Fig 3.2.1). This lag phase was followed by an increase in hydroperoxide formation over the 24 hour incubation period, with final concentration measured as 1.3 μM.

The level of protein hydroperoxides formed in the treated cells was significantly different from the non-treated samples at all time points after 6 hours of incubation ($p \leq 0.001$). This result is in agreement with previous findings in the lab in that AAPH causes significant protein hydroperoxide formation in the cells compared to control cells.

3.2.2 Measurement of PrOOH in THP-1 cells using new XO reagent

The same experiment was repeated as in Section 3.2, using the new stock Xylenol Orange from Sigma-Aldrich, as the Sigma stock had run out and the product had been discontinued. Samples were removed from the appropriate wells after 0, 2, 4, 6, 8, 18, and 24 hours, and analysed for protein hydroperoxides using the standard FOX assay (Section 2.2.1).
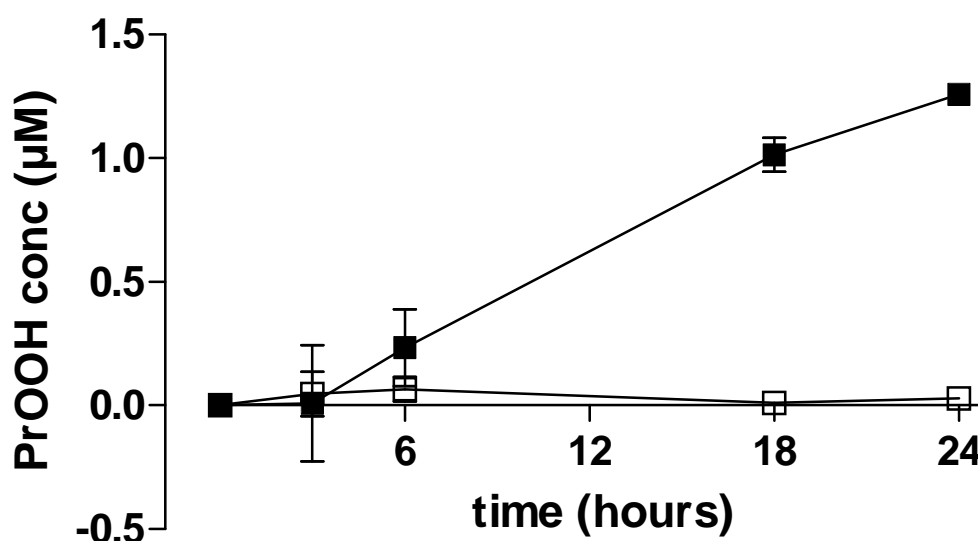

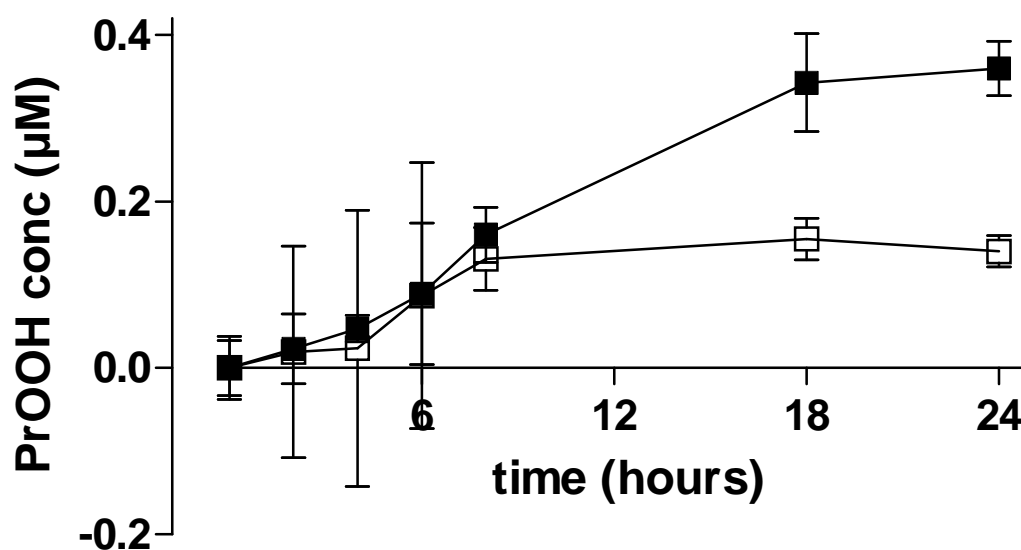**Figure 3.2.2 Protein hydroperoxide formation on THP-1 cells measured by standard FOX analysis using new XO reagent.** THP-1 cells (0.5×10^6 cells/mL) were incubated in the presence (■) and absence (□) of 10mM AAPH in EBSS at 37°C, 5% CO_2. At indicated time points, samples were taken for standard FOX analysis of protein hydroperoxides. Each value shown is the mean $\pm$ SEM of three replicates.

Cells not exposed to AAPH show little increase in protein hydroperoxide levels. Cells incubated show some increase in protein hydroperoxide levels after 6 hours, but to a much lower extent than the previous data (Fig 3.2.1). The lag phase is less obvious in this graph due to the much lower values measured during the assay. After 24 hours incubation in 10mM AAPH, the final concentration measured in treated cells was only 0.3μM, approximately ¼ of the final amount seen previously.

The same setup and protocol was repeated, with the same results obtained at least three times, the low values still obtained and with consistent trends of protein hydroperoxide formation.

3.2.3 Protein hydroperoxide formation in BSA, analysed with new stock Xylenol Orange

In order to test whether the shift in PrOOH concentration was XO or cell-dependent, the FOX assay was re-performed. BSA (2mg/mL) was incubated at 37°C in PBS (pH 7.4) for up to 5 hours in the presence or absence of 10mM AAPH. Three replicates of the samples were analysed at 0, 2.5 and 5 hours after incubation began.

Figure 3.2.3 AAPH-induced formation of protein hydroperoxides on BSA

BSA (2mg/mL) in PBS pH 7.4 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 of protein hydroperoxides. Each value graphed is the mean ± SEM of three replicates.

The treated BSA samples still show a good linear increase in protein hydroperoxide levels over time, but measurement after 5 hours incubation in AAPH showed a final concentration of only 3.3μM. The non-treated protein remained at low levels of protein hydroperoxide formation, as expected. The data shown above was a consistent result when repeated, suggesting that the new XO was less reactive than the previous stock from Sigma.

3.2.4 Protein hydroperoxide formation in BSA using fresh AAPH

There was some concern that the AAPH stock had become degraded in the fridge, so the previous experiments were repeated using unopened AAPH stored in the freezer. The sample was kept on ice in the dark and made up in PBS immediately prior to the assay.
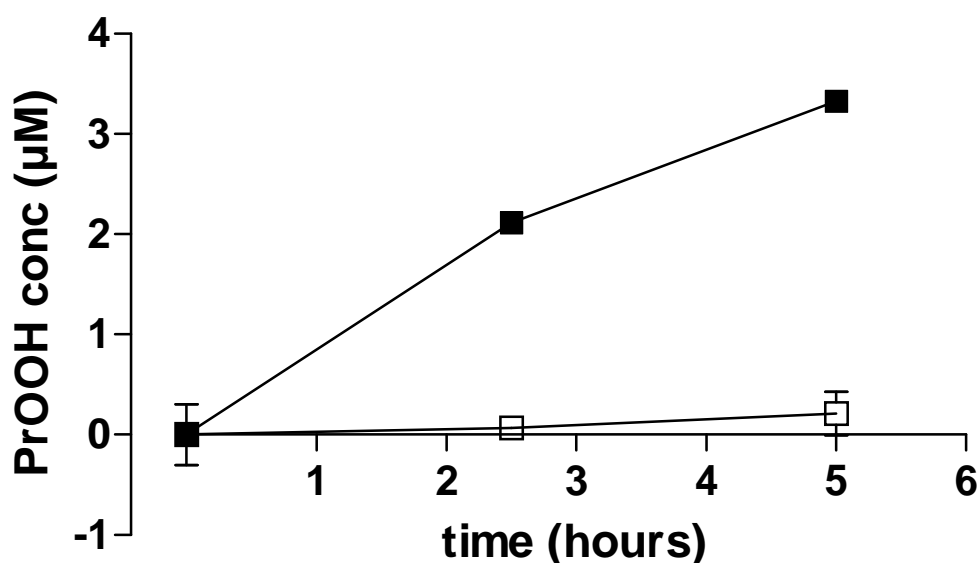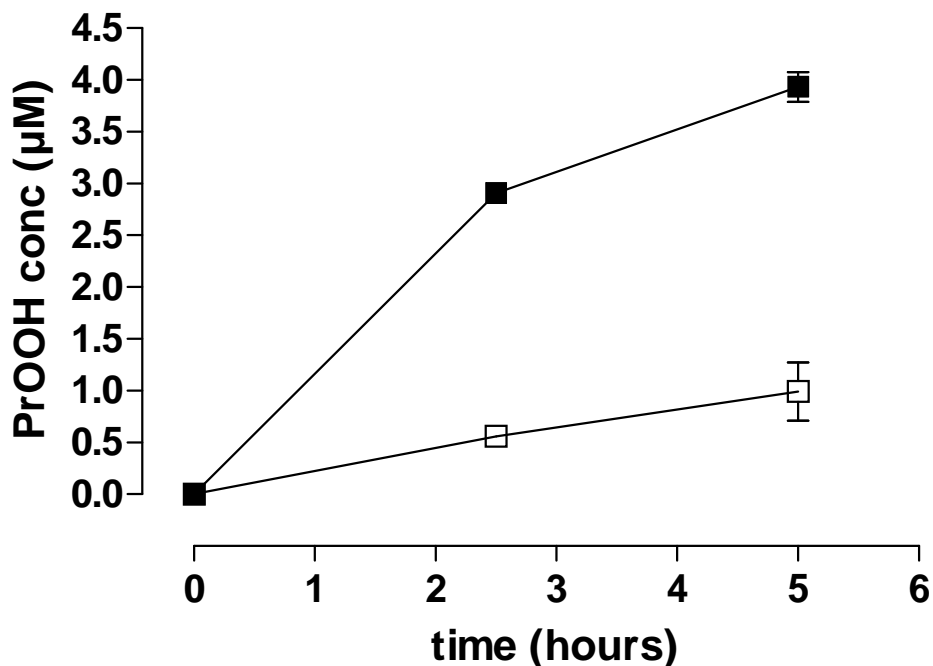

Figure 3.2.4 AAPH-induced formation of protein hydroperoxides on BSA

BSA (2mg/mL) in PBS pH 7.4 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 of protein hydroperoxides. Each value graphed is the mean ± SEM of three replicates.

There is little difference between using the frozen AAPH compared to stock kept in the cell culture fridge (4°C). The linear increase is still observed with the BSA incubated with AAPH, reaching a value of 3.9μM after 5 hours incubation, and the BSA incubated without AAPH has formed very little protein hydroperoxide. The measured protein hydroperoxide levels are still lower than the values obtained with the original Xylenol Orange obtained from Sigma.

3.2.5 Effect of AAPH on combination of BSA and THP-1 cells

In order to understand the decrease in protein hydroperoxide levels, a comparative setup between BSA and cells was set up to see if the cells themselves were interfering with the FOX assay. Cells were set up as described previously (Section 3.2) and analysed using Sigma-Aldrich stock of Xylenol Orange. BSA (2mg/mL) was incubated at 37°C in PBS (pH 7.4) for up to 18 hours alone, and up to 24 hours in the presence of THP-1 cells. BSA was added to cells with and without 10mM AAPH to provide thorough comparative controls. Three replicates of each sample was removed and analysed as each time point occurred.
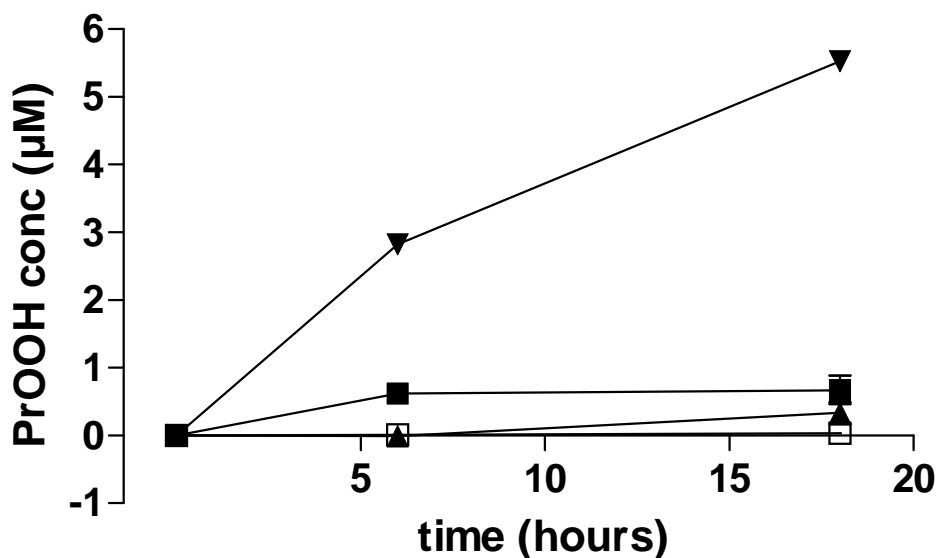
Figure 3.2.5 Direct comparison of protein hydroperoxide formation in BSA and THP-1 cells. THP-1 cells (0.5×10^6 cells/mL) were incubated as follows: **(A)** the presence of 10mM AAPH (▲), **(B)** Cells + 2mg/mL BSA (□), **(C)** Cells + 2mg/mL BSA + 10mM AAPH (■), and **(D)** 2mg/mL BSA + 10mM AAPH (▼) 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.

The BSA samples alone (no cells) show a linear increase in protein hydroperoxide formation, at similar values to what had previously been seen with the Xylenol Orange obtained from Sigma-Aldrich. Cells incubated with 2mg/mL BSA but no AAPH do not show any change in protein hydroperoxide levels, as expected. Cells

incubated with AAPH, without BSA, show a slight increase in protein hydroperoxide levels, and when the data of this treatment alone is enlarged (Fig 3.2.6) it shows that the values are similar to those seen previously (Fig 3.2.2). Cells incubated with AAPH and BSA show higher levels of protein hydroperoxide formation than without BSA, but significantly lower levels than BSA + AAPH without cells.
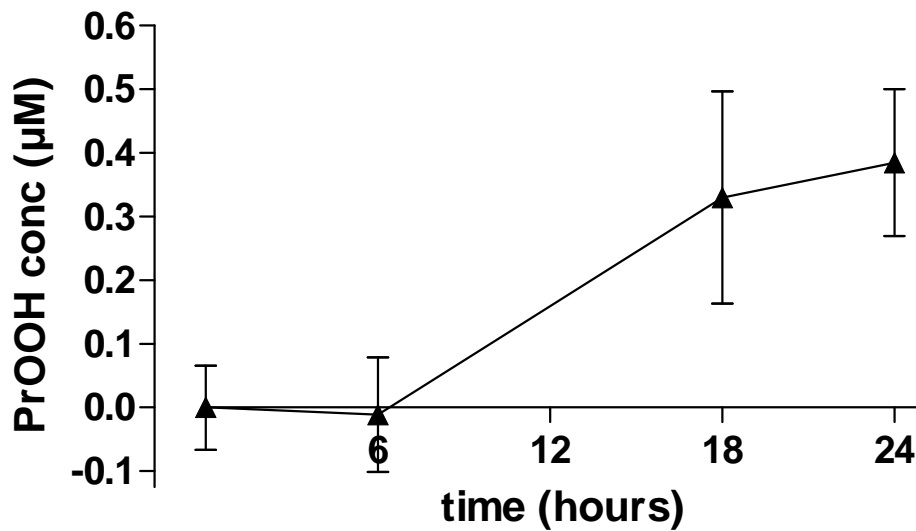

Fig 3.2.6 Enlargement of data plot from Fig 3.2.4, THP-1 Cells + 10mM AAPH.

This section is taken from Fig 3.2.4 by removing the other three data plots. Parameters were left unchanged.

3.2.6 Relationship between the absorbance of the Fe-XO complex and the concentration of Fe^{3+}

The FOX assay relies on the protein hydroperoxides ability to oxidise Fe^{2+} into Fe^{3+} which in turn bind to Xylenol Orange to form the Fe-XO complex, which is measured in the assay (*Gay, Collins, Gebicki 1999). This principle was tested in order to determine if the iron in solution was causing the decreased protein hydroperoxide values following the protocol set up by Gebicki's group (Gay, Collins, Gebicki 1999).
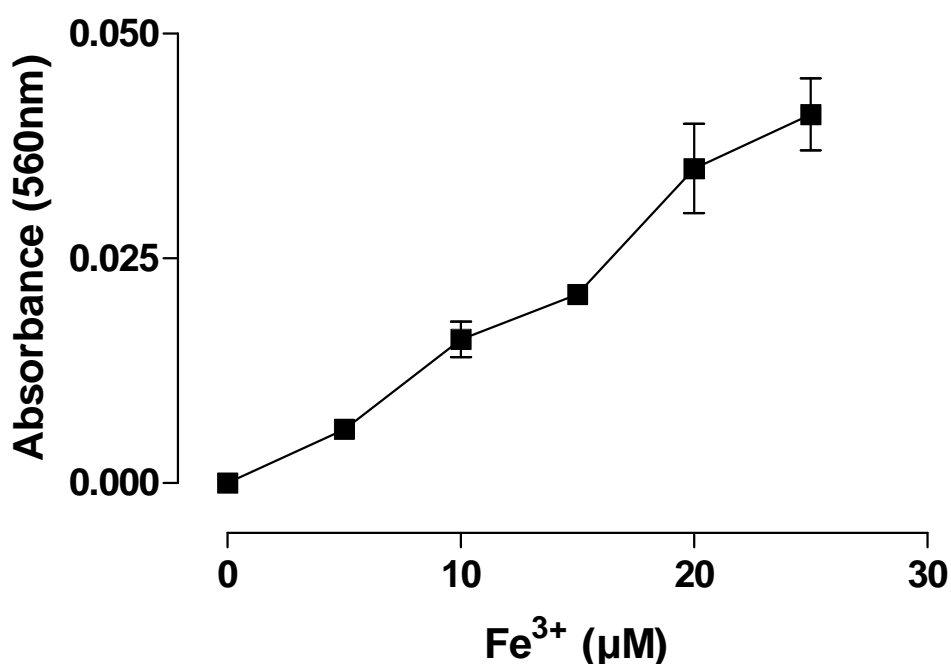
Figure 3.2.7 Relationship between the absorbance of the Fe-XO complex and the concentration of Fe^{3+}. Solution contained 180μM xylenol orange and increasing amounts of Fe^{3+}. Each value shown is the mean ± SEM of three replicates.

The absorbance measurements show good linearity as the concentration of Fe^{3+} increase. The experiment was repeated with similar results obtained. This shows that the iron in solution is not responsible for the decrease in protein hydroperoxide measurements.

3.2.7 FOX measurement of Hydrogen Peroxide

This assay is used to test the accuracy of the Fe-XO complex when measuring hydroperoxides (*Gay, Collins, Gebicki, 1999). It generates a Molar absorption co-efficient which is used to analyse pure and complex protein samples when analysing using the FOX assay method.
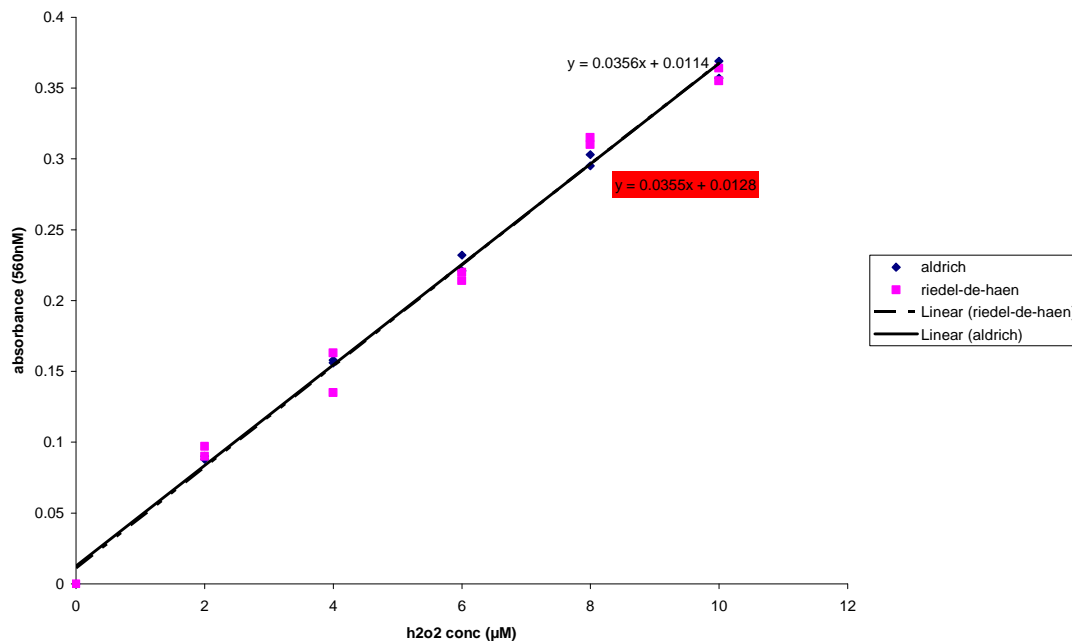
Figure 3.2.8 Standard curves of the ferric-xylenol orange (Fe-XO) complexes generated by H_2O_2. The complexes were formed during incubation of H_2O_2 in 25mM H_2SO_4 with 100μM Fe^{2+} and either 250μM Sigma-Aldrich xylenol orange, or 250μM Riedel-de-haën xylenol orange. Each value shown is the mean ± SEM of three replicates.

This result shows that there is little to no difference between the Sigma-Aldrich and Riedel-de-haën xylenol orange products, in terms of forming the Fe-XO complex to measure the amount of protein hydroperoxides within solution. The extinction co-efficient published by Gebicki's group showed the Aldrich product had an absorption co-efficient of 34,360 $M^{-1}cm^{-1}$ (*Gay, Collins, Gebicki, 1999). There is quite a big difference between each of the products, signifying the importance in determining a correct value for each type when using them to measure protein hydroperoxides with the most accuracy.

A 0.5g sample of xylenol orange was sent to Gebicki's lab at Macquarie University in order to determine the absorbance of the product with BSA protein using the iodometric assay (Gebicki & Guille, 1989). It was found that the xylenol orange from

Sigma-Aldrich has much poorer absorbance than the Sigma product, only 62% of that obtained with the original Sigma XO. This is likely responsible for the low protein hydroperoxide values obtained in the samples when measured using the FOX assay. From this result it was decided to back up the use of the FOX assays ability to measure cellular PrOOH with the measurement of protein carbonyls.

3.2.8 The Carbonyl assay

The Carbonyl assay has been widely used as an effective tool in measuring the amount of protein hydroperoxides in cells.

Cells were set up as described in section 3.2 and removed from incubation in 10mM AAPH after 0, 8, 18 and 24 hours. The samples were then treated as described in Section 2.2.8.
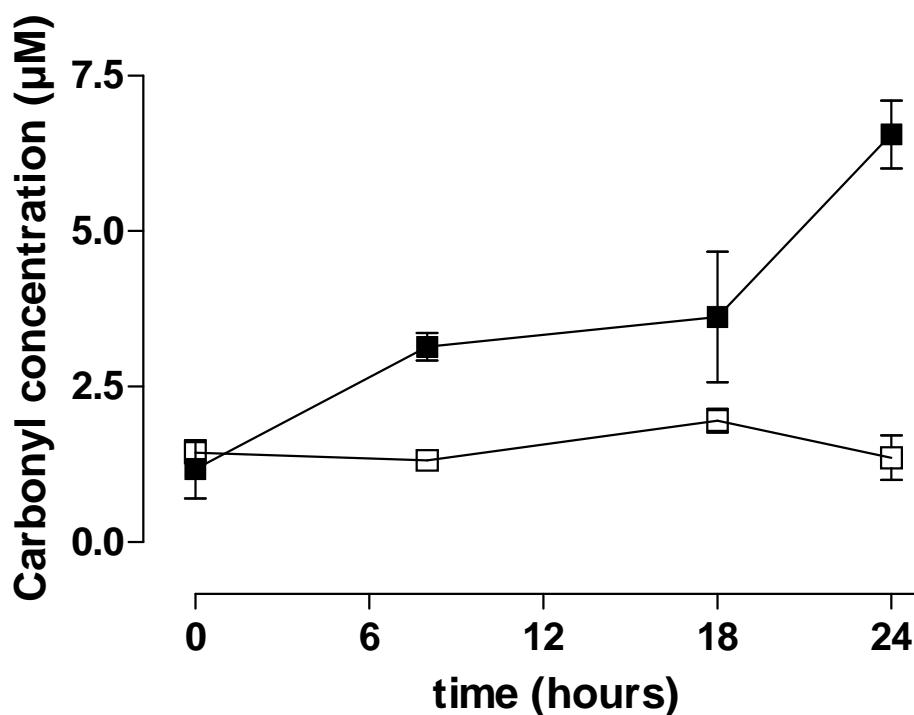
Figure 3.2.9 Carbonyl formation in THP-1 cells when incubated with AAPH.

THP-1 cells (0.5×10^6 cells/mL) were incubated with (■) or without (□) 10mM AAPH in EBSS at 37°C, 5% CO_2. Each value shown is the mean ± SEM of three replicates.

This result shows there is a significant increase in carbonyl formation in cells treated with 10mM AAPH ($p \leq 0.001$). There is a slight immediate increase in carbonyl formation, which increases from 3.1μM up to 6.5μM after 24 hours. Cells left

untreated show little change over the 24 hour incubation period. This trend differs from the FOX assay measurement of protein hydroperoxide formation (Figure 3.2.1), with a longer lag phase in the AAPH-treated cells.

3.2.9 Protein analysis using SDS-Page

SDS-Page is a useful tool to show the degradation of protein into smaller subunits. THP-1 cells were removed from growing media and incubated in fresh EBSS with 10mM AAPH for 0, 8, 12 and 24 hours. One sample set was treated with 5% ethanol for 12 hours. After the incubation period, the cells were run through SDS-Page gel as method described in Section 2.1.2 F. After washing with Coomassie blue de-stain the, the gel was photographed under yellow light and the bands compared with the marker for identification of target proteins.

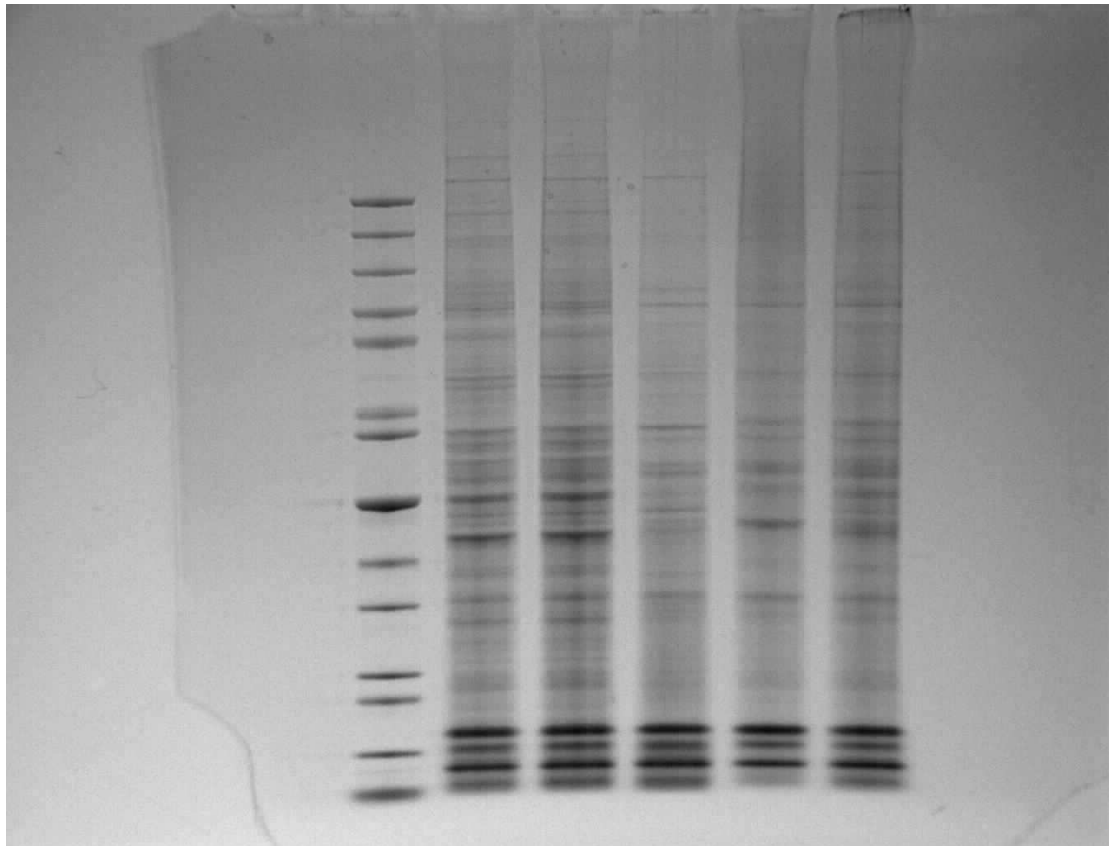**Figure 3.2.10 SDS-Page gel of THP-1 cells treated with AAPH.** THP-1 cells (0.5×10^6 cells/mL) were incubated for up to 24 hours with 10mM AAPH in EBSS at 37°C, 5% CO_2. Lane 1: marker; Lane 2: THP-1 cells with no AAPH added; Lane 3: incubation = 8 hours; Lane 4: incubation = 12 hours; Lane 5: incubation = 24 hours; Lane 6: THP-1 cells with 5% ethanol for 12 hours.

The bands at 43kDa and 50kDa are present in samples incubated for 0 & 8 hours, but these bands are not apparent in samples incubated 12 & 24 hours, or the ethanol sample. Most of the bands in the gel become light and blurred as the incubation time increases.

3.3 Altering Glutathione levels in THP-1 cells

Glutathione is one of the main intracellular antioxidants and may initially protect THP-1 cells from forming protein hydroperoxides. In order to test this theory, the nascent production of GSH was either promoted or prevented by pre-incubating the cells with either NAC or BSO, respectively.

3.3.1 Measuring Glutathione by HPLC

After pre-incubating the cells with either BSO or NAC (Section 2.1.2 A), glutathione levels were measured in the cells (Section 2.1.2 D). Two different concentrations of both BSO and NAC were used in order to determine what was best for use on THP-1 cells. These values were chosen after regarding the research of several other groups manipulating GSH levels in leukocytes (Dobashi et al, 2001; Friesen et al, 2004; Kim et al 2004). Viability of the cells after treatment with these chemicals was determined by Trypan blue analysis (Section 2.2.1) in order to keep cells in good condition for further experiments. Samples were incubated for 24 hours with all treatments before analysis by HPLC.
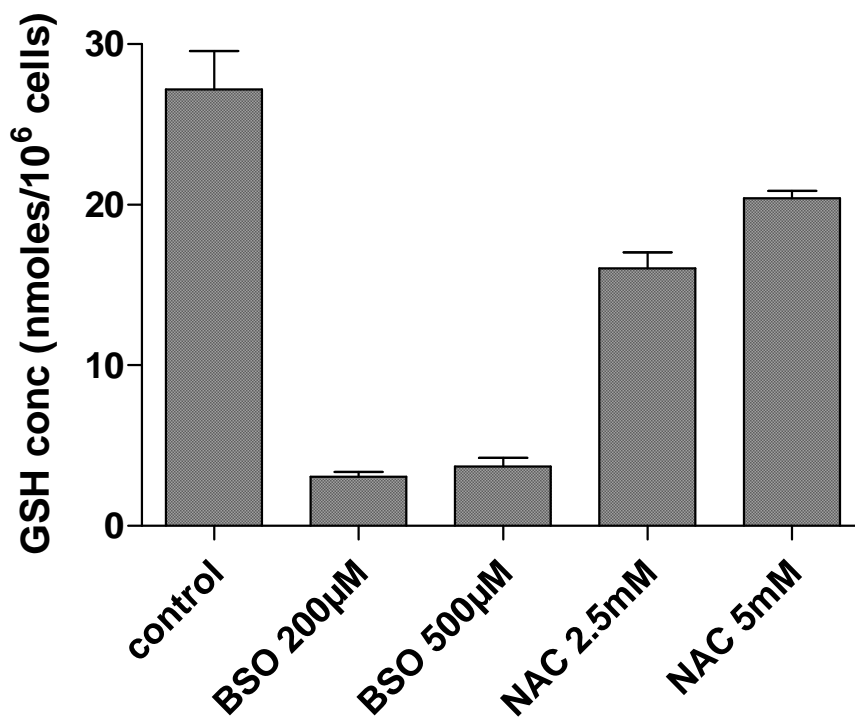
Figure 3.3.1 GSH concentration of THP-1 cells modified with BSO and NAC.

THP-1 cells ($0.5×10^6$ cells/mL) were incubated for 24 hours with BSO (200μM and 500μM) or NAC (2.5mM and 5mM) in RPMI 1640 at 37°C, 5% CO_2. Each value shown is the mean ± SEM of three replicates.
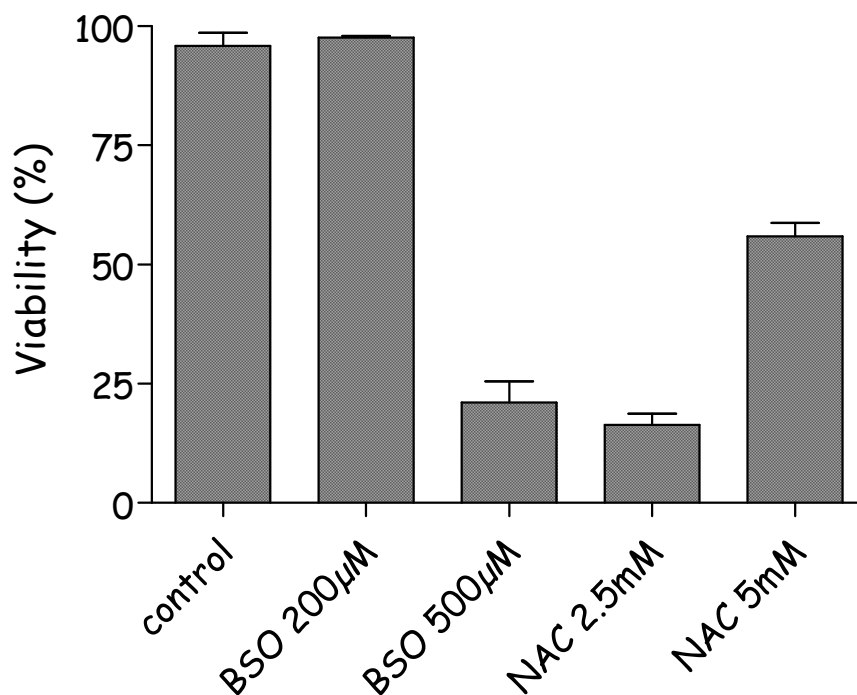
Figure 3.3.2 Trypan Blue analysis of THP-1 cells, modified with BSO and NAC.
THP-1 cells (0.5×10^6 cells/mL) were incubated for 24 hours with BSO (200μM and 500μM) or NAC (2.5mM and 5mM) in RPMI 1640 at 37°C, 5% CO_2. Viability is measured as percentage of live cells. Each value shown is the mean ± SEM of three replicates.

After 24 hours incubation in BSO, GSH levels were decreased as expected. There is little difference between using 200μM and 500μM BSO as the GSH levels are depleted to such low levels in even the lower BSO concentration. The viability of the 200μM sample is much higher than the 500μM sample. The NAC samples did not react as expected, and have actually decreased GSH levels instead of increased them. The viability of these samples was also greatly affected, with the 2.5mM sample getting to less than 25% that of control cells, and the 5mM samples only slightly higher than that. The effect of NAC on cells over a shorter time period was explored in the latter experiments (Section 3.3.3).

3.3.2 Effect of BSO on THP-1 cell viability

After determining the concentration and incubation period required for GSH depletion with BSO, the effect of GSH depletion on THP-1 cells incubated over a 24 hour time period with 10mM AAPH was investigated.

THP-1 cells were incubated in 200μM BSO for 24 hours incubation in 10mM AAPH for 0, 8, 18 and 24 hours. Cell viability was established by MTT viability assay (Section 2.1.2 C), and results are expressed as percentage of control cells.
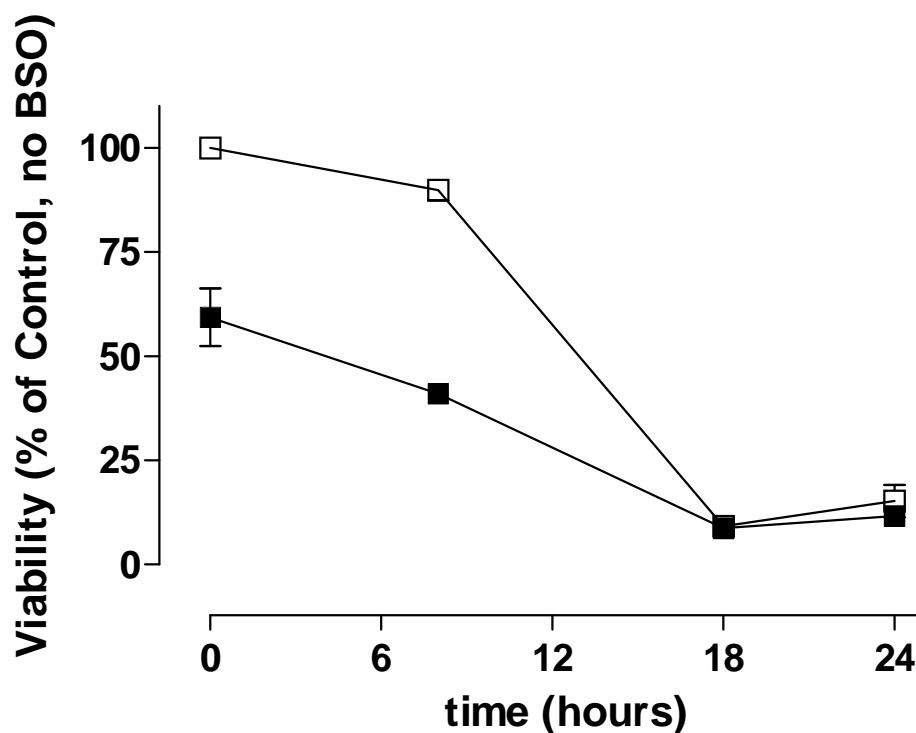
Figure 3.3.3 MTT viability test of the effect of BSO on AAPH-treated THP-1 cells. THP-1 cells (0.5×10^6 cells/mL) were incubated for 24 hours with (■) or without (□) 200μM BSO in RPMI 1640 at 37°C, 5% CO_2. Cells were then incubated for up to 24 hours with 10mM AAPH in EBSS at 37°C, 5% CO_2. Viability is expressed as percentage of MTT-viable cells. Each value shown is the mean ± SEM of three replicates.

To confirm this result, cells from the same AAPH treatment were also analysed by the trypan blue exclusion assay (Fig 3.3.4).
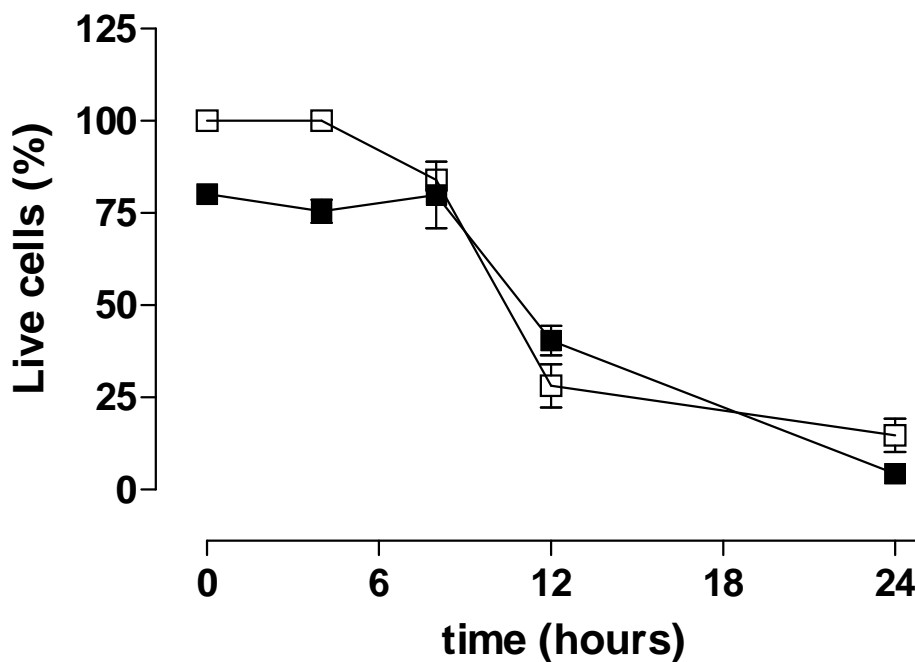
Figure 3.3.4 Trypan blue analysis of the effect of BSO on AAPH-treated THP-1 cells. THP-1 cells (0.5×10^6 cells/mL) were incubated for 24 hours with (■) or without (□) 200μM BSO in RPMI 1640 at 37°C, 5% CO_2. Cells were then incubated for up to 24 hours with 10mM AAPH in EBSS at 37°C, 5% CO_2. Viability is expressed as percentage of live cells. Each value shown is the mean ± SEM of three replicates.

These results clearly show that THP-1 cells pre-treated with 200μM BSO have significantly lower viability than those not treated ($p \leq 0.001$). This difference remains until after the 6 hour incubation period with 10mM AAPH, where the changes in cell viability become virtually identical, signifying no difference between the differently treated cells after this point in time. The 6 hour lag phase seen in Figures 3.3.3 and 3.3.4 also correspond to the lag phase seen in THP-1 cells when measuring protein hydroperoxides using the standard FOX assay.

3.3.3 Effect of NAC on GSH levels and cell viability in THP-1 cells

After assessing the results Fig 3.3.2, further investigation was required to determine the incubation period and concentration needed to increase the cellular GSH levels with NAC. Dobashi et al (2001) used NAC to increase GSH levels in THP-1 cells, with an incubation period of 2 hours. The THP-1 cells were thus set up using this time as a guide, and their viability tested using MTT analysis (2.2.2 C).
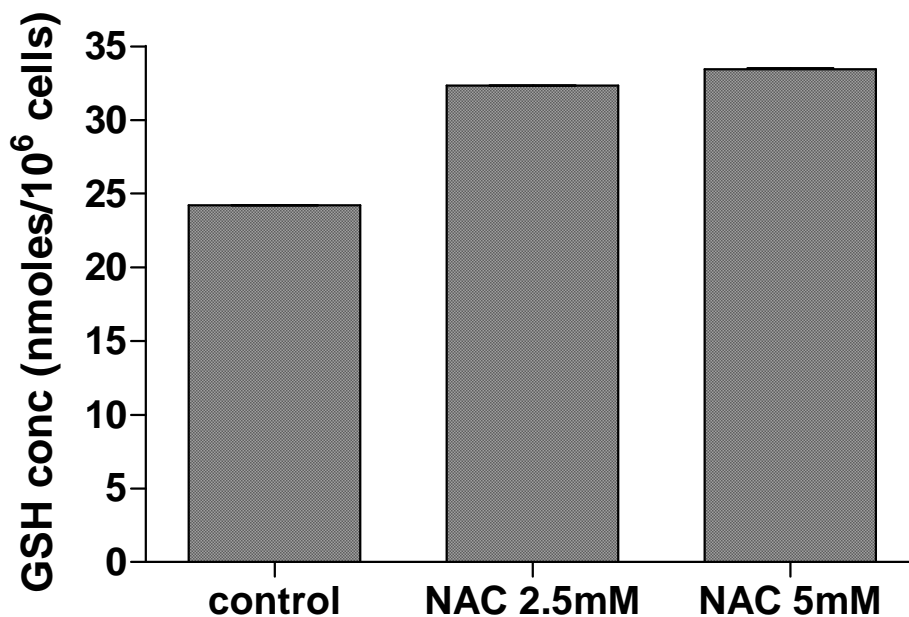
Figure 3.3.5 GSH measurement in THP-1 cells modified with NAC. THP-1 cells $(0.5 \times 10^6$ cells/mL) were incubated for 2 hours in NAC (2.5mM or 5mM) in RPMI 1640 at 37°C, 5% CO_2. Each value shown is the mean ± SEM of three replicates.

This experiment was repeated several times with similar results obtained, showing a significant increase in GSH levels when THP-1 cells were treated with 2.5mM NAC for 2 hours, but little increase from 2.5mM to 5mM NAC. From this result it was decided to use 2.5mM NAC in all further experiments.

A time course was set up to investigate changes in GSH levels occurring during incubation with AAPH. Cells were incubated for 0, 4, 8, 12 and 24 hours in 10mM AAPH, then measured using the MBB method as described in Section 2.1.2 D.
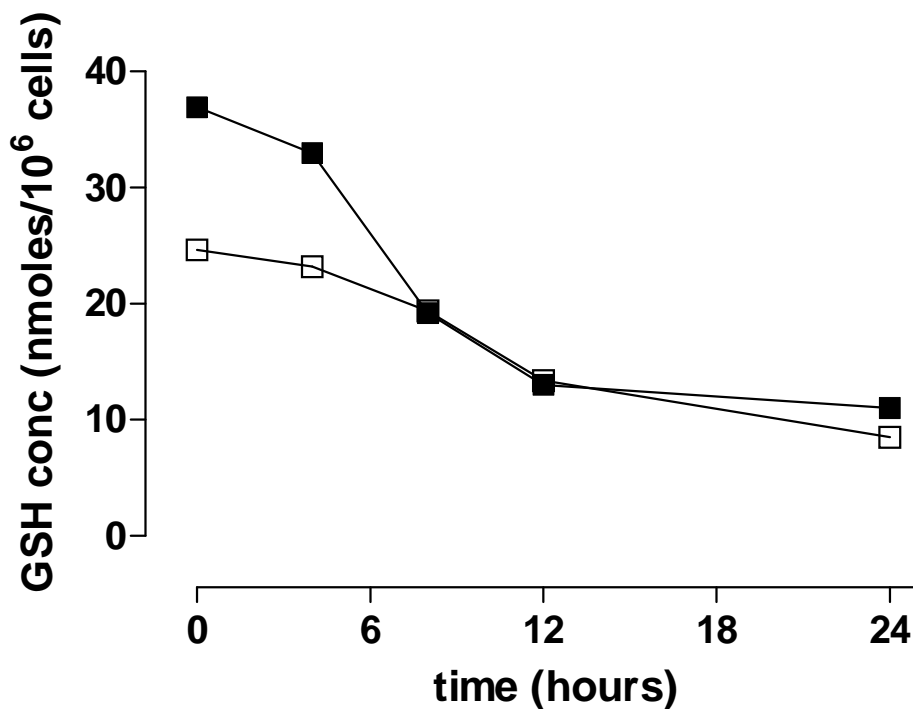
Figure 3.3.6 GSH measurement in THP-1 cells modified with NAC. THP-1 cells (0.5×10^6 cells/mL) were incubated with (■) or without (□) 2.5mM NAC for 2 hours in RPMI 1640 at 37°C, 5% CO_2. Cells were then incubated for up to 24 hours with 10mM AAPH in EBSS at 37°C, 5% CO_2. Each value shown is the mean ± SEM of three replicates.

Figure 3.3.6 shows that GSH levels begin at higher levels in cells modified with 2.5mM NAC, and after 6 hours incubation in 10mM AAPH the GSH levels fall from 36.9 nmoles/10^6 cells to 19.2 nmoles/10^6 cells. The unmodified data set shows a significantly lower ($p \leq 0.001$) initial GSH concentration of 24.6 nmoles/10^6 cells, which drops to GSH levels similar to treated cells (19 nmoles/10^6 cells). After 24 hours, both data sets show very low levels of GSH at all.

In order to assess the condition of the cells undergoing this treatment, a Trypan blue analysis was carried out (Figure 3.3.6).
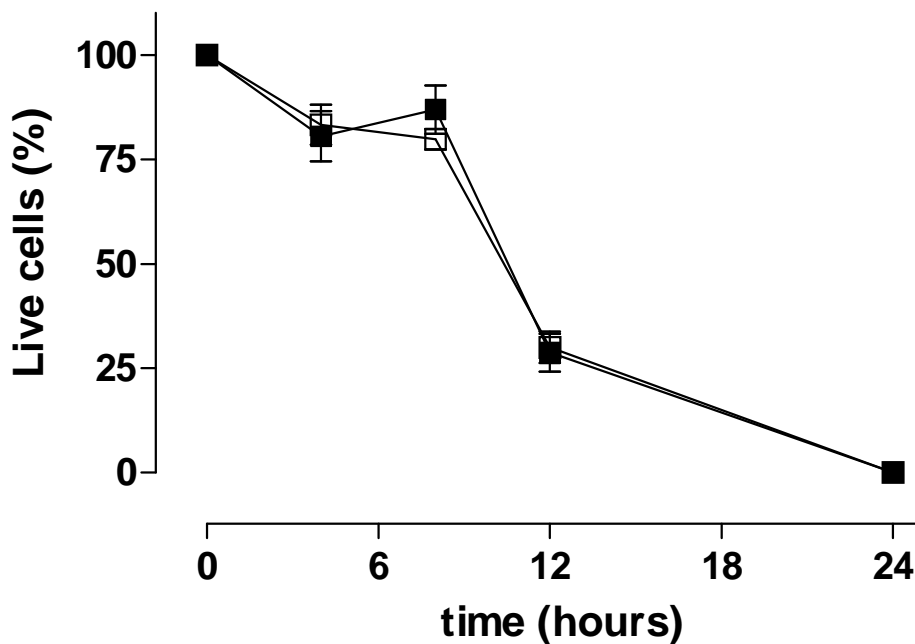
Figure 3.3.7 Trypan blue analysis of THP-1 cells modified with NAC. THP-1 cells
$(0.5\times10^6$ cells/mL) were incubated with (■) or without (□) 2.5mM NAC for 2 hours in RPMI 1640 at
37°C, 5% CO_2. Cells were then incubated for up to 24 hours with 10mM AAPH in EBSS at 37°C, 5%
CO_2. Viability is expressed as percentage of live cells. Each value shown is the mean ± SEM of three
replicates.

The Trypan Blue test shows approximately 80% cells were viable until after 6 hours
incubation in 10mM AAPH. From then on the amount of live cells decreases
dramatically, with no live cells after 24 hours incubation. Both data sets follow the
same trend, with no significant difference between cells modified with/without
2.5mM NAC for 2 hours prior to incubation in 10mM AAPH ($p\leq0.001$).
NAC has proven a difficult chemical to use in modifying THP-1 cells. Often GSH
levels did not significantly change when measured by the MBB method (Figure
3.3.8). It often appears the NAC is not "uploading" into the cells and therefore not
altering GSH levels when required. As no significant effect on GSH loss or cell
viability was observed, no further investigation of NAC loading was carried out.
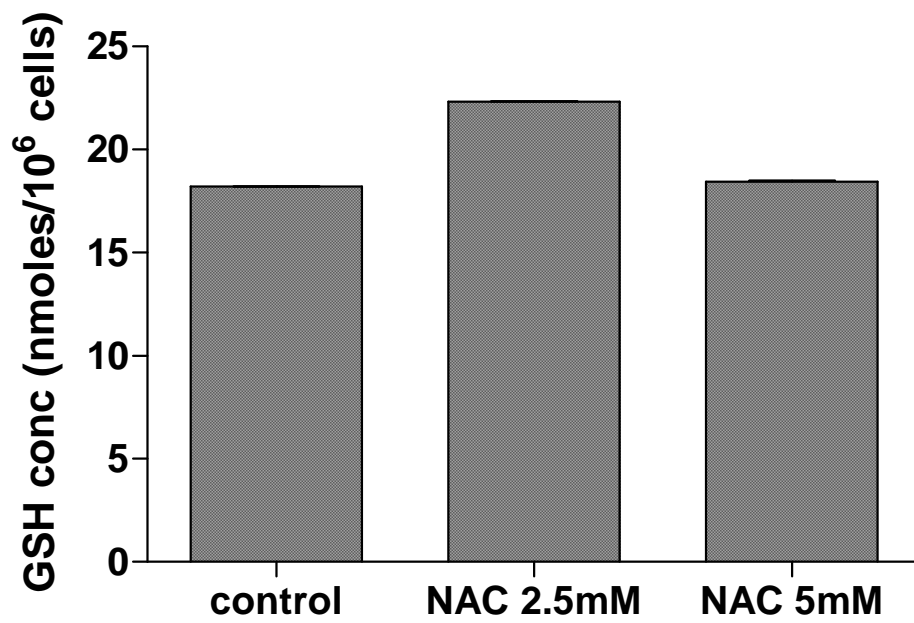
Figure 3.3.8 GSH levels measured in THP-1 cells when modification with NAC has failed. THP-1 cells (0.5×10^6 cells/mL) were incubated for 2 hours in NAC (2.5mM or 5mM) in RPMI 1640 at 37°C, 5% CO_2. Each value shown is the mean ± SEM of three replicates.

3.4 Manipulating the rate of apoptosis with BSO

3.4.1 Using the FOX assay to assess difference in protein hydroperoxide formation

Using the results in Section 3.3.2 as a guide, the FOX assay was used to investigate any changes that may occur when cells modified with BSO form protein hydroperoxides. Cells were incubated for 24 hours prior to the FOX assay with/without 200μM BSO. Each sample was incubated for up to 24 hours in 10mM AAPH

The cells were removed from incubation with AAPH at 0, 8, 18 and 24 hours, and then treated as described in Section 2.2.3.
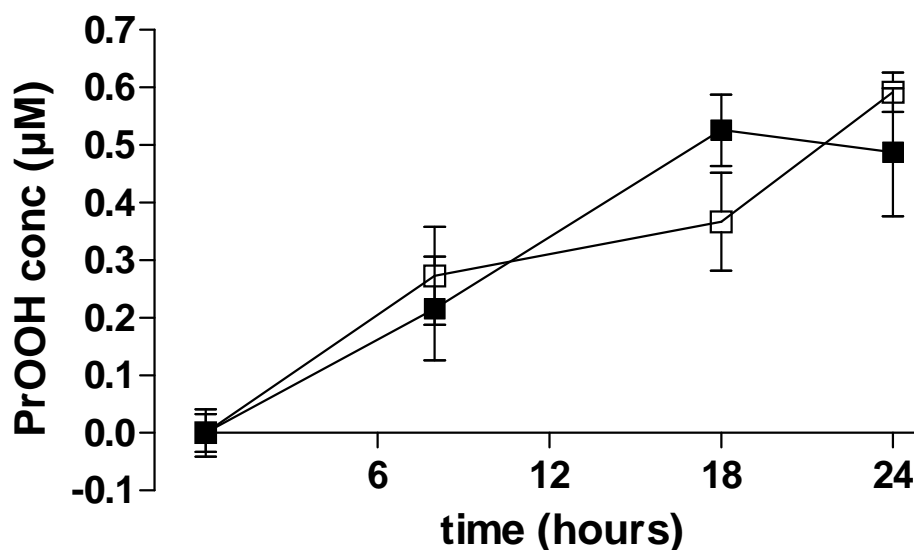
Figure 3.4.1 Effect of BSO on protein hydroperoxide production in THP-1 cells.

THP-1 cells (0.5×10^6 cells/mL) were modified for 24 hours with (■) or without (□) 200μM BSO in RPMI 1640 at 37°C, 5% CO_2. Following this, cells were incubated in 10mM AAPH for up to 24 hours in EBSS at 37°C, 5% CO_2. Each value shown is the mean ± SEM of three replicates.

The above figure shows there was no significant difference detected between THP-1 cells treated with or without 200μM BSO. This result was repeated several times with similar results obtained each time.

3.4.2 Using the carbonyl assay to examine the effects of BSO

Due to problems encountered earlier with the FOX assay, it was decided to check the result in Figure 3.4.1 by using the carbonyl assay and examining any changes between THP-1 cells treated with/without BSO.

Cells were modified with 200μM BSO in RPMI 1640 for 24 hours before incubating in EBSS with 10mM AAPH for up to 24 hours. Cells were removed at 0, 8, 18 and 24 hours and measured for carbonyl formation using method described in section 2.2.8.
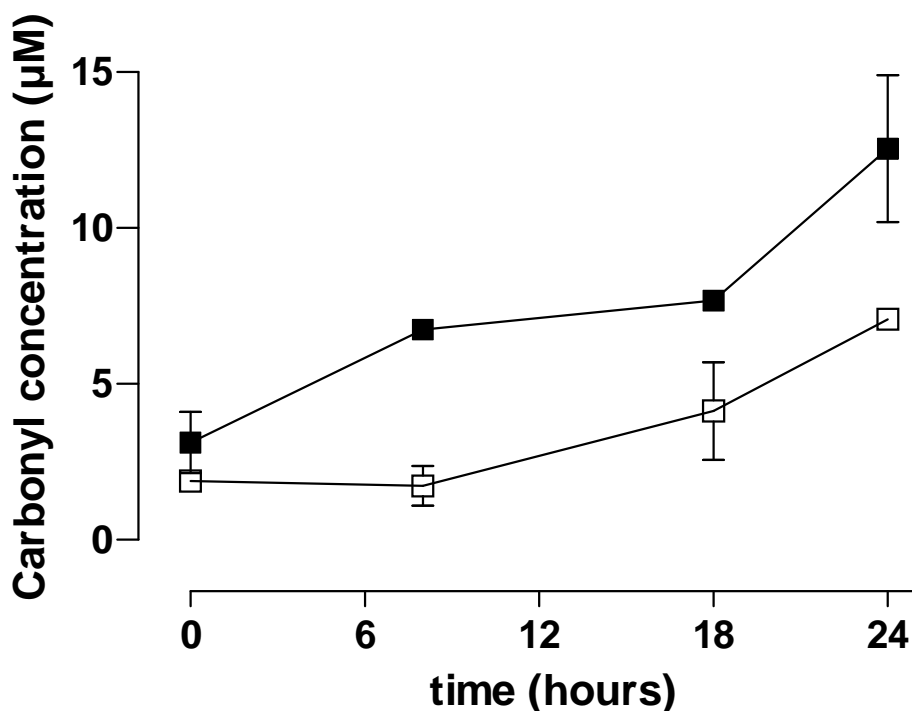
Figure 3.4.2 Effect of BSO on carbonyl content in THP-1 cells. THP-1 cells (0.5×10^6 cells/mL) were modified for 24 hours with (■) or without (□) 200μM BSO in RPMI 1640 at 37°C, 5% CO_2. Following this, cells were incubated in 10mM AAPH for up to 24 hours in EBSS at 37°C, 5% CO_2. Each value shown is the mean ± SEM of three replicates.

An increase in carbonyl concentration was seen in cells treated with BSO, with a lag phase that coincides with that seen in Figure 3.2.9. The concentration of carbonyls is significantly larger than the non-treated group ($p \leq 0.001$), and increases from 3.1μM to 12.5μM. It is interesting to notice the initial concentration of the two populations also differs, showing that BSO alone has an effect on the carbonyl content of THP-1 cells. This set up was repeated with similar results obtained.

3.4.3 Measuring apoptosis by FACS analysis

Samples were double-labelled for flow cytometric analysis with annexin V and propidium iodide (PI) staining in order to detect the very early events during apoptosis. THP-1 cells were incubated with/without 200µM BSO for 24 hours prior to the experiment, after which they were incubated in EBSS with 10mM AAPH for 0, 8, 12 and 24 hours. Samples were analysed according to the method described in the ApoAlert Annexin V-FITC Apoptosis Kit (Section 2.1.2 I) and measured in the flow cytometer at the Haematology Research Lab, Christchurch School of Medicine, University of Otago.
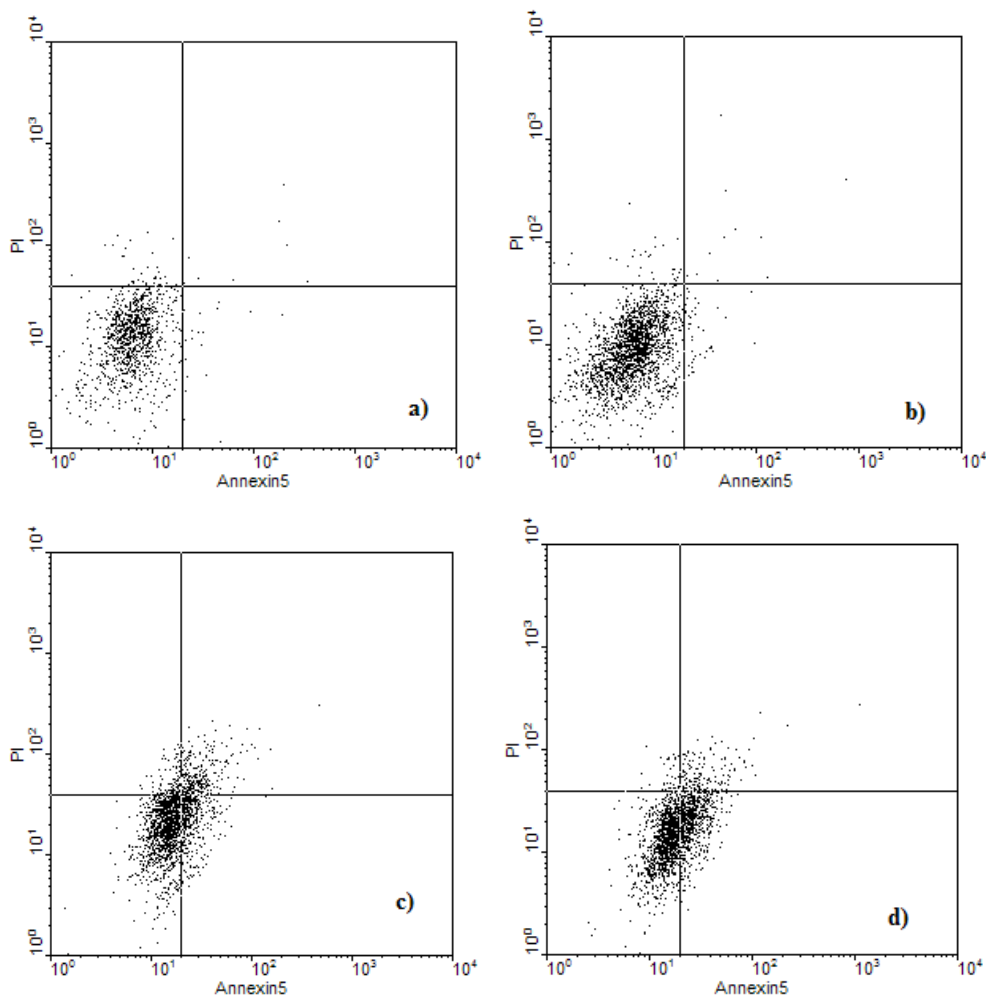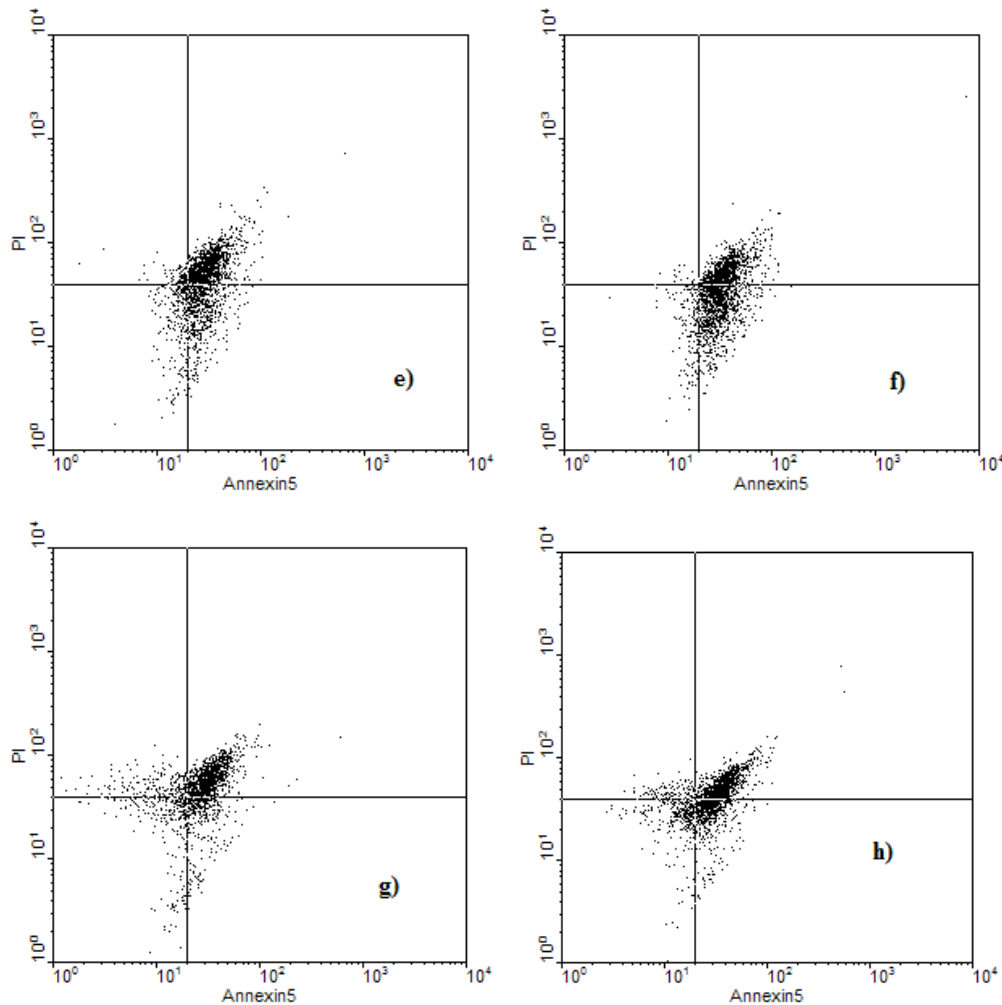
Figure 3.4.3 Flow cytometry dot plots showing the effect of AAPH on BSO treated/untreated THP-1 cells. THP-1 cells at 0.5×106/mL in EBSS were incubated for A) 0 hours, B) 0 hours + BSO, C) 8 hours, D) 8 hours + BSO, E) 18 hours, F) 18 hours + BSO, G) 24 hours and H) 24 hours + BSO with 10mM AAPH. The cells were collected and washed in PBS before staining with Annexin V-FITC and propidium iodide (PI). 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 and cells showing only increased PI binding were classified as necrotic.

Flow cytometry analysis showed a significant increase in Annexin-V, but little to no change in PI staining, after 8 hours incubation in AAPH (figure 3.4.3 A-D) in both samples with/without BSO added. By 18 hours incubation, both samples show a large percentage of cells show Annexin-V staining, and PI staining is also more significant than earlier time points (figure 3.4.3 E-F). By 24 hours, both samples show there are few cells that remain un-stained by PI or Annexin-V,

signifying that there are few cells left unaffected by AAPH treatment (figure 3.4.3 G-H).

At each time point the samples show little to no difference between BSO treated or un-treated cells. This fits with the results seen in figure 3.4.1. The pattern in staining with PI or Annexin-V shows that an apoptotic population forms in approximately 8 hours of AAPH incubation, which confirms the data discussed earlier (section 3.2).

4 Discussion

4.1 AAPH induced oxidative damage to proteins

4.1.1 The effect of AAPH on BSA

The use of AAPH as a free radical initiator has been widely used due to the nature of the highly reactive peroxyl radical (ROO˙) (Gebicki and Gebicki 1993; Gieseg, Duggan et al. 2000; Li, Kondo et al. 2001). It is a water-soluble, thermally-dependant and labile azo-initiator, and reacts with cell components on the extracellular membrane. The concentration used in all experiments within this project was determined through the studies of previous students, who found the optimal concentration to be 10mM (Cassidy 2003, Duggan 2000).

After 5 hours exposure to 10mM AAPH, the pure protein BSA showed a significant increase in protein hydroperoxide (PrOOH) production (Fig 3.1.1). Oxidative damage occurred in a short period of time, indicated by PrOOH formation at a rapid, linear rate. The non-treated BSA samples showed no significant PrOOH production, signifying that AAPH alone induces ROOH production at a linear rate, and in this case causes oxidative damage to proteins. This data shows that the FOX assay is effective in showing the result of AAPH-induced oxidative damage when measuring pure protein samples, and provides a reliable measure of PrOOH production.

4.1.2 AAPH induced oxidative damage to THP-1 cells

The formation of PrOOH in THP-1 cells appears to begin after a small lag phase between 3-6 hours (Figure 3.2.1). After this time point a linear increae in PrOOH was observed, reaching a final concentration of 1.3μM after 24 hours incubation. Earlier studies on the similar U937 cell line did not show any lag phase (Duggan 2000; Cassidy 2003), but more recent projects with THP-1 cells have also shown a 6 hour lag phase (Kappler 2005). It is possible that the earlier experiments missed the lag phase when measuring their samples due to using larger time intervals. Du and Gebicki have theorised that cultured cells may acquire reactive protein peroxide groups immediately after exposure to biologically significant amounts of hydroxyl radicals (Du & Gebicki 2004). This group exposed U937 cells to far more reactive

hydroxyl radicals generated by γ irradiation, and measured PrOOH by the FOX assay. This indicated that normal cellular antioxidant defence mechanisms are unable to prevent protein oxidation. However, the theory does not conform to the data acquired by Platt & Gieseg where there was evidence that thiol groups offer complete protection to proteins of U937 cells from AAPH-generated peroxyl radical-mediated PrOOH formation (Platt, Gieseg 2002). The data in the present study with THP-1 cells agrees with Platt & Gieseg, as the first signs of PrOOH do not appear until 3-6 hours of AAPH treatment. The difference in PrOOH formation between the two cell lines may be attributed to the lower expression of GSH levels in U937 cells compared to THP-1 (Ferret, Soum et al. 2000). As GSH is the most predominant antioxidant in cells, it is possible that it is protecting the proteins from oxidative damage for a short period of time, but that the levels of ROS eventually overcome the protective properties of GSH and PrOOH begins to form. This means that in U937 cells where GSH is lower, the protective effect is less significant and proteins would oxidise at a faster rate than in THP-1 cells. This statement is also supported by the findings discussed in section 4.2.2. There are other cellular factors that may play a role in causing a lag phase; other radical acceptors and redox enzymes may dampen the effect of oxidative damage on proteins as they may have a scavenging effect, therefore reacting with any ROS before oxidative attack on proteins (Holmgren 1979; Iwata, Hori et al. 1997).

4.1.3 Testing new XO stock

Sigma discontinued the manufacture of their XO product, so the product was substituted with XO from Aldrich. This new XO stock showed a similar trend in PrOOH formation in THP-1 cells, but showed much lower levels of PrOOH, only 0.3μM after 24 hours incubation in AAPH (figure 3.2.2). The assay was thus repeated with the pure protein BSA in order to determine the assay was working as previously (Figure 3.2.3). The amount of PrOOH in this data is also much lower than seen in previous work, with a final PrOOH concentration of 3.3μM after 5 hours incubation in AAPH.

In order to determine the cause of this lower measurement, changes to the assay were performed by a process of elimination. The freshness of the AAPH kept in the fridge was checked by using unopened frozen AAPH to see if this had degraded at all.

Figure 3.2.4 shows the results of this, with only a slight increase in PrOOH formation, up to 3.9μM after 5 hours incubation. Following this, a direct comparison of BSA and THP-1 samples and a combination of the two was tested, in order to see if the proteins within the THP-1 cells were being sufficiently exposed to enough AAPH to cause PrOOH production. The data shows that BSA alone followed the same trend as previously seen, and continued with a linear increase up to 5.5μM PrOOH after 18 hours incubation in AAPH. The cells alone can be examined more clearly in the enlarged figure 3.2.4, still showing a low final PrOOH concentration of 0.3μM after 18 hours incubation in AAPH. It is interesting to note that the combined BSA/THP-1 data plot in figure 3.2.5 shows much lower PrOOH production than BSA alone (0.6μM after 18 hours), although this is twice as high as AAPH incubation with cells alone. This may be due to a scavenging effect, whereby the cells are dampening the total PrOOH concentration due to the possession of the antioxidant GSH, therefore preventing high levels of oxidative damage and lowering the total PrOOH measured in the sample. Although interesting, this does not provide an explanation for the lower PrOOH concentrations seen in THP-1 cells alone when using the Aldrich brand XO. Figure 3.2.7 shows the absorbance readings increase in a linear fashion as the concentration of iron increases, indicating the iron in solution is not responsible for the decreased PrOOH concentrations seen in the THP-1 cells. A standard curve of the Fe-XO complex with Hydrogen peroxide was used to test the reliability of the Fe-XO complex that measures PrOOH in solution. Two brands of XO were used; the Aldrich sample used previously, and a purified sample from Riedel-de-haën (Figure 3.2.8). These samples show a higher absorption co-efficient value of 35,550 M^{-1} cm^{-1} than the value published by Gebicki's group where the Aldrich sample was shown to have an absorption co-efficient value of 34,360 M^{-1} cm^{-1} (Gay, Collins et al. 1999). This result signifies the importance of using the correct value when calculating the PrOOH concentration within samples.

On testing a 0.5g sample of the Aldrich brand XO using the iodometric assay they originally set up (Gebicki & Guille, 1989), Gebicki's lab at Macquarie University found that it had a much poorer absorbance of 62% compared to high absorption seen in the Sigma brand XO. Due to the lower reliability of the assay with the new XO, the carbonyl assay was then used to back up the trends seen in previous assays, and to examine the effects of oxidative damage on proteins in THP-1 cells.

Data in figure 3.2.9 shows carbonyl formation in THP-1 cells incubated with AAPH occurs later than PrOOH production measured by the FOX assay. The lag phase lasts for at least 18 hours, followed by a large increase at some point between 18 and 24 hours to a carbonyl concentration of 6.5μM. Carbonyl derivatives may result from the direct oxidation of lysine, arginine, proline, and threonine amino acid residues (Berlett & Stadtman 1997). This will cause cleavage of peptide bonds in the amino acid chain that these residues surround, rendering the protein non-functional. Degradation of the proteins by specific proteases is very important in order to keep oxidised proteins from accumulating within the cell and preventing disruption of normal cell activity (Friguet, Szweda et al. 1994).

Protein degradation was looked at using an SDS-Page gel. Figure 3.2.10 shows a gel run on THP-1 cells incubated with AAPH for up to 24 hours, and in ethanol for 12 hours. When looking at bands in the 42 and 50kDa region, the loss of clear bands after 12 and 24 hours incubation in AAPH, and 12 hours in ethanol, is clearly evident indicating that proteins of this molecular weight have been broken down into smaller subunits after constant attack by AAPH over time. This would allow for an increase in PrOOH formation on these smaller subunits as the cells protective mechanisms will be less effective on these smaller breakdown products. It also indicates that proteins of THP-1 cells are able to resist protein oxidation for at least 8 hours. This is fitting with the data analysed earlier by the FOX assay (figure 3.2.1).

4.2 Glutathione levels can be manipulated in THP-1 cells

4.2.1 Modifying GSH levels in THP-1 cells

GSH has been modified by many groups in order to monitor the effects in apoptotic behaviour within cells (Dobashi et al. 2001; Friesen et al. 2004; Kim et al. 2004). Using these groups methods as a guide, GSH levels were altered by adding NAC or BSO to THP-1 cells prior to AAPH addition and its effects on protein oxidation and cell viability were studied.

Cells were treated with NAC or BSO in order to increase or decrease the intracellular GSH concentration, respectively (figure 3.3.5). Results show that 24 hour treatment with BSO causes a significant decrease in GSH levels, with intracellular GSH concentration shifting from 27 nmoles/10^6 cells down to 3 nmoles/10^6 cells.

Incubation of THP-1 cells in NAC for the same time period also caused a decrease in GSH, down to 16-20 nmoles/10^6 cells. After regarding this result, a shorter incubation period of 2 hours in NAC was chosen using Dobashis groups' studies as a reference (Dobashi et al. 2001). Figure 3.3.5 shows the result of this shorter incubation, resulting in an increase of intracellular GSH from 14 nmoles/10^6 cells to 22 nmoles/10^6 cells. The NAC concentrations of 2.5mM and 5mM show very little difference in GSH increase, thus we decided to use 2.5mM NAC in future experiments.

Cell viability was important to maintain after altering GSH concentrations, and this was assessed using trypan blue analysis. Results seen in figure 3.3.2 show BSO concentrations affect THP-1 cells significantly, as using the higher 500μM concentration greatly lowers the number of live cells after 24 hours. Incubation with 200μM BSO does not appear to affect cell viability, as the number of live cells remains unchanged after 24 hours. Using this result as a guide, THP-1 cells were treated with 200μM BSO then incubated in 10mM AAPH for 24 hours. Figures 3.3.3 and 3.3.4 show assessment of cell viability using both MTT and trypan blue analyses, with both results confirming similar trends; that THP-1 cells treated with BSO undergo AAPH-induced apoptosis at a faster rate than cells left untreated. Cells treated with BSO are less-viable than non-treated cells until 8 hours, where both data sets start to follow similar trends, with little to no difference in cell viability between the two.

The same analyses were used when treating THP-1 cells with/without 2.5mM NAC. Figure 3.3.6 confirms that the samples have different GSH concentrations, with NAC causing an increase in intracellular GSH from 24 nmoles/10^6 cells to36 nmoles/10^6 cells. However, results in figure 3.3.7 shows there is little to no difference in cell viability when THP-1 cells treated with/without 2.5mM NAC undergo AAPH-induced apoptosis. NAC proved to be inconsistent to upload into THP-1 cells, and several attempts to increase GSH failed (figure 3.3.8). Its use in THP-1 cells was discontinued in this study due to the unreliability of the treatment.

4.2.2 BSO alters the amount of oxidative damage in THP-1 cells

Treatment of THP-1 cells with 200μM BSO did not cause a change to levels of protein oxidation measured by FOX analysis (figure 3.4.1). Both BSO-treated and untreated cells follow the same trend, and differences in the concentration of PrOOH are not apparent. However, figure 3.4.2 shows that treatment with BSO causes THP-1 cells to form carbonyl groups at a faster rate than non-treated cells, and without any apparent lag phase. The carbonyl content increases from 3.1μM to 12.5μM in this data set, compared to the lower increase seen in the non-treated cells from 1.8 μM to 7μM. This result signifies that the reduction of intracellular GSH by BSO treatment causes a rise in the amount of carbonyl formation; a measurement that indicates the increase of ROS-mediated protein oxidation (De Zwart, Meerman et al. 1999; Levine, Williams et al. 1995).

These results show that reduction of the intracellular antioxidant GSH by BSO removes one of the major defence mechanisms for THP-1 cells against oxidative injury. GSH works as a cofactor for glutathione peroxidases whose role is to detoxify hydrogen peroxide and lipid peroxides within the cell (Meister & Anderson 1983; Meister 1994). Without the presence of the GSH scavenger, cells have an increased susceptibility to the damaging effects of ROS. Its removal also affects the cells reducing potential, which exacerbates further oxidative stress on the cell, ultimately leading to irreversible apoptotic events. The two suggested mechanisms for GSH depletion are shown in figure 4.2.2. Glutathione levels are also depleted by expulsion from the cells via specific transporters (Reed 1990). Previous research has shown that the intracellular concentration of GSH drops during prolonged treatment with AAPH, without the recycling of GSH via the catalytic activity of glutathione reductase (Kappler 2005). Some studies have also suggested that as it can only be supplied to the cell at a limited rate, the catalytic recycling of GSH may be considered unfeasible at a certain point of PrOOH production (Luperchio, Tamir et al. 1996). This would result in the loss of cell viability as GSH levels drop down to concentrations where they have an inconsequential scavenging effect, leading ultimately to cellular apoptosis (Pascoe & Reed 1987; Salgo, Squadrito et al. 1995).
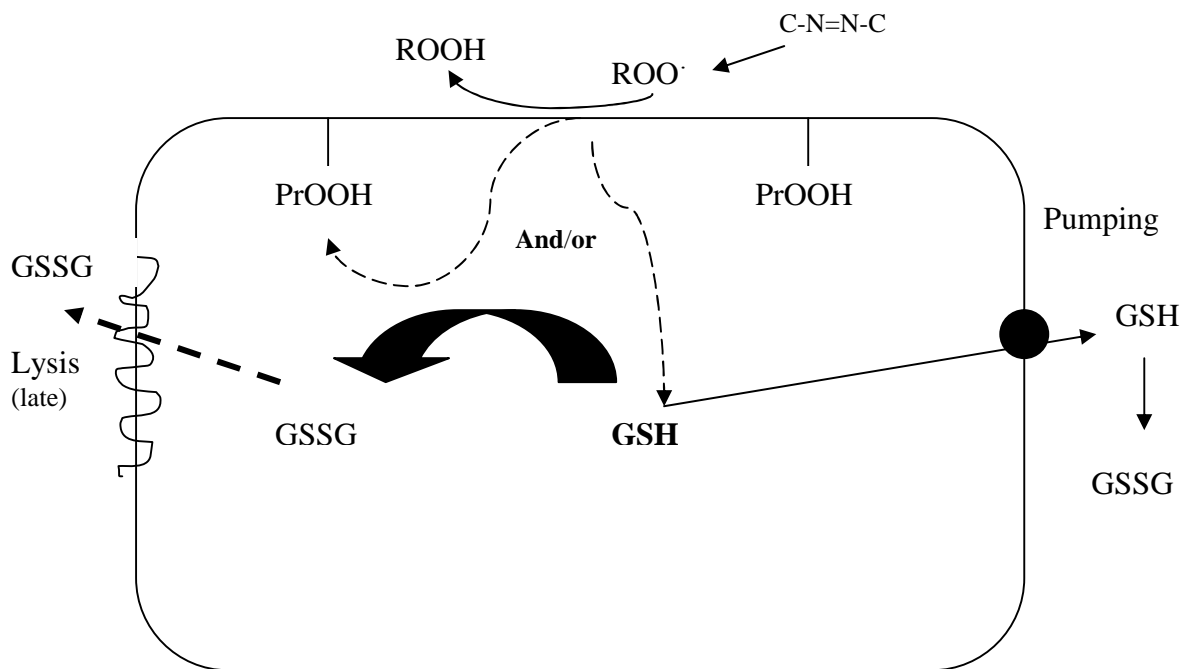
Figure 4.2.2 Possible mechanism of GSH depletion on THP-1 cells incubated with AAPH

When undergoing apoptosis, cells express phosphatidylserine (PS) at their external surface. The FACS analysis method takes advantage of this principle by staining these residues with Annexin-V with high affinity when there are calcium ions present. PI is a dye that binds to the DNA of cells much in the same method as trypan blue, in this way marking the cells as having damaged membranes. The results in figure 3.4.3 show that Annexin-V staining increases as cells are exposed to AAPH over time. This occurs to a small degree after 8 hours incubation, indicating that some PS exposure is occurring and apoptosis is happening in some of the cells. After 18 hours exposure, more than half of the cells were measured as apoptotic, and many show signs of PI staining signifying necrotic death induced by AAPH exposure. By 24 hours, there are few cells left unstained by either of the two markers, with most of the cells residing in the upper right quadrant, i.e. they are both PI positive and Annexin-V positive. This situation suggests that the cells may be undergoing secondary necrosis (Vermes, Haanen et al. 1995). Necrosis can follow apoptosis if the incubation is carried out for long enough or if there are insufficient quantities of other cells to engulf that clear debris including those apoptotic cells (Fadeel & Kagan 2003). There is no significant difference in PI or Annexin-V staining between BSO treated/untreated cells at any of the measured incubation time points. This indicates that even though GSH levels are

lowered (as shown in figure 3.3.1) THP-1 cells still undergo apoptosis at the same rate as normal, unmodified THP-1 cells. These results also confirm data found previously in the lab, where a low caspase-3 activity baseline was observed in both cells treated with AAPH and in control cells. Caspase-3 activity was therefore not present in THP-1 cells during AAPH-induced oxidative stress indicating cell death was either caspase independent apoptosis or necrosis (Kappler 2005).

4.3 Summary

This study examined the effect of AAPH on the leukemic-like cell line THP-1. The formation of PrOOH and progression of cell death was monitored when GSH levels were altered from normal basal levels.

The results have shown that the major intracellular antioxidant GSH plays a role in delaying the effects of ROS-mediated free radical attack on THP-1 cells, acting as a scavenger against oxidative damage, and slowing the onset of PrOOH formation. However, once the GSH is depleted beyond a recyclable concentration, PrOOH formation rapidly increases, and cell viability drops to a point of no return. Removal of GSH by BSO reduces the lag phase of PrOOH onset seen in normal cells as the scavenging effect GSH has in protecting oxidative damage to proteins is lost.

SDS-Page analysis shows degradation of proteins after 8 hours incubation in AAPH, fitting with the lag phase seen in PrOOH measurements and reaffirming the ability of GSH to protect THP-1 cells from oxidative damage induced by AAPH.

Flow cytometric analysis showed an increase in PI and Annexin-V staining as the cells were incubated for longer periods of time in AAPH. After 8 hours, apoptosis is indicated by positive staining of cells with Annexin-V and negative staining for PI. Double labelling of cells by 24 hours indicates apoptosis and secondary necrosis, fitting with earlier studies in the lab (Kappler 2005).

GSH has a protective effect on proteins of THP-1 cells against oxidative attack for a limited amount of time. After this point, PrOOH formation causes the activation of apoptotic events. The stress placed on the cells from these events ultimately leads to necrotic death in THP-1 cells.

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