Gas Chromatographic-Mass Spectrometry analysis of Volatile Organic Compounds from Cancer Cell Cultures - The Effect of Hypoxia

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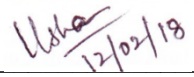
Statement of Authorship and Originality

Except where explicit reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis by which I have qualified for or been awarded another degree or diploma. No other person's work has been relied upon or used without due acknowledgment in the main text and bibliography of the thesis. No editorial assistance has been received in the production of the thesis without due acknowledgement. Except where duly referred to, the thesis does not include material with copyright provisions or requiring copyright approvals.

Signed: 

About This Thesis

This thesis contains 6 chapters in total, with Chapter 1 being a literature overview and Chapter 2 being a summary of materials and methods. The three results chapters contain all my own work. Results chapter contributions are summarised in Table A. Chapter 6 contains the final discussions and conclusions, linking together the work undertaken in this thesis.

Table A: Contributions to the thesis results chapters

Chapter	Title	Extent of Candidates Contribution
3	Gene expression analysis – Oxygen Gradient Vs Normoxia	Designed the study and carried out all experiments. Wrote the chapter. 85%
4	Quantification of Lipid Perioxidation activity and Reactive Oxygen Species in A549 cells.	Designed the study and carried out all experiments. Wrote the chapter. 85%
5	Comparative VOC analysis from the headspace of A549 cells cultured under two different physiological conditions.	Designed the study and carried out all experiments. Wrote the chapter. 85%

Each results chapter is presented in a publication format, with figures and figure legends shown at the end of each chapter. Part of Chapter 1 has been published as a review paper (https://www.ncbi.nlm.nih.gov/pubmed/24861817) of which I was the first author. This resulted in some content overlap and repetition in introductory concepts and methodologies. Each chapter also has a separate references section.

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Thesis Summary

Early diagnosis of lung cancer improves patient outcomes which has led to a search for non-invasive diagnostic tests suitable for population screening. Volatile organic compounds (VOCs) in exhaled breath have shown potential, however, confirmation of the metabolic origins and disease specificity of candidate markers is required. Cell culture metabolomics can identify disease biomarkers and their origins. To date VOC profiles from in vitro cultured cancer cells have little similarity to cancer breath VOC profiles. In vivo, cancer cells experience hypoxia whereas in vitro cells are cultured under normoxic conditions. Since hypoxia influences cell metabolism, we hypothesize that cancer cells cultured under hypoxic conditions will have altered cell metabolism and produce VOC profiles more typical of cancer breathe. This study investigates the effect of hypoxia on metabolic reprogramming in A549 lung cancer cells cultured under standard normoxic (atmospheric oxygen) or hypoxic (2% oxygen) conditions. Results from quantitative RT-PCR demonstrated a significant upregulation in hypoxia of the glucose transporter (*GLUT1*) and the key TCA regulatory gene *PDHK1*, demonstrating that hypoxia plays a pivotal role in regulating metabolism in A549 cells. A ratio-metric assessment of Lipid Peroxidation (LPO) and the production of reactive oxygen species (ROS) showed an increase in LPO and a slight decrease in the production of ROS in hypoxic cultures, the combined effect of which may serve to equip the cells to adapt to and proliferate under low oxygen. Finally, the comparison of endogenous VOCs produced by A549 cells under hypoxic and normoxic conditions identified twelve VOCs unique to cells grown under hypoxic conditions including n-pentane, a marker of LPO and cancer, and 3-methyl hexane, which has been reported as a biomarker of cancer. This data is consistent with the hypothesis that a hypoxic tumour microenvironment may influence cell metabolism leading to a unique and diagnostic cancer VOC profile.

Abbreviations

GLUT1	Glucose Transporter
HK1	Hexokinase 1
HK2	Hexokinase 2
ALDOA	Aldolase A
ENO1	Enolase 1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
ACTB	b-Actin
EEF2	Eukaryotic Elongation Factor 2
PFKL	Phosphofructo Kinase L
PGK1	Phosphoglycerate Kinase 1
PKM2	Pyruvate Kinase M2
LDHA	Lactate Dehydrogenase A
PDH	Pyruvate Dehydrogenase
PDHK1/ PDK1	Pyruvate Dehydrogenase Kinase 1
Ac-CoA	Acetyl Co-Enzyme A
TCA	Tricarboxylic Acid
MCT4	Mono Carboxylate Transporter 4
CAR9	Carbonic Anhydrase 9
mTOR	Mammalian Target of Rapamycin
PPP	Pentose Phosphate Pathway
GCMS	Gas Chromatography-Mass Spectrometry
CAR/PDMS	Carboxen polydimethyl Siloxane
VOCs	Volatile Organic Compounds
ppbv	Parts per billion volume
ppmv	Parts per million volume
SPME	Solid phase micro extraction
PLOT	Porous Layer Open Tubular column
TIC	Total Ion Current
SIFT-MS	Selected Ion-Flow tube Mass Spectrometry

IMS	Ion Mobility Spectrometry
PTR-MS	Proton Transfer Mass Spectrometry
FAIMS	Field Asymmetric Ion-Mobility Spectrometer
DNA	Deoxyribonucleic Acid
CYP	Cytochrome P450
VEGF	Vascular Endothelial Growth Factor
HIF	1a Hypoxia Inducible Factor 1a
HRE	Hypoxia-Responsive Element
OXPHOS	Oxidative Phosphorylation
ATP	Adenosine Triphosphate
A549	Adenocarcinomic Human Alveolar Basal Epithelial cells
ROS	Reactive Oxygen Species
mROS	Mitochondrial Reactive Oxygen Species
mtDNA	Mitochondrial Deoxyribonucleic Acid
LPO	Lipid Peroxidation
HCC	Hepatocellular Carcinoma
qPCR	Quantitative PCR
GOI	Gene of interest
TBHP	Tert-butyl hydrogen Peroxide
ROI	Region of interest
SOD	Superoxide dismutase
H2O2	Hydrogen peroxide
PET	Positron emission tomography
ANOVA	Analysis of Variance

Chapter 1 - **Literature Review**

Abstract

Lung cancer is the leading cause of cancer deaths. Unfortunately, lung cancer is often diagnosed only when it becomes symptomatic and is at an advanced stage when few treatment options are available. Hence, a diagnostic test suitable for screening widespread populations is required to enable earlier diagnosis. Analysis of exhaled breath provides a non-invasive method for early detection of lung cancer. Analysis of volatile organic compounds (VOCs) by various mass spectral techniques has identified potential biomarkers of disease. Nevertheless, the metabolic origins and the disease specificity of VOCs need further elucidation. Cell culture metabolomics can be used as a bottom-up approach to identify biomarkers of pathological conditions and can also be used to study the metabolic pathways that produce such compounds. This review summarizes the current knowledge of lung cancer biomarkers in exhaled breath and emphasizes the critical role of cell culture conditions in determining the VOCs produced *in-vitro*. Hypoxic culture conditions more closely mimic the conditions of cancer cell growth *in vivo*. We propose that since hypoxia influences cell metabolism and so potentially the VOCs that the cancer cells produce then cell culture metabolomics projects should consider culturing cancer cells in hypoxic conditions.

Introduction

Lung cancer is one of the five most commonly diagnosed cancers and is the leading cause of cancer-related deaths throughout the world [1-4]. The five-year survival rate for lung cancer patients is poor, largely due to symptoms of lung cancer usually becoming apparent only once the disease has reached an advanced stage. Methods for detection of lung cancer are generally invasive and not suited to widespread population screening; hence there is a need for a non-invasive, accurate and rapid screening test for early detection.

The exhaled breath of lung cancer patients contains volatile organic compounds (VOCs), some of which may be useful biomarkers of the disease. These may provide a non-invasive means to screen for lung cancer using techniques such as gas chromatography-mass spectrometry (GC-MS). Previous results indicate that lung cancer can be diagnosed in this way with some accuracy [5-29]. The diagnostic VOCs identified in these studies were mostly alkanes. However, most lung cancer VOCs that have been reported are not disease specific and their metabolic origins remain unknown. Knowledge of all the VOCs produced by lung cancer cells should lead to a panel of diagnostic biochemical markers that can be measured in combination to increase the sensitivity and specificity of lung cancer diagnosis.

The use of cell culture metabolomics allows for both the discovery of novel biomarkers of pathological conditions and investigation of the metabolic pathways that produce them. However, previous studies have found poor correlations between the VOCs from cancer cells in culture and those found by breath analysis (see section 2 of this review). We propose that one reason for this discrepancy is the use of normoxic *in-vitro* culture conditions that have traditionally been used for growing cancer cell lines. *In vivo* cancer cells experience low oxygen or hypoxic conditions as a consequence of the diffusion limit of oxygen within tissues, which has been measured to be around 150 μm [30, 31]. Consequently, once a tumour grows to greater than 300 μm diameter or approximately 15 to 20 cells across, the cells in the centre will be experiencing hypoxic conditions. VOCs however are generally hydrophobic and therefore lipid soluble and so should pass freely from hypoxic regions of the tumour to enter the circulation to travel to the lungs for release by breath. There have been several excellent recent reviews of the VOCs associated with lung cancer [32, 33]. In this chapter, I review the current state of knowledge about

biomarkers of lung cancer in exhaled breath but with an emphasis on the critical role of cell culture conditions in in-vitro studies in determining the VOCs produced.

Breath Analysis

Pros and Cons of Breath Analysis

Breath analysis provides a non-invasive window to observe biochemical processes of the body [34]. Ancient physicians knew that the smell of human breath can indicate a certain disease state which could be a useful diagnostic tool. For example, diabetes is associated with the sweet smell of acetone in breath, renal failure results in a urine-like smell and fishy odour in breath is linked to liver disease [35] (for review see **Table 1.1** [36]).

Table 1.1 : *Volatile compounds associated with disease*

Volatile compound(s)	Disorder(s)
Ethane and pentane	Oxidative Stress
Methylated Hydrocarbons	Lung or Breast Cancer
Isoprene	Cholesterol metabolism
Acetone	Diabetes Mellitus, ketonemia
Dimethylsulfide, methyl mercaptane, ethyl mercaptane	Liver damage
Ammonia, dimethylamine, trimethylamine	Uremia, Renal damage

Analysis of exhaled breath has many advantages compared to other diagnostic techniques such as bronchoscopy or medical imaging. It is non-invasive and painless and exhaled air can be sampled as often as necessary without restriction; particularly important for the critically ill and for large scale screening in healthy populations for cancer and various other diseases such as renal and liver diseases.

Currently, clinically available breath tests include: Breath-alcohol test which determines ethanol concentration [37], the nitric oxide (NO) test to detect asthma, and diagnosis of *Helicobacter pylori* infection by ^{13}C-urea or ammonia breath tests [38].

Water soluble and non-volatile chemicals in exhaled breath tend to condense with the water vapour whereas VOCs tend to remain in the gas phase. Quantitative analysis of breath condensate is hampered by a number of problems including the unclear relationship between assumed alveolar or airway

concentrations and substance concentrations in the condensate [39] and the limited stability of some compounds. In contrast the kinetics of VOCs can be approximated according to substance solubilities and most exhaled VOCs are stable [34]. Consequently, targeting the volatile component of breath for analysis reduces many issues associated with analysis of breath condensate.

VOCs are only a small fraction of the total chemical compounds present in human breath and occur in low concentrations in the nmol/L – p/mol/L range [34, 40]. Despite this, in 1971 Pauling et al. detected the presence of large numbers of VOCs using microanalysis of breath by newly developed capillary Gas Chromatography (GC) [41]. To date, there are more than 800 VOCs detected in the exhaled breath in picomolar concentrations [19, 42] and there have been many studies that aim to correlate single substances or sets of exhaled markers with clinical conditions [43-47].

The basic research in breath analysis relies on the advances of analytical technology to detect and identify the VOCs. The sample of exhaled breath is analysed using various high-performance equipment such as Gas Chromatography Mass Spectrometry (GC-MS), selected Ion-Flow Tube Mass Spectrometry (SIFT-MS), Ion-Mobility Spectrometry (IMS) and Proton Transfer Mass Spectrometry (PTR-MS). Though GCMS remains a gold standard to perform qualitative and quantitative analysis of trace compounds, it is not currently a portable device and also requires trained personnel to operate and interpret the data [48, 49]. On the other hand, real time analysis with absolute quantification of VOCs can be performed using SIFT-MS but it is both expensive and not ideal for broad profiling [50, 51]. Although high specificity can be achieved using PTR-MS and IMS, there is a high risk of signal interference [52-55].

These analytical methods are potentially useful, but not well suited to point of care diagnostics in clinical practice. However portable analytical devices are being developed using Field Asymmetric Ion-Mobility Spectrometer or FAIMS technology and Aspiration Ion-Mobility Spectrometry or AIMS [56]. Arasaradnam et al have found that FAIMS technology can be used to differentiate between patients with colorectal cancer and healthy controls with 80% sensitivity and 60% specificity [57]. Also, standardisation of protocols for collection and analysis of exhaled breath must occur in order to achieve consistency in VOC profile analysis [58-60].

Biological Origins of VOCs

Breath VOCs may be endogenous (generated within the body) or exogenous (absorbed as contaminants from the environment). The origins of many endogenous VOCs have now been identified through an improved understanding of the mechanisms and kinetics of their synthesis [5] (and for review see [32]) (**Table 1.2**). Alkanes and methylated alkanes in breath are markers of oxidative stress [61-67], which are the products from the reaction of lipids with reactive oxygen species (ROS). ROS comprise of oxygen free radicals and hydrogen peroxide and are constantly produced in the mitochondria from where they can leak into the cytoplasm [62]. Cellular anti-oxidant defences such as glutathione (in reduced form) usually protect cells from ROS, but when these defences are insufficient, ROS causes peroxidative damage to proteins, polyunsaturated fatty acids, and DNA [64]. These peroxidative changes to DNA bases may be carcinogenic [68, 69]. Considerable evidence supports the hypothesis that oxidative stress appears to be increased in some cancers [63] including lung cancer [19].

Table 1.2: *VOCs – Biological Origin*

VOCs	Biological Basis
Acetaldehyde	Ethanol metabolism [70, 71]
Acetone	Decarboxylation of acetoacetate and acetyl-CoA
Ethane & Pentane	Lipid Peroxidation[34]
Ethylene	Lipid Peroxidation [72]
Hydrogen & Methane	Gut Bacteria [73]
Isoprene	Cholesterol biosynthesis [74]
Methylamine	Protein metabolism [72]

Breath methyl alkanes are products of lipid peroxidation of polyunsaturated fatty acids in cell membranes, a process that also generates alkanes such as ethane and pentane that are found in exhaled breath [67]. Alkanes are metabolised to alkyl alcohols by cytochrome P450 (CYP) – mixed function oxidase enzymes [75] and a number of studies have demonstrated that these enzymes are activated in lung cancer [76-79]. For example, poly-aromatic hydrocarbons in tobacco smoke induce CYP 1A1 and CYP 1A2 activity, resulting in the accelerated drug metabolism and activation of some procarcinogens [80]. Consequently, the biotransformation of volatile alkanes and mono methylated alkanes that are

produced by oxidative stress may be accelerated by CYP enzymes that have been activated in patients with lung cancer so producing aldehydes, alcohols and ketones in measurable quantities in breath [81].

Acetaldehyde is related to ethanol metabolism [70]. Sources of endogenous acetone often are associated with oxidation of free fatty acids or ketosis as a result of starvation or untreated diabetes [82, 83], and isoprene, an unsaturated hydrocarbon, is endogenously produced as a by-product of cholesterol biosynthesis via the mevalonate pathway in mammals [84-86] and has been reported on breath using various analytical techniques [59, 71, 87, 88]. Methylamine is often linked with protein metabolism as mentioned in **Table 1.2**.

VOCs Identified in Breath of Cancer Patients

A number of studies have detected chemical compounds in breath samples from patients with and without lung cancer [19, 41, 79, 80, 89, 90]. Although the VOCs identified as markers of lung cancer differ between reports, the results have all shown significant variations between exhaled breath of lung cancer patients and healthy volunteers [89]. The source and physiological function of most lung cancer VOCs, however, are still unknown [90]. Some of them could be of exogenous origin and so be inhaled, absorbed from the lungs and metabolised in the body, and the metabolites excreted by expiration. Other VOCs that are of endogenous origin may be generated as products of internal metabolic processes [89]. VOCs found in the breath of lung cancer patients include a wide range of aldehydes, alkanes and methylated alkanes containing C_2-C_{11} carbons.

A recent review by Saalberg (2016) summarises the compounds which have been identified as biomarkers of lung cancer in breath and in-vitro by various research groups, however some of the VOCs stated as biomarkers may be questioned as being of endogenous origin [91]. For example, benzene, ethyl benzene, toluene and xylene isomers which are known to originate exogenously (mainly petroleum and cigarette smoke by-products present in the environment) and styrene, which is also a petroleum product as well as a common GC column bleed product, have all been reported as biomarkers of cancer [20, 22, 23, 27, 66, 92, 93]. Also, some studies have reported other compounds related to cigarette smoking as markers of lung cancer, including alkenes and aromatic compounds such as acetonitrile, 2-methyl furan, 2,5-dimethyl furan, furan, 1,3-cyclohexadiene, 1,3-cyclopentadiene, 2-

methyl-1-butene and 1,4-pentadiene, as well as N, N–Dimethyl acetamide, a known contaminant found originating from the Tedlar bags used for breath sampling [94, 95].

As analytical technology rapidly advances, so has the detection of compounds in breath. Many compounds have been detected whose biochemical origin is unknown and many VOC metabolites reported as biomarkers have been found not to be disease specific. Hence, validation of the biomarkers is a necessary step in developing a specific and sensitive test for the early detection of lung cancer. *In-vitro* analysis of established cancer cell lines is an approach that should help identify endogenous VOCs and define the underlying mechanisms that lead to quantitative or qualitative changes in their concentrations in lung cancer breath.

Cell Culture Metabolomics

Validation of Biomarkers

The integrated analysis of metabolomics and other 'omics' technologies may provide sensitive ways to detect changes related to disease and discover novel biomarkers [96]. Subtle changes in metabolism can be detected by analyses of the products of cellular processes which in turn can lead to development of prognostic models useful for early detection of cancer.

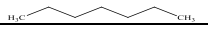
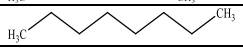
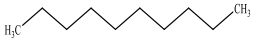
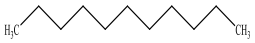
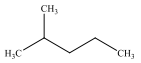
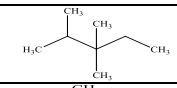
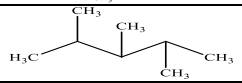
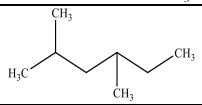
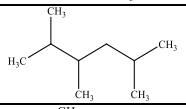
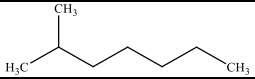
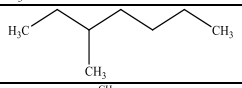
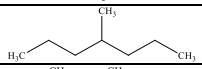
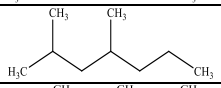
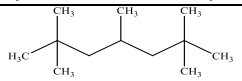
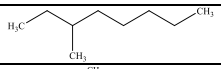
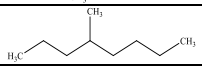
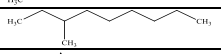
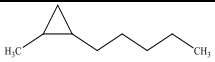
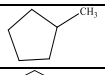
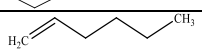
The metabolome is downstream of the transcriptome and proteome, and is considered to be complementary to genomics, transcriptomics, and proteomics [97, 98]. Understanding the metabolome may also assist in identifying intermediate or provisional cancer biomarkers for establishing preventive or therapeutic approaches for health [96].

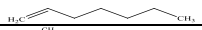
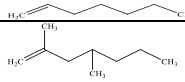
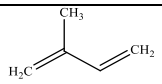
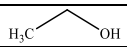
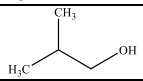
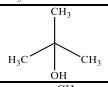
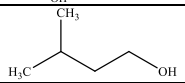
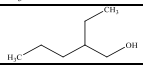
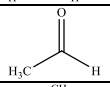
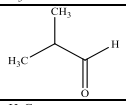
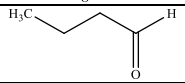
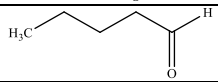
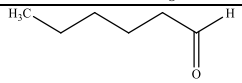
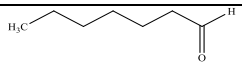
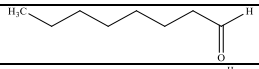
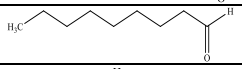
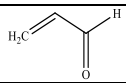
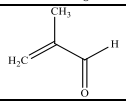
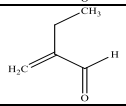
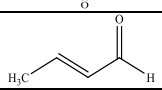
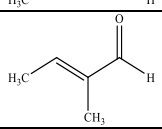
VOCs in breath can derive from cancer cells, healthy cells, immune cells and microbes [99]. Several studies have investigated the release of VOCs from human cancer cells in vitro [93, 99-102], for example headspace on-line measurements by selected ion flow tube mass spectrometry were able to detect acetaldehyde release from the lung cancer cell lines SK-MES and CALU-1 [102].

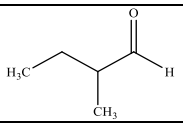
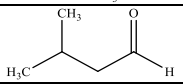
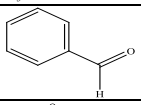
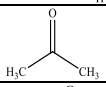
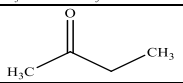
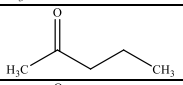
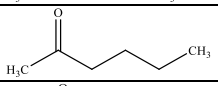
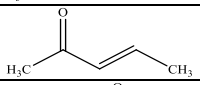
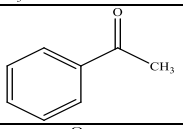
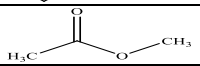
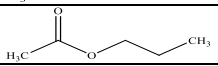
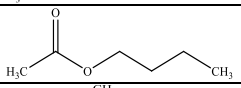
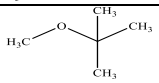
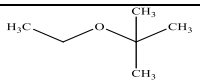
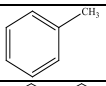
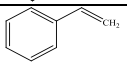
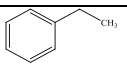
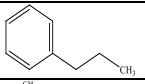
If some breath markers of lung cancer do derive from the cancer cells themselves, then there should be an overlap between the set of VOCs produced by cancer cells in culture and the VOCs detected in the breath of lung cancer patients. Comparison of the VOC profiles of breath analysis and cell cultures

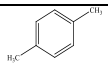
(**Table 1.3**) reveals that, of the 68 VOCs detected in either breath or cell culture, 16 VOCs were detected in both. There were an additional 17 VOCs detected only in breath and 22 found only in lung cancer cell cultures and 13 VOCs found only in controls (non-transformed cell lines). This poor relationship indicates that *in-vitro* culture of lung cancer cells is not a good model for the production of VOCs in breath of lung cancer patients. A more detailed examination of the compounds identified shows that of the 16 compounds common to both cell culture headspace and lung cancer breath, five were straight chain alkanes and methylated alkanes, which is consistent with lipid breakdown associated with oxidative stress [37, 89]. Interaction of reactive oxygen species (ROS) with polyunsaturated fatty acids such as linoleic acid and palmitic acid in the cell membrane results in a series of reactions called lipid peroxidation. During the process of peroxidation of polyunsaturated fatty acids volatile alkanes are formed that can be excreted in breath unchanged or distributed throughout the body, partly metabolized, and then excreted in breath.

Table 1.3: *Comparison of VOCs found in breath and in-vitro analysis of cells cultured under standard normoxic conditions.*

Class	Compound	Structure	Breath	*In-vitro* (Normal Cells)	*In-vitro* (Cancer Cells)	References
Hydrocarbons						
Alkanes *(Straight chain)*						
	Pentane		+[a]	–	–	[5, 20, 103]
	Heptane		+	–	–	[5, 20]
	Octane		+	↑ b(hFB)	↑ (A549)	[20, 100]
	Decane		+	–	↑	[19, 20, 93, 104, 105]
	Undecane		+	–	↑	[19, 94, 101]
Alkanes *(branched)*	2-methyl pentane		+	–	–	[20, 99, 103]
	2,3,3-trimethylpentane		–	↑ (hFB&HBEpC)	↑ (NCI-H2087)	[100, 101]
	2,3,4-trimethyl pentane		–	↑	–	[100]
	2,4-dimethyl hexane		–	↑	–	[100]
	2,3,5-trimethyl hexane		–	↑	↑ (Calu-1)	[100, 101]
	2-methyl heptane		+	–	–	[19]
	3-methyl heptane		–	↑	–	[100]
	4-methyl heptane		–	↑	–	[100]
	2,4-dimethyl heptane		+	–	↑ (Calu-1)	[7, 19, 101]
	2,2,4,6,6-pentamethyl heptane		+	–	–	[20]
	3-methyl octane		+	–	–	[19]
	4-methyl octane		+	–	↑ (Calu-1)	[7, 101]
	3-methyl nonane		+	–	–	[19]
Cycloalkanes	1-methyl-2-pentyl cyclopropane		+	–	–	[19]
	Methyl cyclo pentane		+	–	+	[19, 93]
	Cyclo hexane		+			[19]
Alkenes	1-hexene		+	–	+	[19, 66, 93]

Class	Compound	Structure	Breath	*In-vitro* (Normal Cells)	*In-vitro* (Cancer Cells)	References
	1-heptene		+	–	–	[19]
	2,4,dimethyl-1-heptene		–	↑	↑(A549)	[100]
	2-methyl -1,3-butadiene (Isoprene)		+	–	+	[19, 20, 93]
Alcohols	Ethanol		+	–	↑(A549)	[27, 100]
	2-methyl-1-propanol		–	↑(hFB)	–	[100]
	2-methyl-2-propanol		–	↑(HBEpC)	–	[100]
	3-methyl-1-butanol		+	↑(hFB)	–	[92, 100]
	2-ethyl-1-hexanol		–	↑(hFB)	↑(NCI-H2087)	[99]
Aldehydes	Formaldehyde		+	–	–	[33]
	Acetaldehyde		+	↓	↓(NCI-H2087, CALU-1)	[99, 101, 102]
	2-methyl propanal		–	↓	↓(A549,NCI-H2087, Calu-1)	[99-101]
	Butanal		+	–	↓(A549)	[21, 100]
	Pentanal		+	–	–	[17, 21, 27]
	Hexanal		+	↓	↓(NCI-H1666, Calu-1)	[17, 19, 21, 27, 92, 93, 106, 107]
	Heptanal		+	–	+	[17, 19, 21, 92, 106, 107]
	Octanal		+	↓	–	[17, 21]
	Nonanal		+	–	–	[17, 21]
	prop-2-enal		+	–	↓(Calu-1)	[22, 101]
	2-methylprop-2-enal		–	↓(HBEpC)	↓(A549,NCI-H1666,Calu-1)	[100, 101, 106]
	2-ethylprop-2-enal		–	–	↓(A549,Calu-1)	[100, 101]
	2-butenal		–	↓	–	[100]
	2-methyl-2-butenal		–	–	↓(A549,Calu-1)	[100, 101]

Class	Compound	Structure	Breath	*In-vitro* (Normal Cells)	*In-vitro* (Cancer Cells)	References
	2-methyl butenal		–	↓	↓ (NCI-H2087)	[99, 100]
	3-methyl butenal		–	↓	↓	[99-101, 106]
	Benzaldehyde		–	↓	↓ (Calu-1)	[100, 101]
Ketones	Acetone		+	↑	↑ (A549)	[27, 100]
	2-butanone		+	–	↓ (Calu-1)	[101, 108]
	2-pentanone		+	↑	↑	[7, 15, 27, 100]
	2-hexanone		–	↑	–	[100]
	3-pentene-2-one		–	↓	–	[100]
	1-phenyl ethanone		+	–	–	[19]
Esters	Methyl acetate		–	↑	–	[100]
	n-propyl acetate		–	↑	–	[100]
	n-butyl acetate		–	↓	–	[100]
Ethers	Methyl-tert-butyl ether		–	↑ (HBEpC)	↑ (A549) ↓ (Calu-1)	[98, 99]
	Ethyl-tert-butyl ether		–	↑ (hFB)	↑ (A549) ↓ (Calu-1)	[100, 101]
Aromatics	Benzene		+	↑ (hFB)	+	[6, 19, 20, 93, 100, 104, 105]
	Toluene		+	–	–	[20]
	Styrene		+	–	+	[6, 19, 20, 93, 100, 104, 105]
	Ethyl benzene		+	–	–	[92, 104, 109]
	Propyl benzene		+	–	+	[20, 93, 105]
	Trimethyl benzene isomers		+	–	+	[19, 20, 93]

Class	Compound	Structure	Breath	*In-vitro* (Normal Cells)	*In-vitro* (Cancer Cells)	References
	Xylene isomers		+	–	–	[19]
Heterocyclics	Tetrahydro Furan		–	–	↓ (Calu-1)	[101]
	Pyrrole		–	–	↓ (A549)	[100]
Nitriles	Acetonitrile		–	–	↓ (Calu-1)	[101]

All Compounds in breath are reported as present or not present in lung cancer patients breath.
[b]Compounds in cell culture are either reported as increased or decreased arrows in quantity compared to medium controls or shown as "+" where reported without quantitation (cell lines indicated as hFB – Human fibroblasts, HBEpC – Human Bronchial Epithelial Cells)

The remainder of the compounds common to breath and cell culture were alkenes, aldehydes and aromatic compounds, some of which are also associated with lipid peroxidation [34, 71]. Of the 22 compounds found only in cell culture most were alcohols, ketones, esters and ethers (**Table 1.3**) suggesting that the VOCs produced by cancer cell culture are mostly oxidised breakdown products. Other analytical methods such as PTR-MS and SIFT-MS have also identified alcohols and aldehydes including isopropanol, formaldehyde and acetaldehyde in breath of lung cancer patients [8, 102].

The increased oxidation of alkanes to alcohols, esters and ketones in cell culture is perhaps, not unexpected when the environment in which cells are usually grown is considered. Most laboratories culture cells in air with 5% carbon dioxide, i.e. there is approximately 20% oxygen in the atmosphere surrounding the cells. This is in contrast to the *in-vivo* environment.

Hypoxia

Hypoxia in Cancer and Hypoxia-Inducible Factor

Oxygen availability alters gene expression and metabolism in cells, hence raising the possibility that hypoxia will change the pattern of VOCs produced by the cancer cells. Tumours possess extensive regions of hypoxia relative to the corresponding normal tissue [110]. A number of adaptive responses are initiated during cellular hypoxic stress, including the activation of a group of transcription factors called Hypoxia Inducible Factors (HIFs). Hypoxia-Inducible Factor-1α (HIF-1α) has been extensively studied as an endogenous hypoxia marker and its mechanism of accumulation under hypoxia is well understood [111, 112]. Another study emphasises the importance of hypoxia in tumours and in the

treatment of the disease [113]. HIF-1α regulates an increased production of *VEGF* [114]. *VEGF* induces neovascularisation but in tumours this happens in an irregular fashion and at a slower pace when compared to the proliferation rate of the tumour [115, 116]. This can result in poor blood supply and so further hypoxia.

As described in **Figure 1.1**, in normoxia HIF-1α and HIF-1β subunits are constitutively expressed. While HIF-1α is rapidly degraded by the proteosomal system, the amount of HIF-1β remains constant. In hypoxia HIF-1α escapes degradation, binds with its partner HIF-1β, and together they bind to a hypoxia-response element (HRE) in target genes in association with co-activators such as CBP/P300. This triggers the expression of multiple target genes that enable the tumour cells to adapt to and overcome the conditions of decreased oxygen by increasing oxygen transport, stimulating angiogenesis and regulating glucose uptake and metabolism [117].

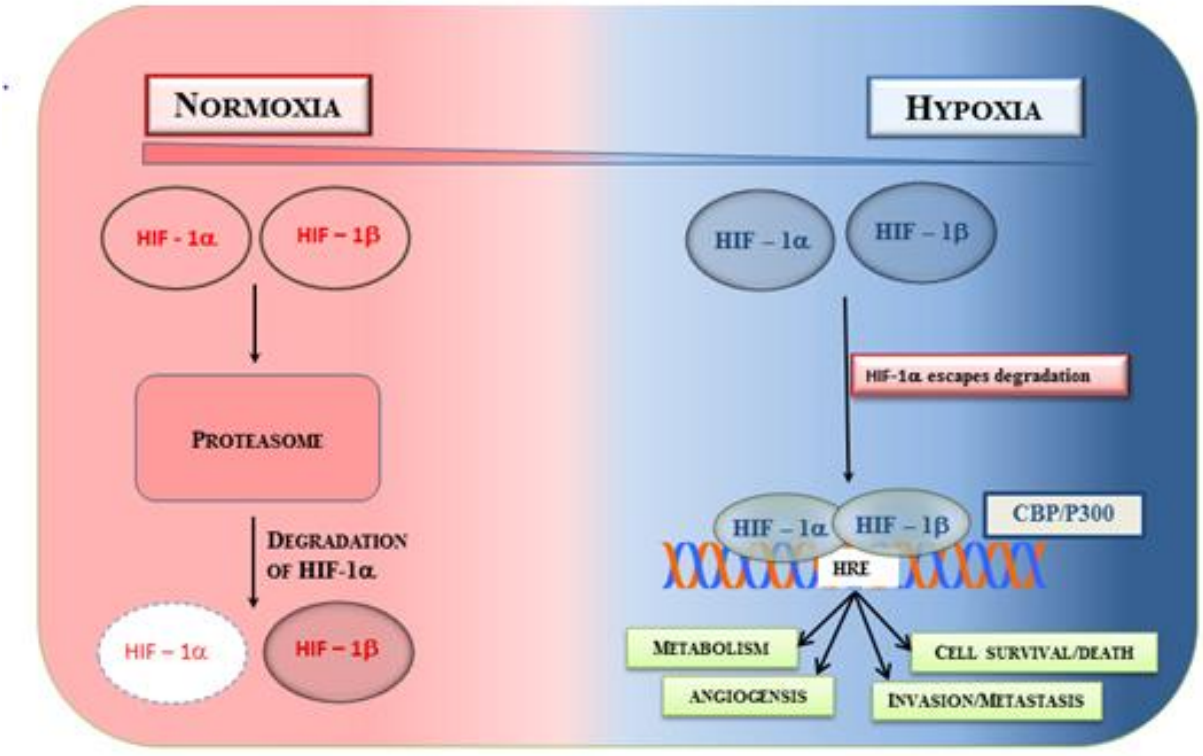


Figure 1.1: *Hif-1a in normoxia and hypoxia*

Activation of the hypoxia-inducible factor (HIF-1α) transcription factor in normoxia and hypoxia. Figure adapted from Brahimi-Horn [118]

Tumour Energy Metabolism – Hypoxia

Warburg Effect

The induction of aerobic glycolysis is one of the strategic metabolic changes that meets the bio-energetic demands of the proliferating tumour [119]. Otto Warburg established that there is enhanced glycolysis in tumours despite the availability of oxygen, a phenomenon known as the "Warburg Effect" [120]. Also, he hypothesised that this phenomenon of "aerobic glycolysis" is due to dysfunctional mitochondria which may contribute to the malignant phenotype [121]. In recent years, it has been established that the activation of oncogenes or tumour suppressors may influence the switch from "cellular respiration" to "aerobic glycolysis" which in turn contributes to the Warburg effect [122-124].

Non-transformed cells also convert glucose to pyruvate but then metabolise pyruvate through the tricarboxylic acid (TCA) cycle and mitochondrial oxidative phosphorylation (OXPHOS) as shown in **Figure 1.2** [118]. The mitochondrial pathway requires oxygen and is much more efficient in ATP production than anaerobic metabolism; producing 38 versus 2 ATP molecules per molecule of glucose [118]. However, in an expanding tumour mass, characterised by low levels of oxygen and a high glucose consumption rate, anaerobic glycolysis can become the predominant pathway of ATP generation [125]. In addition to glycolysis, a recent study has shown that under hypoxia, autophagy is present and is also required to support ATP production [126].

Recent studies also show that increased mitochondrial reactive oxygen species (mROS) (explained in detail in section 4.2) may cause mutations in mitochondrial DNA (mtDNA) resulting in attenuation of mitochondrial function [124, 127].

A mechanistic link between apoptosis and metabolism has also been demonstrated by Iansante (2015) in human hepatocellular carcinoma (HCC). In their studies they show that PARP14 (poly (ADP-ribose) polymerase), an anti-apoptotic protein promotes aerobic glycolysis by regulating the activity of pyruvate kinase, a key regulator of the Warburg effect [128]. In summary, the activation of oncogenes or tumour suppressors along with the stabilisation of HIF can increase "aerobic glycolysis" through enhanced glycolytic flux and/or dysfunctional mitochondria.

Gene Expression – Hypoxia

Under hypoxia, the metabolic switch from a respiratory phenotype to a glycolytic phenotype is achieved by HIF 1 induced up-regulation of glycolytic enzymes and down-regulation of mitochondrial oxidative metabolism [117, 129, 130]. Some of the genes which are activated under deprived oxygen conditions (HIF target genes) and involved in the process of enhanced glucose consumption are listed in **Table 1.4**. When there is excess of glucose available, energy inefficient aerobic glycolysis is preferred over OXPHOS by the cells as glycolysis has the potential to produce ATP at a faster rate, providing the required energy to the rapidly growing tumour [131, 132]. Metabolic reprogramming thus serves two purposes, to rapidly provide ATP from glucose to supply energy to rapidly growing tumour cells, and to protect cells under low oxygen tension.

***Table 1.4**: Target genes of HIF involved in glucose metabolism, cellular growth and proliferation under hypoxia.*

Gene	Pathway involved	Effect of Hypoxia
GLUT1 Glucose transporter	Glucose metabolism	Overexpressed in tumours [133]
Pyruvate Kinase M2 (*PKM2*) Glycolytic enzyme	Glucose metabolism and regulation of HIF1 transcription	Increased expression [134] Decreased expression [135, 136]
Lactate Dehydrogenase-A (*LDHA*)	Glucose metabolism	Increased expression [137]
Pyruvate dehydrogenase kinase 1 (*PDK1/PDHK1*)	Glucose metabolism (Switches metabolism of glucose from OXPHOS to aerobic glycolysis)	Increased expression [138]
Mammalian target of Rapamycin (*mTOR*)	Cellular growth and proliferation	Increased expression [139, 140]

Glucose Transporter – GLUT1

Glucose is an essential and critical carbon source for the biosynthesis of various macromolecules (lipids, proteins and nucleic acids) as well as providing an important substrate for ATP generation [141-143].

Glucose enters the cell through a family of membrane bound facilitated diffusion channels known as Glucose transporters or "*GLUTs*" [144]. Aberrant PI3K/AKT signalling induces the expression of high affinity glucose transporters *GLUT1* and *GLUT3* that are often found upregulated in tumours [133, 145-148]. Transcriptional oncoproteins such as MYC along with HIF 1a induce and activate the expression of all the enzymes in the glycolytic pathway including glucose transporters [149-151].

Pyruvate Kinase –M2 (PKM2)

Pyruvate kinase M2, an isoform of the rate-limiting enzyme pyruvate kinase that catalyses the generation of pyruvate and ATP from phospho-enol pyruvate and ADP is involved in glycolytic energy production and plays a key role in metabolic adaptation under low oxygen tension [137, 152, 153]. *PKM2* is also known to regulate the flux of carbons into both non-oxidative and oxidative branches of the pentose phosphate pathway, the former producing Rib-5-P which is a key intermediate in nucleotide biosynthesis [154, 155] and the latter producing NADPH for ROS detoxification [156-158]. Hence, *PKM2* not only supports proliferation and tumour growth but also prepares the cancer cells to survive under oxidative stress. Furthermore, it has been established that *PKM2* stimulates metabolic changes which favour proliferation, and is a key mediator and promoter of the Warburg effect [136, 159]. However, the biological impact of *PKM2* in carcinogenesis and tumour growth is controversial [144].

Pyruvate Dehydrogenase Kinase 1 (PDHK1/PDK1)

Recent studies show that HIF1α stabilization under hypoxia leads to the expression of Pyruvate Dehydrogenase Kinase 1(*PDHK1*) [138, 160], that phosphorylates and inhibits pyruvate dehydrogenase, limiting the conversion of pyruvate to acetyl - Co A and so shunting pyruvate away from entering Krebs cycle, resulting in a metabolic switch from OXPHOS to aerobic glycolysis [124] (see **Figure 1.2**). Consequently, *PDHK1* induction decreases tricarboxylic acid (TCA) cycle activity so reducing oxygen consumption.

Lactate Dehydrogenase A (LDHA)

Lactate dehydrogenase A (*LDHA*) is an enzyme involved in conversion of pyruvate (generated from glucose) to lactate which is then secreted into the extracellular environment rather than oxidised to completion. By this conversion into Lactate, *LDHA* recovers the NAD+ required to maintain glycolysis

that is crucial to conserve tumour proliferation [161]. *LDHA*, in particular *LDH5* tetramer is known to be overexpressed in lung cancer (NSCLC) under hypoxia [162].

Mammalian Target of Rapamycin (mTOR)

Mammalian target of Rapamycin (*mTOR*) is a kinase which is activated during cell stress conditions and integrates growth signals to enhance cell survival [144, 163, 164]. Protein translation, a vital process for tumour growth is enhanced through Akt-mediated *mTOR* activation [165-167]. It is constitutively expressed in advanced breast cancer [139, 140].

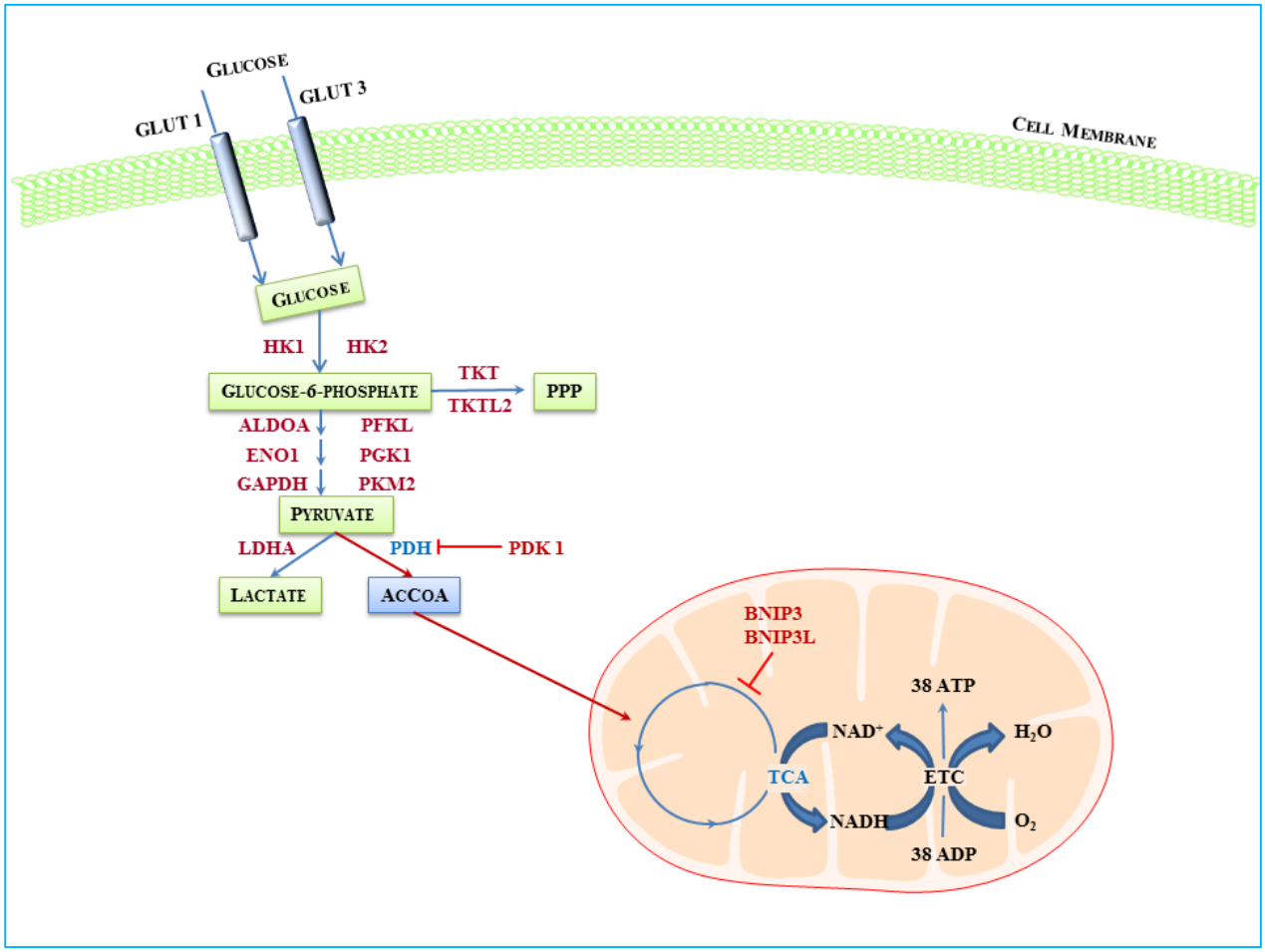


Figure 1.2*: *Metabolic Reprogramming in Hypoxia

Activation of HIF1-α/β activates pyruvate dehydrogenase kinase 1(*PDK1*), an inhibitor of pyruvate dehydrogenase (PDH) which leads to the shunting of pyruvate away from the TCA cycle and instead it is converted to Lactate. Figure adapted from Semenza et al. [168]. Hexokinase 1 and 2 (*HK1*, *HK2*), Aldolase A (*ALDOA*), Enolase 1 (*ENO1*), Glyceraldehyde-3-phosphate (*GAPDH*), Phosphofructokinase L (*PFKL*), Phosphoglycerate kinase 1(*PGK1*), Pyruvate Kinase M2 (*PKM2*), Lactate dehydrogenase A (*LDHA*), Acetyl-CoA (*AcCoA*), BCL2 family of mitochondrial proteins (BNIP3, BNIP3L).

Reactive Oxygen Species

Reactive Oxygen Species such as superoxide anion, hydrogen peroxide, hydroxyl radicals, singlet oxygen, and lipid peroxyl radicals are ubiquitous molecules derived from oxygen that have accepted extra electrons and have the capacity to oxidize other molecules [169]. The formation of free radicals or oxidants is a well-established physiological event in aerobic cells [170]. Most of these chemically reactive molecules are short-lived and react with surrounding molecules while the antioxidants are generated to remove these oxidising agents. An imbalance between oxidant and antioxidant defences causes oxidative stress. This may in turn provoke oxidation of polyunsaturated fatty acids in cellular membranes. Increased ROS levels are associated with several pathophysiological states, such as neurodegeneration, cancer, mutagenesis, cardiovascular diseases and aging [171-173]

Sources of ROS

ROS production is an important component of pathogen killing in phagosomes [174] and peroxisomes [175, 176]. In addition, there are three other important sources of ROS – mitochondria, endoplasmic reticulum (ER) and cell membranes.

The process of generation of ROS begins when molecular oxygen (O_2) is reduced to form the superoxide anion (radical form) (**Figure 1.3**) which can cause potential damage if left unchecked. Superoxide dismutase (SOD1 – cytosolic, SOD2 – mitochondria and SOD3 – extracellular) are a class of metallo proteins that catalyse the conversion of the radical form (superoxide anion molecules) into much less damaging non-radical forms such as hydrogen peroxide (H_2O_2) and water. This hydrogen peroxide combined with superoxide anion are precursors of hydroxyl radicals generated via the Fenton Reaction.

Hydrogen peroxide diffuses easily across the cell membranes and is relatively stable. Several studies identified that H_2O_2 acts as a crucial signalling molecule involved in controlling various processes including apoptosis and cell proliferation [177] by inactivating phosphatases. To maintain redox homeostasis, hydrogen peroxide is scavenged by anti-oxidant enzymes such as catalases and glutathione peroxidases (GTPX). Clearly the mitochondria are the major cellular sources of Reactive Oxygen species (ROS) as by-products of mitochondrial respiratory chain [178]. Also, it has been established that mitochondria derived ROS (mROS) are involved in oxygen sensing [179]. Another source of ROS

results from oxidative protein folding in Endoplasmic reticulum (ER), along with an isoform of NAD(P)H oxidase – NOX4, present on the ER that produces hydrogen peroxide from molecular oxygen via a two electron reduction [176, 180]. Other NADPH oxidases (NOX1-5) and Dual oxidases (DUOX1-2) localised to various cellular membranes form the third functional source of ROS either in the form of superoxide anion or hydrogen peroxide [181-185]. NOX is also known to promote elevated glycolytic activity by providing additional NAD^+, [186]. Various endogenous sources of ROS and the role of hypoxia in production of ROS are illustrated in **Figure 1.3**.

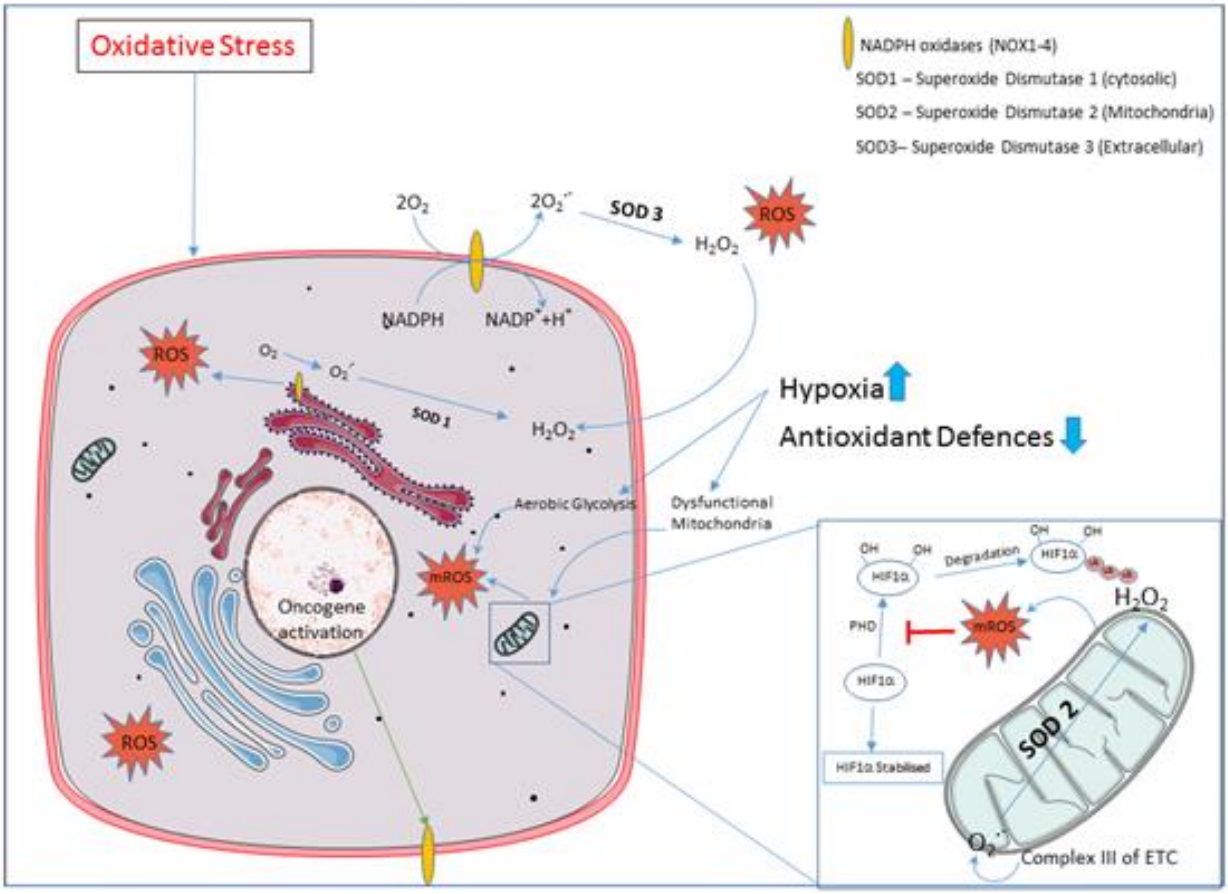


Figure 1.3: *Sources of ROS*

Intracellular sources of ROS under oxidative stress and hypoxia on ROS production. Increased hypoxia (indicated as ⬆) leads to aerobic glycolysis and decreased mitochondrial activity and there is decreased antioxidant defences (indicated as ⬇) during oxidative stress. Stabilisation of HIF1a by ROS from the mitochondrial complex III of electron transport chain which leads to the induction of a series of signalling cascades that aid tumour cells to survive and proliferate. This figure was produced using Servier Medical Art. www.servier.com.

ROS and Hypoxia

ROS is known to inhibit prolyl hydroxylases (PHDs) which promotes the ubiquitination of HIF1a through hydroxylation. Seminal studies by Brunelle et al. demonstrated that not only H_2O_2 but also increased oxidative stress is required for stabilisation and accumulation of HIF1a which then initiates transcriptional targets of hypoxia-response genes that are involved in metabolic reprogramming to promote survival during hypoxia [187, 188]. Several studies have established that ROS derived from mitochondria complex III play a key role in tumorigenesis by inducing hypoxia-mediated transcription [179, 185, 189-193]. Recent studies demonstrated that mitochondrial generated ROS (mROS) play an essential role in stabilising HIF-1a subunit using ρ^o cells (cells lacking mitochondrial DNA) which were incapable of stabilising HIF-1a under hypoxia [189, 194]. As ρ^o cells lost important components of electron transport chain (ETC) they fail to exhibit mitochondrial respiration and hence do not produce mROS [127, 189]. In summary, oxidative stress leads to increased production of ROS which in turn activates transcription factors HIF and post-translational mechanisms which then produce metabolic changes leading to biochemical responses such as cell proliferation and survival.

Lipid Peroxidation

Lipids are a group of compounds with important biological functions in the human body. They make up between 30 and 80% of biological membranes by mass. Lipid peroxidation (LPO) is an oxidative degradation of polyunsaturated fatty acids. Biological cell membranes are made up of polyunsaturated fatty acids (PUFAs) which are known to be important in several physiological cell functions including cell homeostasis [195]. Polyunsaturated fatty acids are susceptible to oxidative degradation as they contain multiple double bonds. Also, various medical disorders such as obesity, atherosclerosis and cancer are characterised by altered levels of PUFAs or of their metabolites [196].

Process of Lipid Peroxidation

The general mechanism of lipid peroxidation consists of three steps: initiation, propagation and termination [197].

Initiation: Lipid peroxidation is a chain reaction – initiated by the interaction of reactive oxygen species (ROS) such as hydroxyl radical (OH*) with a fatty acid (RH) to generate a lipid (fatty alkyl) radical [198-200].

Propagation: The lipid free radical (R*) reacts rapidly with molecular oxygen to form a much more unstable fatty peroxyl radical (ROO*) which can easily extract a hydrogen from adjacent fatty acid to form hydro peroxides and a new lipid free radical.

Termination: The above chain reaction is terminated by the interaction of two radical species to form non-radical products.

Oxidative degradation of bio-membranes can initiate a complex cascade of events resulting in the formation of toxic end products as shown in **Figure 1.4**. There is abundant evidence that these end products (radicals, lipid hydro peroxides, and reactive aldehyde derivatives) modify proteins both in-vivo and in-vitro [203-207]. Of many end products, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are categorised to have mutagenic and carcinogenic potential [208-218]

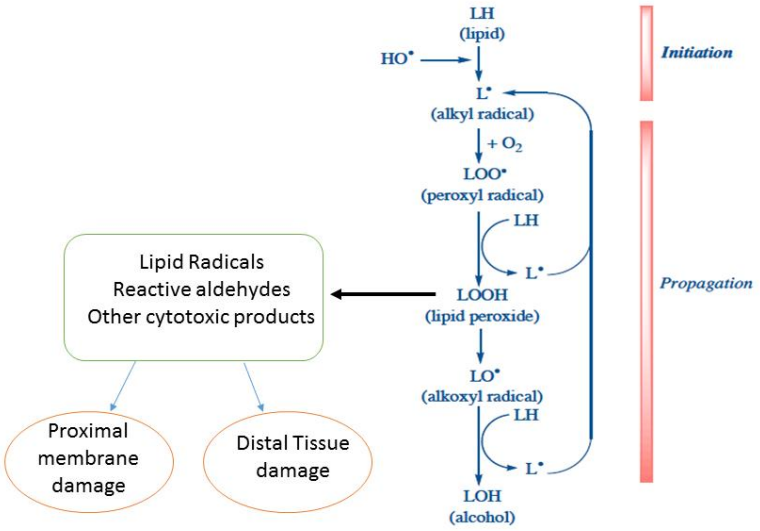


Figure 1.4: *The process of lipid peroxidation*

(figure adapted from Cadenas E) [219]

Historically, ethane (end product of oxidation of ω-3 fatty acid) and pentane (end product of oxidation of ω-6 fatty acid) are known to be the markers of oxidative stress and have been used to assess the extent of lipid peroxidation in subjects by breath test [62].

Altered Cell Metabolism – Effect on VOC Output

Metabolic Reprogramming – VOC profile changes

Metabolic reprogramming of cancer cells is a complex interplay of transcription factors including HIF1a, oncogenes and growth factors. This altered metabolism not only delivers the required conditions for cell survival but may also influence the output of VOCs which can either be investigated *in-vitro* (cell culture metabolomics) or *in-vivo* by breath analysis.

To summarise: oxidative stress causes overproduction of ROS initiating potential oxidative degradation of bio-membranes, DNA damage and proteins which then results in altered cell metabolism that has an effect on VOCs emanating from the cells as described in **Figure 1.5**:

1. Lipid Peroxidation – relatively unstable ROS react with membrane lipids causing irreversible damage by initiating a chain reaction (**Figure 1.4**) which results in the formation of toxic by-products which are capable of modifying proteins.
2. Formation of DNA adducts – activates transcription factors and post-translational mechanisms which lead to altered gene expression. Discrete cohorts of genes can be either up or down-regulated in response to hypoxia.
3. Protein oxidation may also lead to altered cell metabolism.

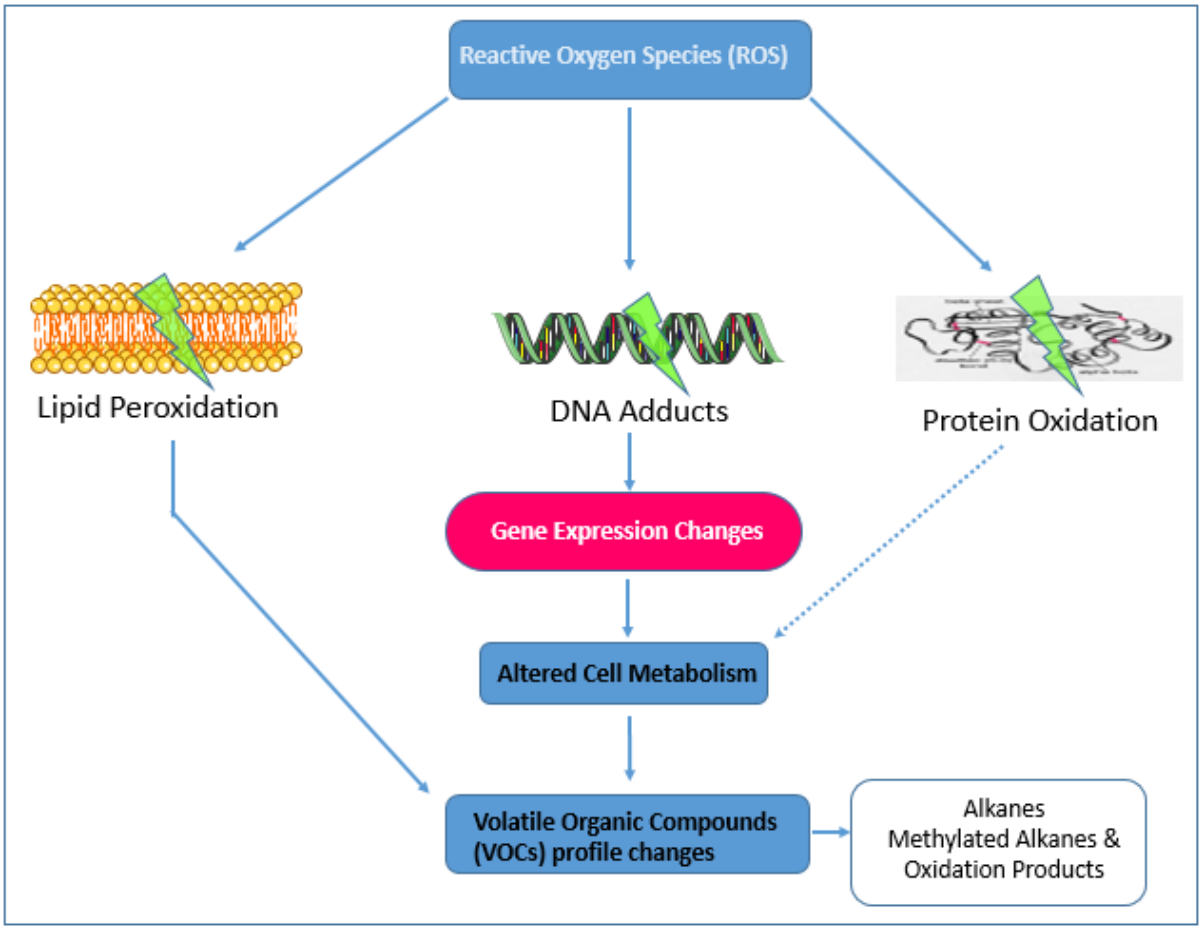


Figure 1.5: *Influence of ROS on cell metabolism*

Downstream effects of ROS lead to altered cell metabolism resulting in changes of VOC profile.

Conclusion

Differences in the VOCs found in breath and in the headspace of cancer cell lines can be attributed to many causes such as different sampling methodology, mass spectral techniques and statistical approaches. Here we propose that cell culture conditions also play a role, as it is known that hypoxia induces autophagy and increased lipid peroxidation. This could explain the presence of alkanes and methylated alkanes found in breath of the lung cancer patients. Little attention has so far been paid to the in vitro culture conditions used to grow cancer cells. The routinely used normoxic culture conditions are likely to produce more alcohols and other oxidised products rather than the methylated alkanes that are more abundant in breath. Hence, oxygen controlled culture conditions should model more closely the in-vivo situation. This approach may help in validating breath VOC markers for diagnosis, clarify further how these compounds are produced and perhaps lead to the identification of novel VOC markers of cancer.

This thesis will investigate the role of hypoxia to mimic the tumour microenvironment in metabolic reprogramming in lung cancer cells in culture, which may have implications for identifying useful markers for early diagnosis in lung cancer patients. This will be investigated based on the following objectives:

1. Gene Expression Analysis of Hypoxia Target Genes.

   To elucidate the role of hypoxia on gene expression and ascertain the ideal culture conditions to better mimic *in-vivo* conditions. To achieve this, the total RNA extracted from the cells cultured under an oxygen gradient (5%, 2% or 1% O_2 plus 5% CO_2) was compared with total RNA from its normoxic (21% O_2 plus 5% CO_2) counterpart. [KPD1]This study will focus on genes and enzymes involved in the glycolytic pathway to determine the metabolic switch from a respiratory to a glycolytic phenotype.
2. Measurement of Lipid Peroxidation (LPO) and Reactive Oxygen Species (ROS) between the two physiological culture conditions

To measure the free-radical mediated damage and to assess the extent of oxidative stress in the cells cultured under low oxygen conditions (2% O_2) that might help to establish the possible biochemical origins of the VOCs.

3. Analysis and comparison of VOCs from cultured cells in-vitro in two different physiological conditions – Normoxia (21% O_2) and Hypoxia (2% O_2) to obtain a VOC profile unique to Cancer Cells cultured in hypoxia. An anticipated outcome of the hypoxic environment would therefore be increased oxidative stress and a large proportion of metabolites being produced as a consequence of lipid metabolism leading to production of alkanes and methylated alkanes, and reduced oxidative degradation.

Hypoxic culture conditions more closely mimic the conditions of cancer cell growth in vivo. Since hypoxia influences cell metabolism, subsequently it may influence the VOCs produced by the cancer cells.

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Chapter 2 - **Materials and Methods**

Introduction

This chapter describes general materials and methods used for the completion of this thesis. Methods specific to particular results chapters are included within the relevant chapter.

Adenocarcinomic Human Alveolar Basal Epithelial cells - A549 Cells

A549 cells were used in all the cell culture experiments and were obtained from the European Collection of Authenticated Cell Cultures through Sigma Aldrich, Australia. A549 cells were originally derived from a 58-year-old Caucasian male and are commonly used in cancer metabolomics studies [1-4].

Cells were grown in HEPES buffered DMEM supplemented with 10% heat inactivated FBS (Gibco ThermoFisher Scientific, Australia), 1% penicillin (Gibco) and 1% streptomycin (Gibco). Cells were grown either in a standard incubator (Brunswick Galaxy 170S) at 37˚C with 5% CO_2 balanced against air (21% oxygen, 74% nitrogen), herein referred to as normoxia, or in a hypoxic chamber (Brunswick Galaxy 48R) at 5% O_2, 2%O_2 or 1% O_2 plus 5% CO_2 balanced against nitrogen at 37^0C, herein referred to as hypoxia. Cell viability was monitored using trypan blue dye exclusion and a Countess cell counter (ThermoFisher Scientific, Melbourne, Australia) after each sampling to determine the number of viable cells. The cell viability was greater than 98% for all the experiments.

VOC Analysis

Sample Preparation for GCMS Analysis

After growing to sub confluence, A549 cells were seeded at 10 million cells per 50 ml media in a 250 ml glass conical flask with stirring to maintain cells in suspension. The flask was sealed with a Teflon stopper and glass insert through which a coated fused silica fibre could be introduced for the sampling of volatile compounds in the headspace above the cell suspension. Flasks were placed on a magnetic stirrer (HD Scientific, Sydney, Australia) and were incubated in normoxia (21% O_2) and hypoxia (2% O_2) for 24hrs. The VOCs extracted from the headspace of culture were compared against the VOCs extracted from the headspace of media without cells. To eliminate the environmental VOCs, the air from both the incubators was sampled and analysed.

GCMS Analysis

Volatile substances in the samples were collected by solid-phase micro extraction (SPME) using commercially available 75µm Carboxen-PolydimethylSiloxane (CAR/PDMS) fibres (Supelco, Sigma Aldrich, Australia). The samples were loaded manually using a SPME holder (Supelco) and released by thermal desorption onto the injection port of the GCMS. The fibres were conditioned prior to use for all the GCMS analyses following the protocol listed by the manufacturer. Additionally, the SPME cleanliness was assessed by performing blank run with and without the fibres before each experiment.

VOC analyses were performed using a Shimadzu QP2010 Ultra gas chromatography mass spectrometry (GCMS) equipped with mass selective detector (single quad mass spectrometer) (Shimadzu Scientific Instruments, Oceania Pty Ltd.).

A 25m x 0.32mm x 5µm PoraBond Q PLOT column was used (Pacific Laboratory Products, Blackburn, Australia) with an initial temperature of 90^{o}C for 5 min followed by a temperature ramp of 5^{o}C/min up to 250^{o}C which was held for 4 min. The temperature was then increased at a rate of 5^{o}C/min to 280^{o}C and held for 3 min. The helium flow rate was 1.48 ml/min. Ionisation of separated compounds was performed by Electron Impact ionisation at 70 e.V. Total ion current (TIC) mode with full scan range of m/z=30 to m/z=600 was employed. The detected substances were identified by spectral match with NIST 2011 Spectral library (Stanton Scientific, Australia) and retention times obtained from the standards where these were available.

Calibration Curve

Preliminary calibration was performed using a specialty mix of alkanes with C_2-C_6 carbon skeleton at a concentration of 100 and 1000 PPM (Scotty Analysed Gases, Supelco Analytical, USA). After the qualitative analysis was performed to determine the VOCs unique to hypoxic conditions, a five-point linear calibration curve was obtained for compounds of interest using a custom mixture of standard compounds (AccuStandard Inc., Connecticut, USA), where available (n-pentane, 3-methyl hexane and 2-nonanone) at concentrations of 10, 30, 100, 300 and 1000 ppm. The standard mixtures were collected using 75µm CAR/PDMS by solid-phase micro extraction and were loaded using the auto sampler (AOC 5000, Shimadzu Scientific Instruments) and released by thermal desorption onto the injection port of

the GCMS. For each compound the regression was linear from 10 to 300 ppm. LOD and LOQ were calculated from the linear regression using the formula LOD = 3 x SD/slope and LOQ = 10 x SD/slope where SD is the standard deviation of the Y-residuals.

Gene Expression

RNA Extraction

Total RNA was extracted from stationary cultures of A549 cells using TRI-Reagent (Sigma Aldrich, Castle Hill, Australia) according to the manufacturer's protocol. Cells were collected in TRI-Reagent and RNA was separated in chloroform by centrifugation at 12,000 RPM for 15-minutes at 4^0C. RNA was precipitated with isopropanol and incubation on ice for 10 minutes. Samples were then centrifuged at 10,000 RPM at 4^0C for 10 minutes. The resulting RNA pellet was washed twice in 75% ethanol before final resuspension in 25 µl of RNAase free water. The quality and quantity of RNA was measured using a Nanodrop spectrophotometer (Thermo Scientific). All RNA samples used had a 260/280 quality ratio of 1.95-2.00.

cDNA Synthesis

cDNA was synthesised following the manufacturer's instructions using a High Capacity cDNA synthesis kit (ThermoFisher Scientific, Australia). A 20 µl reaction was prepared by adding a solution containing reaction buffer, random primers, free nucleotides and reverse transcriptase to a 2 µg of total RNA and incubated according to the protocol in **Table 2.1**: cDNA synthesis temperature conditions. A final working concentration of 5 ng/µl was obtained by diluting the completed reaction to 400 µl in nuclease free water. The resulting cDNA obtained was stored at -20°C.

***Table 2.1**: cDNA synthesis temperature conditions*

	Annealing	**Extension**	**Inactivation**	**Hold**
Temperature	25°C	37°C	85°C	4°C
Time	10 minutes	120 minutes	5 minutes	∞

Primer Design

Primers were designed using the NCBI Primer Blast Tool and purchased from Bioneer Pacific (Kew Australia). All primers were analysed using BLAST to attain target gene specificity and avoid amplification of genomic DNA, with at least one primer in the set spanning an exon-exon junction. To ensure that all the PCR reactions could be run with the same annealing temperature, all the primers were designed to have a melting temperature close to 60°C.

PCR Primer sequences:

ACTB	5´-CGCGAGAAGATGACCCAGAT-3´	5´-GAGTCCATCACGATGCCAGT- 3´
EEF2	5′-AGGCTGCCATGGGCATTAAA- 3′	5′-AGGCGTAGAACCGACCTTTG-3′
GLUT1	5´-TGGCATCAACGCTGTCTTCT- 3´	5´-AGCCAATGGTGGCATACACA- 3´
LDHA	5´-GGAACTGGATCGGTTGGTGT -3´	5´-AAGGGCTGCCATGTTGAAGA- 3´
mTOR	5´-AAGCCGCGCGAACCTC- 3´	5´-CTGGTTTCCTCATTCCGGCT – 3´
PDHK1	5´-CTCAGGACACCATCCGTTCA- 3´	5´-ACCATGTTCTTCTAGGCCTTTCAT-3´
PKM2	5´-ATGCAGCACCTGATAGCTCG- 3´	5´-AGGCTCGCACAAGTTCTTCA – 3´

Real-time PCR

A RealPlex PCR detection system (Eppendorf, North Ryde, Australia) was used to perform quantitative PCR (qPCR) in triplicate on at least three independent RNA preparations. Target cDNA levels were analysed in 10 µl reactions with SensiMix SYBR No-ROX (Bioline, Alexandria, Australia). qPCR was performed using 4 µl of cDNA template (20 ng) and primers at a concentration of 1 µM following the parameters as described below.

PCR Cycle Parameters

The thermal cycling conditions for activation were 2 minutes at 95^0C followed by 40 cycles of 15 seconds at 95^0C for denaturation, 15 seconds at 59^0C for annealing and 10 seconds at 72^0C for extension.

Gene Expression Analysis

The relative expression formula was used to analyse the real-time qPCR data. Eukaryotic elongation factor 2 (*Eef2*) or β-Actin (*ACTB*) were used as reference gene (REF) and the gene of interest (GOI) cycle threshold (Ct) was made relative to the REF gene using the following equation:

$$\mathbf{Ct}_{GOI} - \mathbf{Ct}_{REF} = \Delta\mathbf{Ct}$$

Where $\mathbf{Ct}_{GOI}$ is the cycle threshold for the gene of interest, $\mathbf{Ct}_{REF}$ is the cycle threshold for the gene of reference. The ΔCt values were transformed to fold changes in expression using the relative expression equation:

$$\textbf{Relative Expression} = 2^{-\Delta Ct}$$

To test for statistical significance, two-way ANOVA analysis with post-hoc Bon Ferroni test was performed on ΔCt values in the normoxic versus the hypoxic group as the least transformed relative data ΔCt, is the most appropriate value to analyse [5]. The threshold of statistical significance was set to < 0.05. The standard deviation of the relative expression of at least three independent replicates are shown as the error bars on gene expression graphs.

Confocal Microscopy

A Nikon Eclipse Ti-E confocal microscope with an attached 37°C chamber was used to perform live cell imaging. Cells were washed twice with PBS buffer and 1 ml of fresh warm media (without phenol red indicator) and then imaged. 25 mM HEPES buffer was included in the imaging media to buffer pH fluctuations brought on, by the change from 5% CO2 in the cell culture incubator to the conditions of microscope incubation chamber. To capture total cell fluorescence, cells were imaged using a 60X oil immersion objective over a 15 µm z-range with an image captured every 1 µm.

Lipid Peroxidation Imaging

A ratiometric fluorescent reporter for lipid peroxidation, Image-iT Lipid peroxidation kit (ThermoFisher Scientific, Australia), was used to measure free-radical damage in cells. Upon oxidation, the fluorescence emission peak shifts from ~590 nm (red) to ~510 nm (green). A549 cells grown to

60% confluence were washed twice in PBS and loaded with 1µl of Lipid peroxidation sensor (ThermoFisher Scientific) in 1 ml DMEM medium with 10% FBS and without phenol red and incubated at 37°C for a total of 30 minutes. For a positive control, cells were incubated for 20 minutes with 100 µM of cumene hydroperoxide to induce lipid peroxidation. Imaging of the treated cells was performed as described above. All experiments were performed on at least three independent samples.

Fluorescent ROS Imaging

Increased oxidative stress in live cells can be assessed by the measurement of ROS production using an Image-iT LIVE Green Reactive Oxygen Species Detection kit (Life Technologies) according to the manufacturer's protocol. Cellular ROS was labelled with a final concentration of 25 µM carboxy-H_2DCFDA (5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate), a compound that fluoresces when oxidised (Excitation/ Emission: 492-495 nm/517-527 nm) along with 1 µM of Hoechst 33342 was added in the final 5 minutes of incubation. Cells were washed twice with PBS before adding 1 ml of fresh warm media prior to imaging. A known inducer of ROS production, *tert*-butyl hydrogen peroxide (TBHP) was used for a positive control with a final working concentration of 100 µM and the cells were incubated for 60 minutes and imaged as described above. Cells were imaged using 60X objective with oil immersion and the experiment was repeated on at least three individual samples.

Fluorescence Intensity Analysis

1) *Quantification of Lipid Peroxidation in A549 Cells.*

   The extent of lipid peroxidation was measured using a ratiometric sensor where the fluorescence emission peak shifts from red (590nm) to green (510nm) when oxidised. The pixel density analysis was done on each individual cell using ND2 software, where the minimum ratio was kept at zero and maximum at two. Also, the background was removed by selecting a region without any visible fluorescence for all the groups. Bright spots on the acquired image, where pixel saturation was detected (using ND2 software) have been excluded by drawing regions of interest (ROIs).

2) *Quantification of ROS in A549 Cells.*

Pixel density analysis of ROS production obtained from the confocal experiments was performed using ImageJ software (http://imagej.nih.gov/ij/index.html) on each individual cell for all the groups after background subtraction.

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Chapter 3 - **Gene Expression Analysis – Oxygen Gradient Vs Normoxia**

Abstract

Metabolic reprogramming is a hallmark of cancer. Hypoxia is known to induce similar adaptive changes in cell metabolism that include a switch from oxidative to glycolytic metabolism, which is mediated at the transcriptional level by HIF1. Metabolic adaptation to hypoxia is facilitated by the activation of genes involved in the glycolytic pathway by the HIF1 subunit HIF1a. Furthermore, mitochondrial function is reduced by HIF1 through upregulation of pyruvate dehydrogenase kinase 1 (*PDHK1*), which inactivates the key TCA cycle enzyme pyruvate dehydrogenase (PDH). This shunts pyruvate away from the TCA cycle and towards conversion to lactate by the hypoxia-inducible enzyme *LDHA*. In this study, we reveal the effects of oxygen concentration on gene expression in A549 lung cancer cells. A quantitative RT-PCR analysis demonstrated a significant upregulation of the glucose transporter (*GLUT1*) and the key TCA regulatory gene *PDHK1* in cells cultured under 2% and 1% O_2. This study demonstrates that oxygen concentration *in-vitro* plays a fundamental role in regulating metabolic reprogramming in A549 cells through altering the expression of HIF1 target genes.

Introduction

The biosynthetic pathways in cells are known to be altered to promote adaptation and improve survival under environmental stress. A good example is the adaptive response of cancer cells to hypoxia where the metabolic switch from oxidative phosphorylation (OXPHOS) to aerobic glycolysis is an important step in cell proliferation and survival. In 1920, Otto Warburg discovered that tumours show increased glucose consumption and altered glucose metabolism despite the availability of oxygen, a phenomenon known as the "Warburg Effect" [1]. This principle is utilized in clinical settings, through uptake of isotope labelled deoxyglucose using PET (positron emission tomography) imaging, also known as FDG-PET, to image increased glucose uptake by tumours.

Previous studies have established that HIF 1 is stabilised in hypoxia and is a "master regulator of oxygen homeostasis" [2, 3]. It also plays a crucial role in the pleiotropic response observed under hypoxia [4-11]. HIF 1 not only activates the transcription of genes encoding proteins that mediate glycolysis, glucose entry etc., but in cancer it also promotes invasion and metastasis [11-15] (see **Figure 1.2**)

In A549 cells, despite the low yields of ATP (only 2ATP), aerobic glycolysis is preferred over the TCA cycle (30 or more ATP), and is achieved by increasing both glucose up-take and its conversion to pyruvate. This is attained by increasing HIF-mediated expression of genes and enzymes involved in the glycolytic pathway including the glucose transporter, *GLUT1* [16, 17]. The pyruvate is redirected away from OXPHOS and converted to lactate by increased HIF-mediated expression of two key enzymes – lactate dehydrogenase A (*LDH-A*) [18] and Pyruvate dehydrogenase kinase 1 (*PDHK1*) [2, 3, 19]. Another pathway which is modified by HIF is the mammalian target of rapamycin (*mTOR*). Hypoxia suppresses expression of *mTOR* to save on energy-consuming protein synthesis so enhancing cellular adaptation [7, 20-22]. The glucose metabolism is also controlled by the oncoprotein, MYC which stimulates the transcription of *LDH-A, PDHK1* and Pyruvate kinase M2 (*PKM2*) [23-25] and increased expression of *PKM2* is observed in many cancers including lung cancer [26, 27]. Hence, the Warburg effect is the result of activation of several pathways largely mediated by HIF1 and MYC.

In the present study, we investigate the effect of low oxygen concentrations on gene expression of HIF 1 target genes (*PDHK1, mTOR, PKM2 and LDH-A*) including the glucose transporter *GLUT1* in A549

lung cancer cells. This was achieved by qPCR on A549 cells exposed to 1% O_2, 2% O_2 and 5% O_2 compared to standard cell culture conditions (21% O_2). The results showed that significant gene expression changes were detected at 2% O_2 and 1% O_2 compared to controls.

Methods and Materials

Cell Culture

The lung carcinoma cell line A549 was grown in DMEM with HEPES buffer media supplemented with 10% heat inactivated FBS (Gibco), 1% penicillin (Gibco) and 1% streptomycin (Gibco) at 37˚C with 5% CO_2. The cells were exposed to Nitrogen balanced against air to give Oxygen concentrations of 1% O_2, 2%O_2 or 5% O_2 plus 5% CO_2 at 37^0C in a hypoxic chamber. Normoxia was air (approximately 21% O_2, 74% Nitrogen) and 5% CO_2 at 37^0C in a conventional incubator. Each experiment was performed with a Normoxic control. Cell viability was monitored after each sampling to ensure the presence of viable cells using a Countess cell counter (Life Technologies Pvt. Ltd, Australia.) which was measured to be greater than 98% for all the experiments.

RNA Extraction and cDNA Synthesis

Gene expression analysis was performed as described in Chapter 2: Methods and Materials (*Section 2.4*) in this thesis. Briefly, total RNA was extracted from A549 cells using TRI-Reagent and the phenol/chloroform method. After cDNA synthesis gene expression was measured using specific primers and quantitative real-time PCR. Expression was compared to *β-Actin* (*ACTB*) and *Eukaryotic Elongation Factor* (*EEF2*) as controls.

Statistics

Data from three independent biological replicates of the qPCR experiments were analysed for statistical significance by two-way ANOVA with Bon Ferroni post-hoc tests. Changes were considered statistically significant when the P value was ≤ 0.05. All statistical analysis was performed on ΔCt values as the least transformed data. The standard deviation of the relative expression of at least three independent replicates are shown as the error bars on gene expression graphs.

Results

Cellular exposure to hypoxia results in altered gene expression

To elucidate the role of hypoxia on gene expression in A549 cells, expression levels of hypoxia-associated genes in cells cultured under different oxygen conditions were investigated. The HIF 1 target genes analysed included the GLUT1 glucose transporter and genes involved in the glycolytic pathway (*GLUT1, PDHK1, PKM2, mTOR and LDHA*, see Chapter 1; Section 3.2, **Table 1.4**). Data analysis using either control gene, *ACTB* or *EEF2*, gave similar results. The data shown in this results section were derived using the *ACTB* gene as the control (see Appendix I for *EEF2* data).

The glucose transporter *GLUT1* was increased in expression level in A549 cells cultured under 1% and 2% O_2 compared to cells cultured under normoxic conditions (

Figure 3.1A, 1% O_2 p = 0.04, 2% O_2 p = 0.001). Cells cultured under 1% and 2% O_2 showed between 3 and 5 fold increased expression compared to normoxic controls. There was no statistically significant increase in GLUT1 expression in 5% O_2.

PDHK1 expression was also increased in A549 cells cultured under 1% O_2 and 2% O_2 (3-fold and 5-fold increase compared to controls, P = 0.02 and 0.001 respectively,

Figure 3.1B). Expression levels of *PDHK1* in cells cultured in 5% O_2 were similar to normoxic cultures.

There were no statistically significant changes observed in the levels of expression of *Pyruvate Kinase M2* (*PKM2*), *mammalian Target of Rapamycin (mTOR)* or *Lactate Dehydrogenase A (LDH-A)* (

Figure 3.1C, D and E). Each of these genes had Ct values of 22.1, 23.8, 28.0 suggesting low expression in controls, which did not alter with changes in O_2.

Figure 3.1: *Relative mRNA expression of HIF1 target genes.*

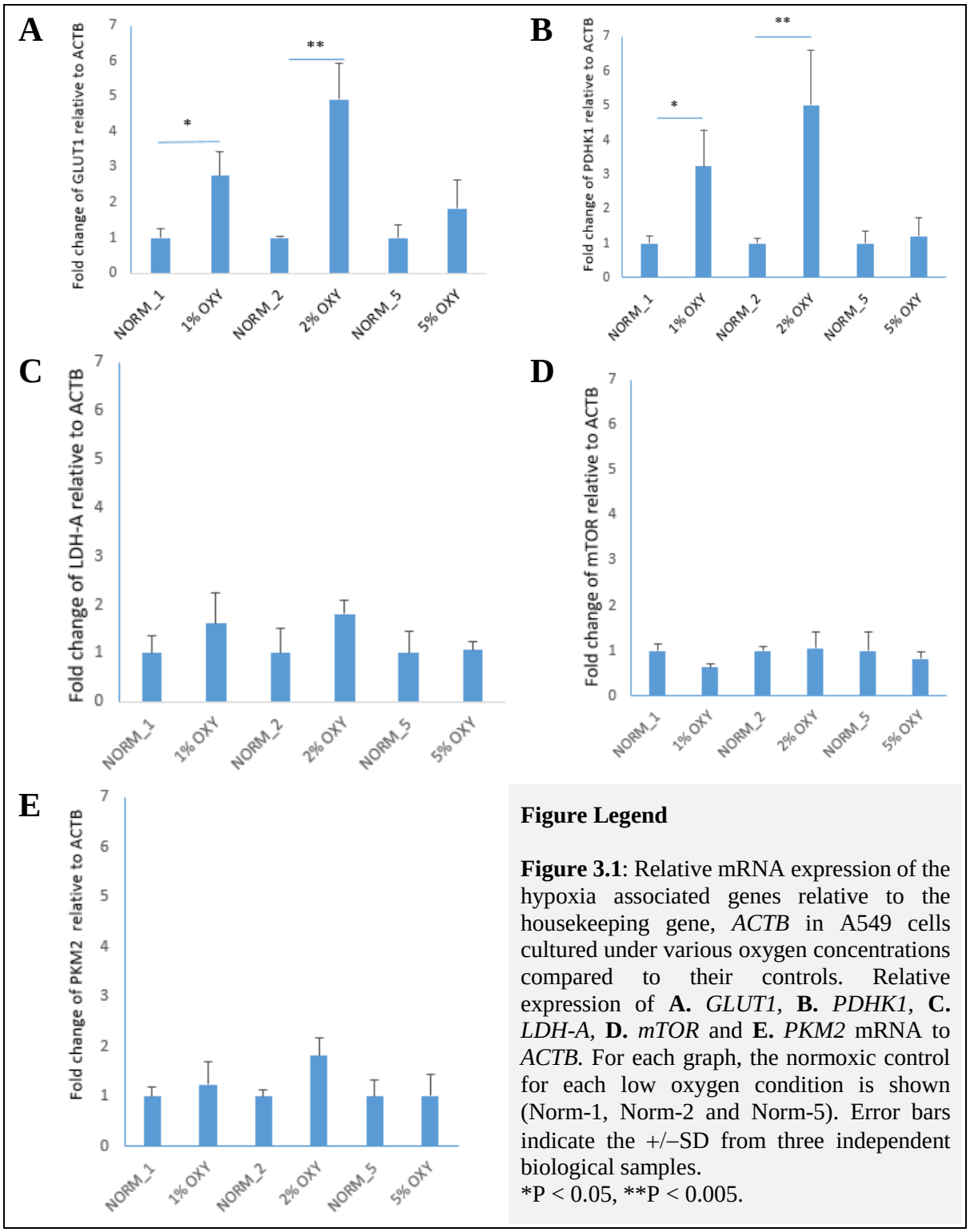

Figure Legend

Figure 3.1: Relative mRNA expression of the hypoxia associated genes relative to the housekeeping gene, *ACTB* in A549 cells cultured under various oxygen concentrations compared to their controls. Relative expression of **A.** *GLUT1*, **B.** *PDHK1*, **C.** *LDH-A*, **D.** *mTOR* and **E.** *PKM2* mRNA to *ACTB*. For each graph, the normoxic control for each low oxygen condition is shown (Norm-1, Norm-2 and Norm-5). Error bars indicate the +/−SD from three independent biological samples.
*P < 0.05, **P < 0.005.

Discussion

Hypoxia results in metabolic reprogramming through HIF mediated changes in a substantial number of genes involved in metabolism [2, 19, 28-30]. Aerobic glycolysis in cancer cells is of particular interest as it plays a crucial role in cellular adaptation and cancer invasion and metastasis [11-15]. HIF 1 plays pivotal roles in the metabolic transformation from respiratory to glycolytic phenotype in three ways; first by increasing glycolytic flux from glucose to pyruvate through activation of upstream glucose transporters and glycolytic enzymes, second by inducing the expression of *PDHK1* that inhibits the pyruvate from entering the TCA cycle and third by inducing the expression of *LDHA* which converts pyruvate to lactate [2, 31]. Cancer cells usually show the Warburg effect where glycolysis is the preferred pathway for glucose metabolism despite the availability of oxygen [32, 33].

Increased glucose uptake is facilitated by high levels of expression of the glucose transporter, *GLUT1*. In the cells cultured under hypoxia a significantly higher level of expression of *GLUT1* was observed, which is consistent with increased uptake of glucose identified in other studies of A549 cells [34]. Furthermore, there was a difference in *GLUT1* expression in cells cultured under differing levels of hypoxia with a 3-fold change in the cells under 1% O_2 compared to a 5-fold change under 2% O_2.

Pyruvate dehydrogenase kinase 1 (*PDHK1*) regulates glucose metabolism through phosphorylation of the pyruvate dehydrogenase (PDH) complex leading to its inactivation. Several studies have reported an increased expression of *PDHK1* in cancer cells including gastric and acute myeloid leukaemia [35, 36]. The suppression of PDH by *PDHK1* results in the reduction of mitochondrial respiration, thereby supporting the cellular adaptation to hypoxia. The activation of *PDHK1* may be a strategic regulatory switch leading to the Warburg effect. The results of this study show overexpression of *PDHK1* in cancer cells cultured under hypoxic conditions (1% O_2 and 2% O_2 only) compared to their normoxic controls. A significant 3-fold increase in the expression of *PDHK1* was detected in cells exposed to 1% O_2 and a significant 5-fold increase in cells exposed to 2% O_2 compared to normoxic controls. This upregulation in hypoxia is consistent with other studies [2, 3].

One interpretation of increased *GLUT1* and *PDHK1* in A549 cells grown in hypoxia is that A549 cells still utilise OXPHOS when cultured in normoxic (environmental air) conditions. Liu et al., using

NSCLC tissues and A549 cell lines, demonstrated a significant upregulation of *PDHK1* and a pivotal role in metabolic reprogramming under hypoxia leading to what they referred to as "enhanced" Warburg effect [37]. Similarly, several studies have established that cancer cells in hypoxia show increased glycolysis or increased *GLUT1* and *PDHK1* expression, [2, 3, 38]. Furthermore, recent studies have established that the overexpression of *PDHK1* attenuates mROS generation and protects cells from hypoxia-mediated apoptosis [2, 39-41]. These results suggest a continued role for mitochondrial OXPHOS in cancer cells alongside the increased preference for glycolysis as an energy source. In this context the increased *GLUT1* and *PDHK1* mRNA expression in hypoxia demonstrated here indicates a further shift towards glycolysis and away from OXPHOS in A549 cells.

Although, an upregulated expression of *PKM2, mTOR* and *LDHA* has been indicated in the previous studies on various tumours (Chapter 1, Table 1.4), in A549 cells only *PKM2* has been reported to be overexpressed. Interestingly, there were no statistically significant changes observed in the expression of the above stated genes in this study on A549 cells. It is noteworthy that a recent study found that *mTOR* responds to reduced pH rather than hypoxia per se as previously believed [42]. The cells in this study were grown in HEPES buffered media to prevent acidification so this may explain no change in *mTOR* expression.

There were no significant gene expression changes observed in the cells exposed to 5% O_2 *in-vitro* possibly due to the fact that 5% of oxygen is close to physiological normoxic conditions. The pO_2 in the muscle cells *in vivo* is close to 5% O_2 [43], whereas in cancer cells the diffusion of oxygen is far less than in healthy tissues due to the high rate of cell proliferation in cancer which results in chaotic vasculature giving rise to hypoxic regions (Section 3.1, Chapter 1 of this thesis). The diffusion limit of oxygen within tissues has been measured to be 150μm [44-47]. This means that once a tumour grows to greater than 300 μm diameter or approximately 15-20 cells across, the cells in the centre experience hypoxic conditions. The findings reported here are consistent with previous studies, where synchronised activation of glucose absorption and metabolic reprogramming occurs in cancer cells and has been identified as a potential therapeutic target [48].

Conclusion

The understanding of how hypoxia effects gene expression and hence tumour progression and survival has attracted substantial interest. There are many *in-vitro* studies analysing gene expression in cancer cells. To ensure such studies are physiologically relevant, there is a need to identify the ideal culture conditions that best mimic *in-vivo* conditions. From the above findings, cells cultured under 2% O_2 have increased *GLUT1* and *PDHK1* expression and possible reprogramming of glucose metabolism. Hence in order to mimic the *in-vivo* conditions found in tumours in cell culture models, it is preferable that the percentage of oxygen should be maintained at less than 5%.

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Chapter 4 - **Lipid Peroxidation and ROS activity in A549 cells cultured under Hypoxia.**

Abstract

The activity of reactive oxygen species (ROS) in cancer cells under low oxygen conditions is well established but the damage it causes still remains unclear. Lipid peroxidation (LPO) is a complicated process that involves the formation and propagation of lipid radicals that cause structural damage and produce a variety of cytotoxic metabolites. These metabolites modify proteins both in-vivo and in-vitro, and can influence cell metabolism. This led to our hypothesis that hypoxic cultures have altered lipid peroxidation and ROS production. Here we investigated whether hypoxia stimulates free-radical mediated damage to lipids. To achieve this, LPO and ROS were measured in A549 cells using fluorescent reporter dyes and confocal microscopy. Importantly, the peroxidation-sensitive change in fluorescence of BODIPY® 581/591 C11 at red (590 nm) relative to that at green (510 nm) facilitated ratio-metric assessment of LPO in cells. An increase in LPO was observed in the cells cultured under low oxygen conditions. However, there was a slight decrease in the production of ROS in hypoxic cultures. In conclusion, hypoxia stimulates LPO free-radicals and attenuates ROS radicals in A549 cells, the combined effect of which may serve to equip the cells to adapt to and proliferate under low oxygen conditions.

Introduction

Lipid peroxidation is the oxidative degradation of cellular lipids that are rich in polyunsaturated fatty acids (PUFAs) by reactive oxygen species. Oxidative modification of PUFAs affects cell membrane properties and signal transduction pathways [1, 2] and is implicated in various pathological processes such as atherosclerosis, cancer, diabetes, acute lung injury [3-7] as well as the neurodegenerative disorders including Alzheimer's and Parkinson's disease [8-10].

Hypoxia is associated with increased lipid peroxidation products in animal tissues (for review see [11]) and several other studies have reported increased levels of MDA (malondialdehyde), a known biomarker of lipid peroxidation, in human breast cancer and lung cancer [12-15].

Free radicals are generated as a by-product of cellular respiration (mitochondrial respiratory chain) [16] and other endogenous sources (see Section 4.1, Chapter 1 of this thesis). Due to their instability and chemical reactivity, they readily react with the cellular membranes which are rich in lipids producing alkyl radical. Once the alkyl radical is generated the cascade of lipid peroxidation follows (see Section 5.1, Chapter 1) causing oxidative damage to the cells under oxidative stress. Anti-oxidant enzymes such as catalases and glutathione peroxidases scavenge ROS to protect cells from cytotoxic effects. Additionally, the pentose phosphate pathway, generates ribose 5-phosphate and nicotinamide adenine dinucleotide phosphate (NADPH) which are vital to defend the cells from ROS [17-19].

It has been established that in many cancer cells, hypoxia induces the expression of an isoform of glycogen phosphorylase (PYGL) that alters glycogen metabolism leading to the generation of glycolytic intermediates and NADPH, a key ROS scavenger [17, 20-22]. Furthermore, other studies have demonstrated that over-expression of uncoupling protein 2 (UCP2) (protein that is expressed in the inner membrane of the mitochondria) inhibits ROS-mediated apoptosis under hypoxia in cancer cells [23-27].

The aim of this study was to determine whether hypoxia alters ROS and lipid peroxidation in A549 lung cancer cells. Changes in free-radical mediated oxidative damage in cancer cells under low oxygen conditions could aid in establishing a biochemical origin of VOCs produced in lung cancer.

Methods

Cell culture

A549 cells were cultured in DMEM supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin and HEPES buffer (Life Technologies) with 5% carbon dioxide in both normoxic (atmospheric oxygen) and hypoxic (2% O2) conditions. Cells were seeded into imaging dishes (Mattek Corporation, Ashland, MA) for confocal microscopy. Imaging was performed as described in Section 2.5, Chapter 2 –Materials and Methods.

Detection and Analysis of Lipid Peroxidation in A549 cells

The cells were stained with BODIPY® 581/591 C11 at a final concentration of 10 μM and fluorescence imaging was performed using 590 nm and 510 nm emission filters using a Nikon Eclipse Ti-E confocal microscope as described in Section 2.5.1 (Chapter 2 –Materials and Methods of this thesis). In order to minimise pixel saturation, initial calibration on cells with the indicator and an inducer of lipid peroxidation was performed and excitation emission intensities adjusted accordingly. The lipid peroxidation was determined by quantitating the pixel density at 510 and 590 nm emission on each cell and calculating the ratio of the emission fluorescence intensities (510nm/590 nm). This was achieved by setting up an automatic calculation of ratios using ND2 software for all the groups. All experiments were repeated at least three times and the results shown are from one typical experiment.

*To determine co-localisation of Lipid Peroxidation and detect any Structural Changes of Mitochondria in A549 cells under hypoxia***.**

A549 cells cultured under hypoxia (2% O2) and Normoxia (21% O2) were incubated for 30 minutes with 200nM MitoTracker (mitochondria probe) to label the mitochondria and 10μM Lipid Peroxidation Sensor. To see if the LPO was co-localised to mitochondria data for both the channels was extracted as red (Far Red) and green (FITC) and the images overlayed using ImageJ software.

Detection and Analysis of ROS in A549 cells

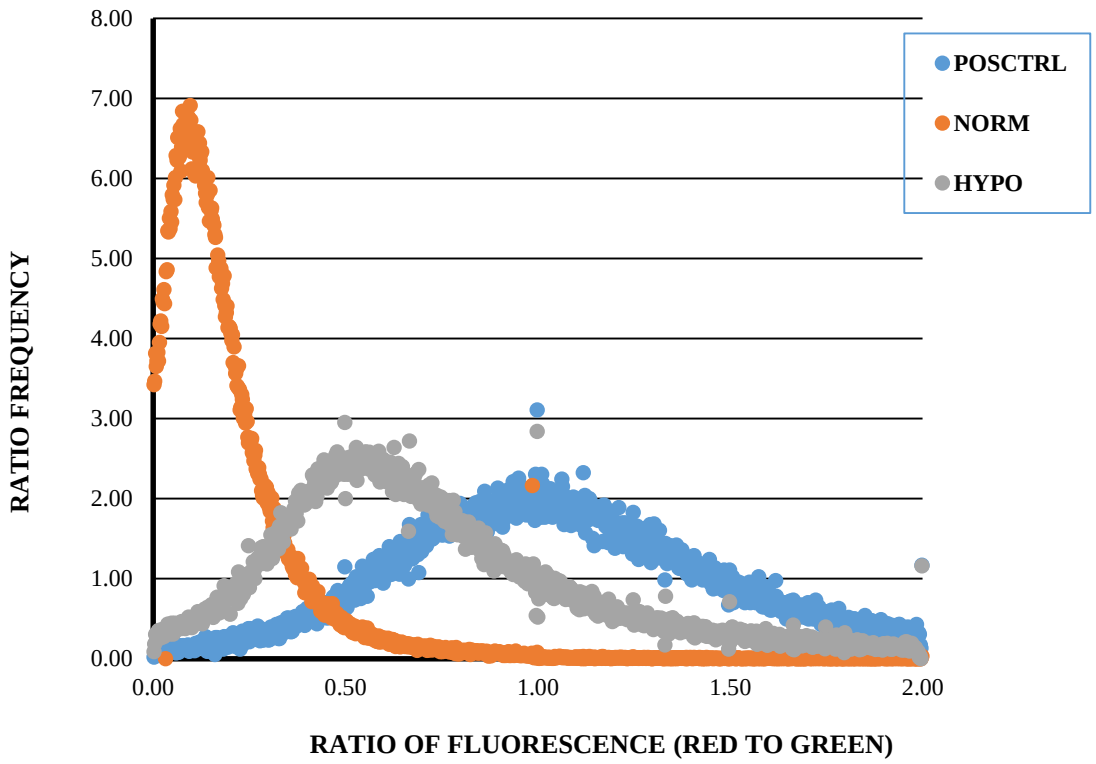
Cellular ROS was detected and measured by staining the A549 cells with H_2DCFDA at a final concentration of 25 μM for 30 minutes. Hoechst 33342 was added five minutes prior to analysis to

identify nuclei (blue) as described in detail in Section 2.5.2 (Chapter 2: Materials and Methods). In order to correct for auto fluorescence, initial calibration on cells without the ROS indicator dye was performed. After confocal imaging, pixel density was calculated on individual cells from both channels (blue and green) for all groups. The integrated optical density (IOD) for all the cells per field of view was quantified using ImageJ software and averaged for each image. Three biological replicates were performed and the data expressed as the mean and standard deviation of the biological replicates. Statistical significance was by Students t-test.

Results

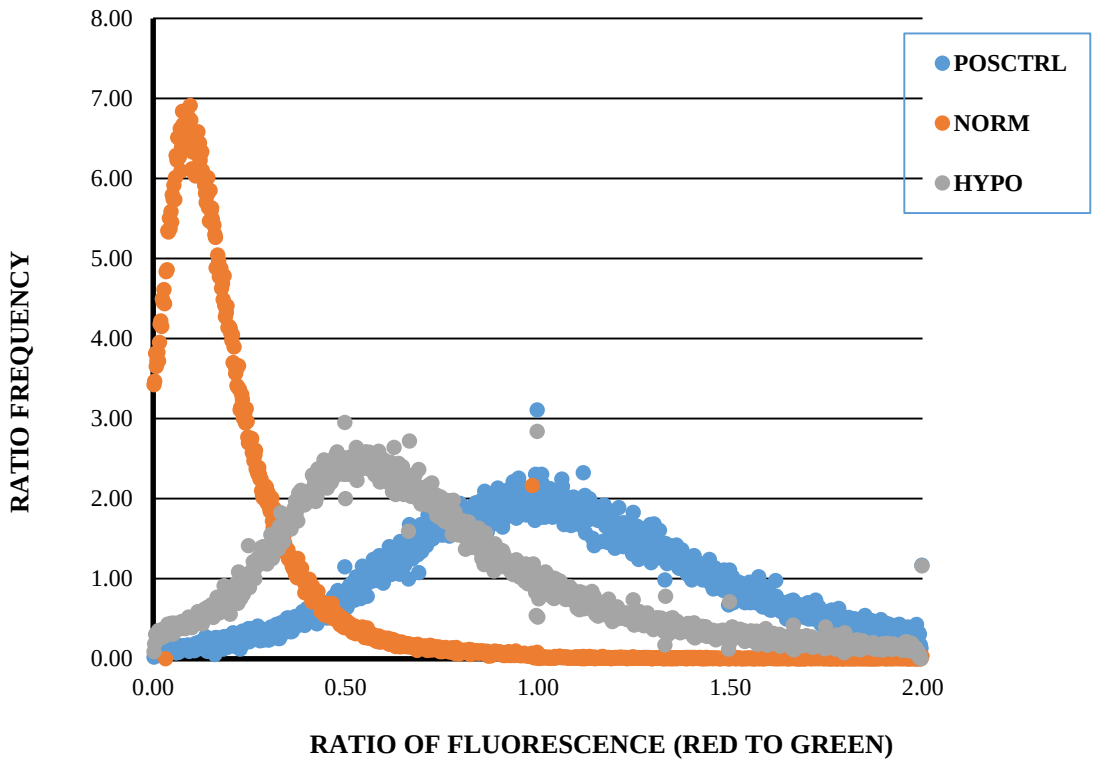
A549 cells cultured under hypoxic conditions show increased Lipid Peroxidation

The effect of hypoxia on peroxidation of unsaturated lipids in A549 cells was assessed. Cells cultured under low oxygen conditions had a visible increase in green fluorescence (510 nm) and decrease in red fluorescence (590 nm), indicative of lipid peroxidation compared to the cells cultured under normoxic conditions. Accordingly, the ratiometric analysis demonstrated a high ratio confirming a higher level of constitutive lipid peroxidation in hypoxic cultures compared to normoxic cultures of A549 cells (



Hypoxic cultures show an increase in lipid peroxidation compared to normoxic cultures. Lipid peroxidation in live cells was assessed by confocal fluorescence microscopy and the pixel density of each cell was measured. A549 cells were incubated for 30 minutes with 10μM Lipid Peroxidation Sensor (a ratio metric dye). Scale bar, 50μm. A high ratio (dark blue/green) indicates high levels of lipid peroxidation and a low ratio (light blue/magenta) indicates no lipid peroxidation (colour legend on the far right in the pictures). **(A)** Positive Control where lipid peroxidation was induced by treating the cells with Cumene Hydroperoxide. **(B)** Under basal conditions, normoxic cultures show no peroxidation whereas hypoxic cultures show constitutive lipid peroxidation **(C). (D)** Scatter plot of normalised pixel frequency against the ratio of fluorescence at 510 nm/590 nm (green/red) indicates a higher pixel density at the low ratio (peak at 0.1) in normoxic cultures compared to hypoxic cultures (peak at 0.5) and the positive control (peak at 1.0).

B-C). This was confirmed by plotting the frequency of pixels at each 510/590 ratio for normoxic and hypoxic cultured cells and positive control cells with induced lipid peroxidation (



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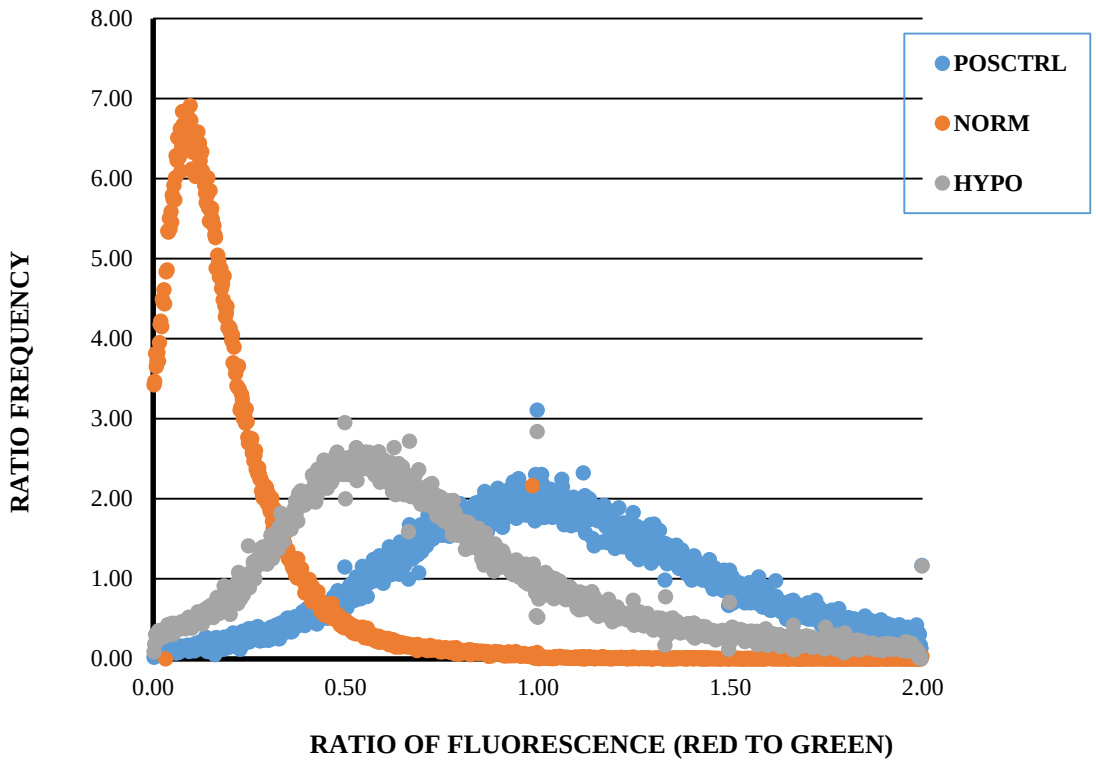
D). A high frequency of pixels was observed at low 510/590 nm ratios (peak at 0.1) for the cells cultured under normoxic conditions which was in contrast to hypoxic cultures, where a high frequency of pixels was observed at higher ratios (peak at 0.5) while a positive control with lipid peroxidation induced with cumene hydroperoxide showed a high frequency of pixels with a peak 510/590 nm ratio of 1.0.



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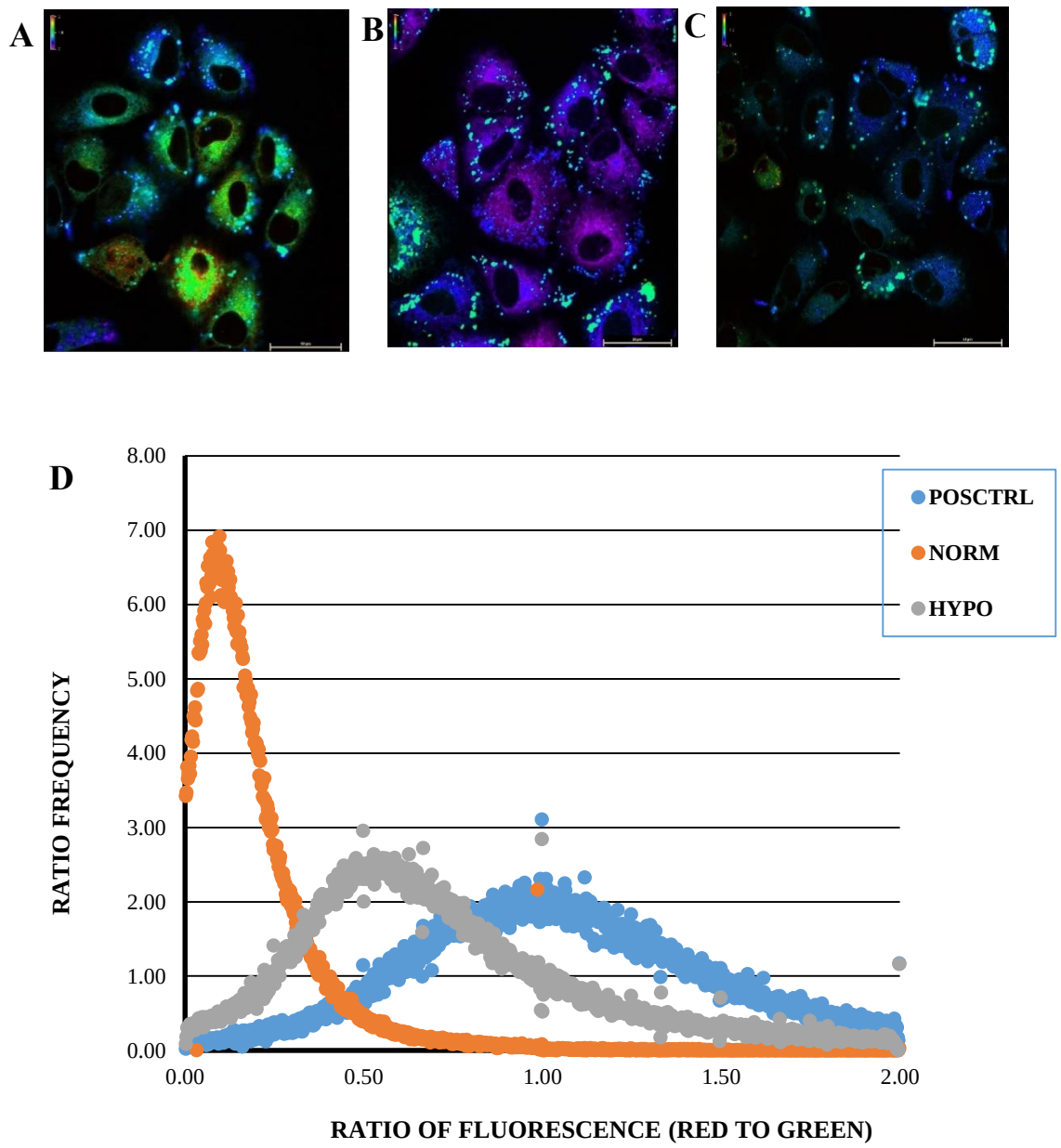
: Lipid peroxidation in A549 cells

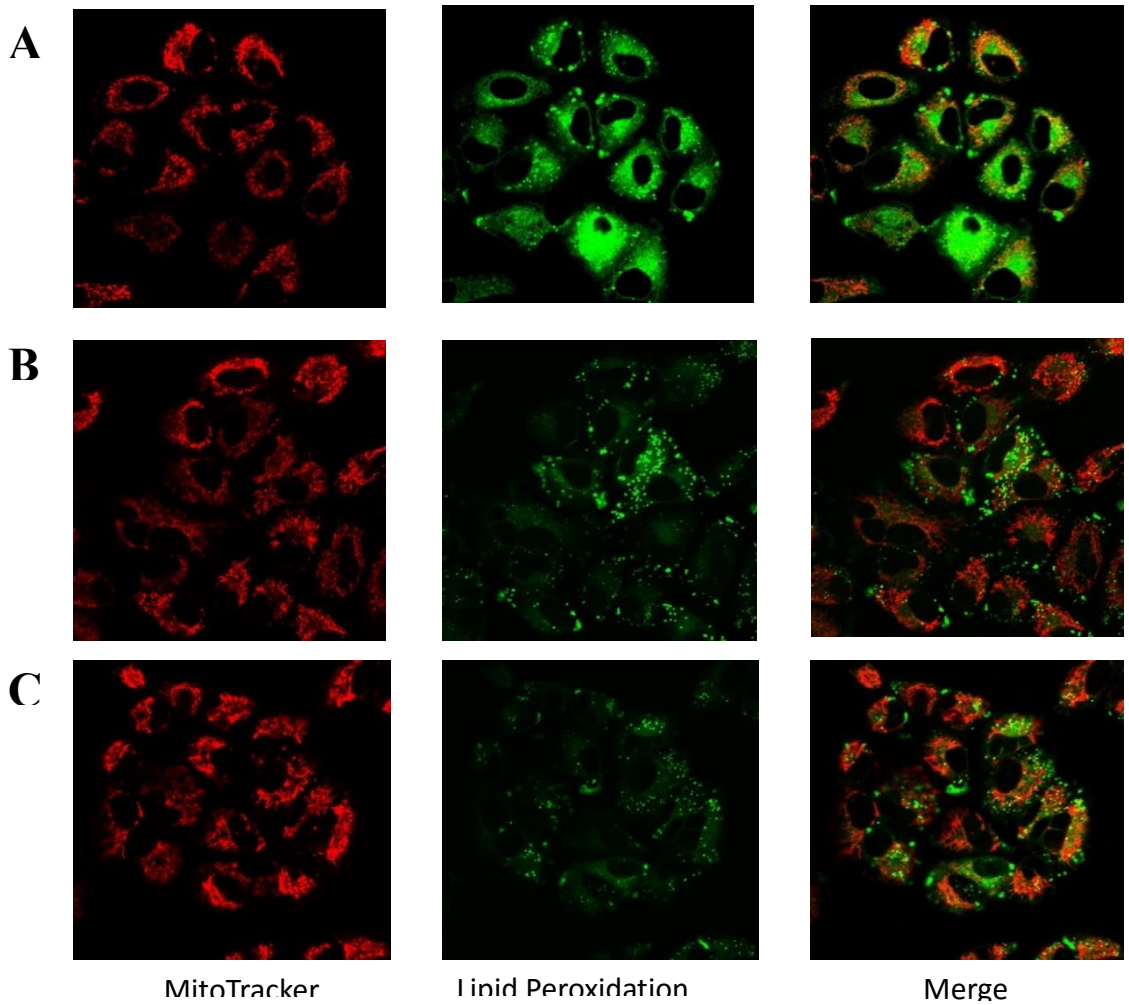


Hypoxic cultures show an increase in lipid peroxidation compared to normoxic cultures. Lipid peroxidation in live cells was assessed by confocal fluorescence microscopy and the pixel density of each cell was measured. A549 cells were incubated for 30 minutes with 10µM Lipid Peroxidation Sensor (a ratio metric dye). Scale bar, 50µm. A high ratio (dark blue/green) indicates high levels of lipid peroxidation and a low ratio (light blue/magenta) indicates no lipid peroxidation (colour legend on the far right in the pictures). **(A)** Positive Control where lipid peroxidation was induced by treating the cells with Cumene Hydroperoxide. **(B)** Under basal conditions, normoxic cultures show no peroxidation whereas hypoxic cultures show constitutive lipid peroxidation **(C). (D)** Scatter plot of normalised pixel frequency against the ratio of fluorescence at 510 nm/590 nm (green/red) indicates a higher pixel density at the low ratio (peak at 0.1) in normoxic cultures compared to hypoxic cultures (peak at 0.5) and the positive control (peak at 1.0).

Co-localisation of LPO and effect of hypoxia on Structural changes of Mitochondria

Increased LPO partially overlapped with mitochondria but was also apparent in other cell compartments. Visual inspection of the images of cells labelled with Mitotracker Deep Red, shows that there were no significant changes in the structure of mitochondria under hypoxia (**Figure 4.1**).

Figure 4.1: *Co-localisation of Lipid peroxidation using mitotracker and structure of mitochondria under hypoxia*



Imaging of mitochondria and co-localisation of lipid peroxidation was determined by confocal microscopy. Scale bar, 50µm. A549 cells cultured under hypoxia (2% O_2) and Normoxia (21% O_2) were incubated for 30 minutes with 200nM MitoTracker (mitochondria probe) and 10µM Lipid Peroxidation Sensor. **(A)** Positive Control where lipid peroxidation was induced by treating the cells with Cumene Hydroperoxide. **(B)** Cells cultured under normoxia (21% O_2). **(C)** Cells cultured under hypoxia (2% O_2). No changes were observed in the structure of mitochondria and the merged images showed lipid peroxidation in punctuate structures throughout the cell with incomplete co-localisation with mitochondria.

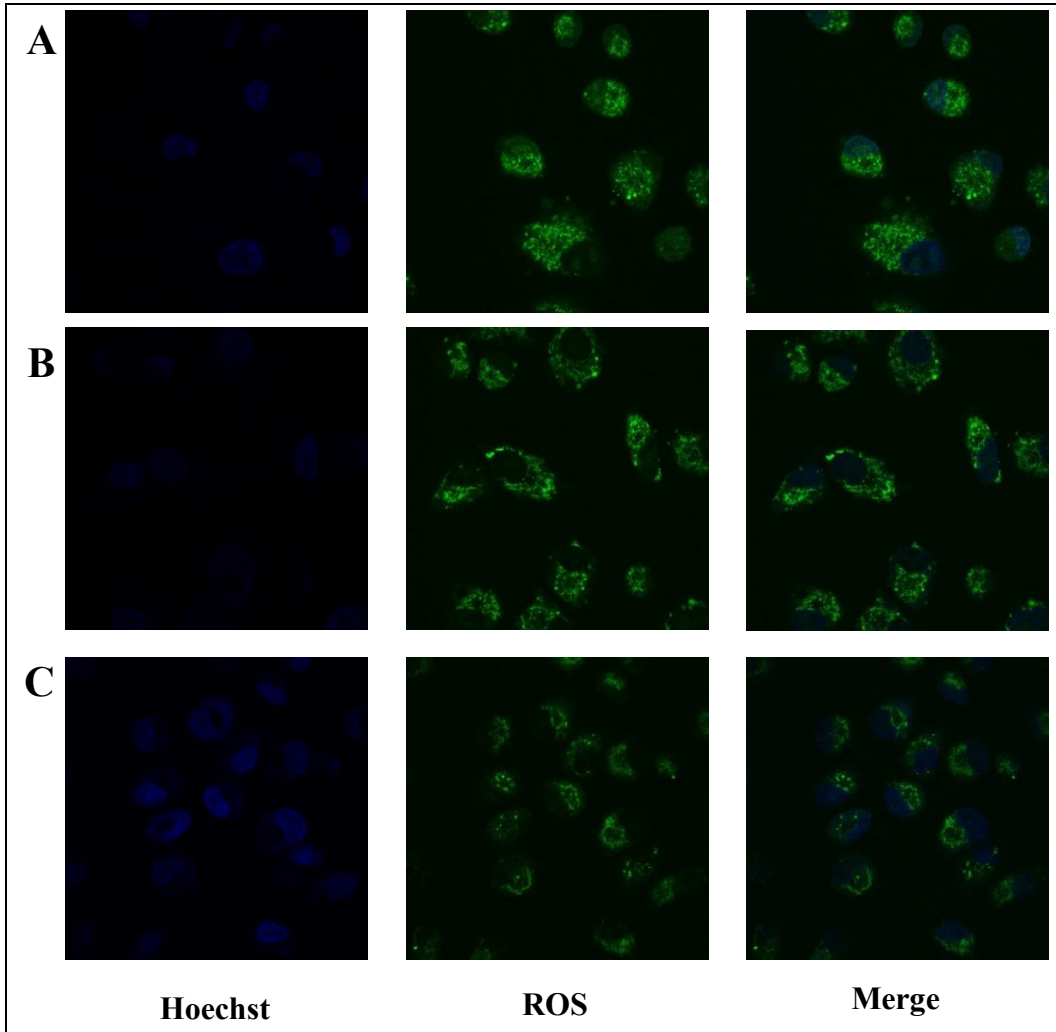
Hypoxic cultures of A549 cells show decrease in cellular ROS compared to Normoxic cultures.

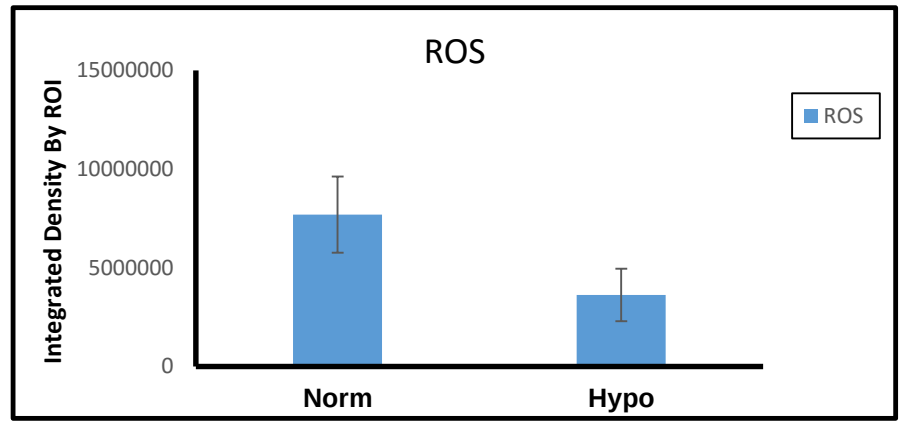
The effect of hypoxia on total ROS was assessed using carboxy-H_2DCFDA by confocal imaging. The cells cultured under normoxic conditions had a greater green fluorescence emission compared to hypoxic cultures as shown in

Figure 4.2 B and C. The integrated density of pixels slightly decreased in hypoxic cultures indicating decline in peroxide radicals (

Figure 4.2 D). As there was auto fluorescence observed in the cells treated with nuclei stain (Hoechst 33342) only, it was treated as negative control and included in the analysis.

Figure 4.2: *ROS production in A549 cells*





Generation of ROS in live cells was assessed by confocal fluorescence microscopy. Scale bar, 50µm. A549 cells cultured under hypoxia (2% O_2) and Normoxia (21% O_2) were stained with a fluorescent ROS indicator (green). **(A)** Positive control where the ROS production was induced by incubating the cells for 1hour with 100µM of *tert*-butyl hydroperoxide (TBHP) before staining and imaging. **(B)** Cells cultured under normoxic conditions. **(C)** Cells cultured under hypoxic conditions. All the groups were also labelled with 1µM of Hoechst (blue) to identify cell nuclei. **(D)** The average pixel intensity (IOD) of all cells in the green channel was measured. Error bars indicate standard deviation of the mean IOD for three biological replicates (n=3). The difference in ROS fluorescence between normoxia and hypoxia was significant ($P<0.05$, Students t-test).

Discussion

Many studies in the past decade have demonstrated increased lipid peroxidation products from cancer cells and tissues in oxidative stress [28-34]. It is also well established that the tumour micro-environment causes oxidative stress resulting in cell cytotoxicity and that cellular anti-oxidant defences are increased as a means of protection [35, 36].

In this study there was an apparent decrease in total ROS in hypoxic cultures compared to the normoxic cultures, which is consistent with the reduction of ROS in hypoxia found previously [37, 38]. In particular Kim et al. demonstrated in a human lymphoma cell line that under hypoxia ROS was attenuated by the overexpression of PDHK1 – a HIF-1 target gene [37] and we found PDHK1 to be increased in expression in A549 cells in hypoxia (Chapter 3 of this thesis). PDHK1 inactivates pyruvate dehydrogenase, so increased PDHK1 activity therefore deprives the tricarboxylic acid (TCA) cycle of Acetyl-CoA and instead promotes the conversion of pyruvate to lactate. This further suggests that in our studies, A549 cells have intact mitochondria and hence hypoxia will induce a metabolic shift of pyruvate away from the TCA cycle.

Cancer cells exhibit metabolic alterations of several critical nutrients and substrates, including metabolic reprogramming of both glucose and glutamine [39]. There is sufficient evidence that increased lipid production and beta-oxidation of fatty acids may be a vital secondary energy source – essential for cancer cell survival [40-46]. Studies by Metallo et al. using isotopic labelled glutamine demonstrated that several mammalian cancer cells (including A549 cells) cultured under hypoxia alternatively produce Acetyl Co-A via reductive carboxylation of glutamine-derived alpha-ketoglutarate (α-KG) (de-novo lipogenesis) in the cytosol [47, 48].

Lipid peroxidation is a chain reaction initiated by the interaction of ROS to generate hydroxyl radical which in turn abstracts hydrogen atoms from unsaturated lipids to produce lipid hydroperoxides [49-52]. A substantial increase in the oxidation of lipids was observed in hypoxia indicating that lipid hydroperoxide free-radical mediated damage was operative. Interestingly, there was no lipid peroxidation in normoxic cultures, despite the slight increase in total ROS, which seems inconsistent.

This may be due to the substantial availability of lipids (from lipogenesis) in the cells cultured in hypoxia which might lead to the initiation of the chain reaction (an autocatalytic cycle) that will propagate until the free radical chain is terminated (which could be challenging to control). Also, the presence of ROS under normoxic conditions may be due to the lesser amounts of active anti-oxidant defences and ROS scavengers such as NADPH but this requires further investigation.

Conclusion

Hypoxia regulates ROS to enhance cell survival and to protect the cell from apoptosis. Lipid peroxidation is significantly increased in hypoxic cultures demonstrating the presence of oxidative degradation caused by free-radicals. Hence, hypoxic culture conditions are likely to alter cell metabolism and possibly VOC output from cells.

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Chapter 5 - GCMS Analysis of Volatile Organic Compounds Produced by Hypoxic A549 cells

Abstract

Lung cancer is often diagnosed at an advanced stage when prognosis is poor. A non-invasive diagnostic test suitable for population screening is needed to enable early diagnosis and improved patient survival. Volatile organic compounds (VOCs) in exhaled breath have potential as diagnostics however confirmation of the metabolic origins and disease specificity of candidate markers is required. Cell culture metabolomics can identify disease biomarkers and their origins. To date VOC profiles from *in vitro* cultured cancer cells show little similarity to breath profiles from cancer patients. One reason for this maybe that *in vivo*, cancer cells experience hypoxia whereas *in vitro* cells are generally cultured in normoxic conditions. Since hypoxia is known to influence cell metabolism we hypothesize that cancer cells cultured under hypoxic conditions will produce VOCs more typical of those found in cancer breath. We present data from A549 lung cancer cells cultured in suspension under standard normoxic (21% oxygen) or hypoxic (2% oxygen) conditions. VOCs were collected using solid phase micro-extraction, analysed by Gas Chromatography-Mass Spectrometry and identified using the NIST11 mass spectral library. Environmental and system contaminants were identified by analysing incubator air (both normoxic and hypoxic) and media-only controls and these compounds were removed from the analysis. Comparison of endogenous VOCs produced by A549 cells under hypoxic and normoxic conditions showed twelve VOCs unique to cells grown under hypoxic conditions including four methylated alkanes and four alkenes. These classes of compounds have been commonly reported on cancer breath. A further four VOCs including two methylated alcohols were unique to cells grown in normoxic conditions, and two ketones were common to both. This data suggests that hypoxic culture conditions influence VOC production and is consistent with our hypothesis that hypoxia produces VOC profiles more similar to the breath of lung cancer patients. These results hold promise for the discovery of new and the validation of current VOC markers of lung cancer.

Introduction

Lung cancer is the most frequently diagnosed cancer and the leading cause of cancer related deaths worldwide [1]. The five-year survival rate for lung cancer is generally poor as the symptoms become apparent only once the disease is well advanced. Better patient outcomes can be achieved with early diagnosis of this disease. Breath analysis appears to be the most propitious approach to detect and identify biomarkers related to a specific ailment, many volatile organic compounds (VOCs) have been analysed to identify new biomarkers of lung cancer [2-28].

Many compounds on breath have been proposed as potential markers of lung cancer [29, 30] but their clinical relevance is still unclear [28]. Validation of the disease relevance of new breath biomarkers will be assisted by an understanding of the metabolic origins of the marker. However, there is currently limited knowledge about the metabolic origins and biochemical pathways that produce breath biomarkers. *In-vitro* studies provide an opportunity to identify disease biomarkers and their origins, however, there are only slight resemblances between the VOC profiles from *in-vitro* studies compared to lung cancer breath. Of the 68 VOCs detected on lung cancer breath and in the headspace of cancer cells in culture, only 16 VOCs are common to both [31-35]

Recent studies have established that *in-vivo* cancer cells experience hypoxia as a consequence of oxygen diffusion limits in the tissues, which has been measured to be around 150 μm [36, 37] resulting in altered cell metabolism [38, 39]. This raises the possibility that cells cultured under controlled low oxygen conditions will produce a pattern of VOCs that may aid in mapping biochemical origins of breath VOCs (Thesis Chapter 1, Section 3). The present study fills a gap in the literature by better mimicking the *in-vivo* conditions and provides new insights into the effects of culture conditions on the VOC profile produced by lung cancer cells.

To investigate our hypothesis that cancer cells cultured under hypoxic conditions will produce VOCs more typical of those found in cancer breath, we first established an experimental *in-vitro* model where the oxygen conditions could be regulated and the VOCs effectively extracted and compared with those VOCs extracted from uncontrolled oxygen conditions (air). This model is based on the observations that hypoxia, a common hallmark of all cancers, causes metabolic alterations and may provide a

metabolic switch from oxidative phosphorylation to aerobic glycolysis and a concomitant increase in lipid peroxidation (see chapter 4).

Methods

Sample Preparation

After growing to sub confluence in plastic culture flasks (Interpath Services, Melbourne, Australia) A549 cells were seeded at 10 million cells per 50 ml into a conical flask fitted with a Teflon stopper with a glass insert, through which a coated fused silica fibre could be introduced for the extraction of volatiles. The flask was placed on a magnetic stirrer (HD Scientific, Australia) and exposed to normoxia (21% O_2) and hypoxia (2% O_2) at 37^0C for 24hrs. The VOCs extracted from headspace of cells were compared against the VOCs extracted from the headspace of media without cells (controls). The air from both incubators was tested to identify system and environmental contaminants.

GCMS Analyses

The methodology used is discussed in detailed in Chapter 2, Section 2.3.2 of this Thesis. Both automatic and manual peak integration were executed for all the TICs and the compounds were identified by spectral match with NIST 2011 spectral library and only those with greater than 95% spectral match were considered positive. Overlapping unresolved peaks were not included in the analysis. Compound identity was further confirmed by retention times and the mass spectral pattern of neat standards where available (n-pentane, 3-methyl hexane and 2-nonanone). The retention times were internally consistent for each VOC and with standards where these were available. Preliminary calibration was performed using 100 and 1000 ppm speciality mix of alkanes with C_2-C_6 carbon skeleton to ensure that the detector was linear. Furthermore, to determine and confirm the candidate compounds of interest, standard curves were performed using a mixture of n-pentane, 3-methyl hexane and 2-nonanone (Accustandard) at concentrations of 10,30,100, 300 and 1000 ppm. For each compound the regression was linear from 10 to 300 ppm. LOD and LOQ were calculated from the linear regression using the formula LOD = 3 x SD/slope and LOQ = 10 x SD/slope where SD is the standard deviation of the Y-residuals. The standard mixtures were collected using 75 µm CAR/PDMS fibre by solid-phase micro extraction and were loaded using the auto sampler (Shimadzu Scientific Instruments) and released by thermal desorption

onto the injection port of the GCMS. Due to unavailability of standards, the other compounds of interest were identified by ion-extraction method using GCMS software and the fragments and their ratios were defined using NIST spectral library. The experiment was repeated on at least three independent samples with control and environmental air sampled from both the incubators for each experiment.

Results

Hypoxia produces unique VOC profiles

Comparison of endogenous VOCs produced by A549 cells under hypoxic and normoxic conditions showed twelve VOCs unique to cells grown under hypoxic conditions as shown in **Table 5.1**. Data shown are for three independent experiments, indicated as Replicate 1, 2 and 3 (Rep1, 2 and 3). Environmental and system contaminants were identified using media-only controls and were disregarded from the analysis (as listed in Appendix II). Pentane was observed along with four methylated alkanes – 3-methyl hexane, 3 - ethyl pentane, 2,3,4 - trimethyl decane and 3,6 - dimethyl undecane (**Table 5.1**). For 3-methyl hexane trace quantities close to the LOD (approximately 17 ppm) and below LOQ (<56 ppm) were detected in hypoxic A549 cultures. For 2 –nonanone, two replicates were below LOQ (<117 ppm), and the third was above LOQ with 127 ppm detected. Another four volatile compounds were identified as alkenes, namely 3,3-dimethyl-1-hexene, 2,5-dimethyl-2-hexene, 3-methyl-2-heptene and 7-methyl-1-undecene. Finally, there were three other VOCs: an alcohol 3-Penten-1-ol, (Z), a ketone identified as 2-Nonanone and a cycloalkane identified as 1,5-diethyl-2,3-dimethyl cyclohexane.

A further four VOCs including two methylated alcohols were unique to cells grown in normoxic conditions. There was minimal overlap of VOCs between normoxic and hypoxic conditions with two ketones identified, which were 1-Hepten-6-one and 2- methyl-3-Octanone.

Of the VOCs identified from the headspace of cultures, seventy-nine VOCs were identified as environmental or system contaminants. Of them, fifty-nine VOCs, were found in the headspace of control cultures (media without cells). The remaining twenty VOCs were found either in the headspace of controls or incubator air and identified as environmental contaminants (see Appendix II of this Thesis).

Table 5.1: *VOCs produced by Hypoxic cultures only*

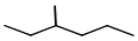
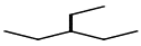
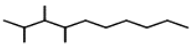
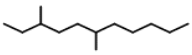
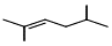
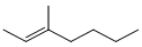
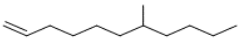
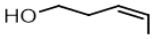
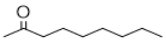
Class	Compound	Retention time	CAS No.	Structure	Rep 1	Rep 2	Rep 3
Alkanes	n-Pentane	13.21	109-66-0		754845	70144	400440
Methylated Alkanes	Hexane, 3-methyl-	24.17	589-34-4		2029387	1043960	2125286
	Pentane, 3-ethyl-	27.29	617-78-7		736698	293839	885762
	Decane, 2,3,4-trimethyl-	38.05	62238-15-7		17928675	12982075	32579041
	Undecane, 3,6-dimethyl-	40.77	17301-28-9		4481110	8071465	4982126
Alkenes	1-hexene, 3,3-dimethyl-	27.06	3404-77-1		644980	318836	900285
	2-hexene, 2,5-dimethyl-	28.16	3404-78-2		642914	294658	691998
	2-heptene, 3-methyl-	28.3	3404-75-9		2090555	-	1626000
	1-undecene, 7-methyl-	38.23	74630-42-5		1654215	1340093	3134859
Alcohol	3-penten-1-ol, (Z)	23.37	764-38-5		2417804	853465	2279754
Ketone	2-nonanone	38.97	821-55-6		1474386	2615736	4812724
Cyclo alkane	1,5-diethyl-2,3-dimethyl cyclohexane	40.4	74663-66-4		8030218	3352667	5728835

Table of areas of VOCs only found in the headspace of A549 cells cultured under hypoxia (2% O_2). Data shown are representative of three independent experiments (n = 3) indicated as Rep 1, Rep 2, Rep 3 in the table.

Discussion

In-vitro studies versus Breath Analysis

This study identified substantial differences between the VOC profiles from A549 cells cultured under normoxic and hypoxic conditions. The cells cultured under hypoxic conditions in particular produced a unique VOC profile consisting of 12 compounds that were not identified in normoxic cultures. These were mostly branched hydrocarbons along with a smaller number of alkenes, ketones, alcohols and a cycloalkane.

n-Pentane and branched hydrocarbons are of particular interest.

Firstly, pentane has been implicated as a volatile product of peroxidation of n-6 PUFAs [40-42], hence considered as an endogenous compound. Pentane has been related to oxidative stress and bronchial asthma [43-46]. Recent studies by Kischkel et al. analysed the VOCs from cancer patients undergoing lung resection and demonstrated a significant decrease in exhaled concentrations of pentane after surgery [47]. This is a significant finding as this compound was only found in the headspace of cells cultured under hypoxia but not in the cells cultured under normoxia.

Another significant finding is that the compounds belonging to the class of branched hydrocarbons were observed – 3-methyl hexane, 3 - ethyl pentane, 2,3,4 - trimethyl decane and 3,6 - dimethyl undecane. Of these compounds, 3-methyl hexane has been found associated with chronic liver disease [48], cholangiocarcinoma [49], and in lung cancer breath [43, 44]. Several of the VOCs from the hypoxic cultures have previously been reported as end-products of lipid peroxidation triggered by reactive oxygen species (ROS) and oxidative stress [50-52]. These results further support the idea that increased lipid peroxidation associated with hypoxia contributes to distinct VOC breath profiles in those with cancer and possibly other chronic conditions. Oxidative stress is a known fundamental mechanism associated with cancer growth and survival and the data shown here supports a role for hypoxia in promoting conditions favourable for lipid peroxidation and the release of methylated alkanes and alkenes.

VOCs from cells cultured under controlled oxygen conditions versus VOCs from cells cultured using traditional culture conditions

Breath analysis has attracted attention in recent years and there are many studies that have detected and identified a variety of VOCs as potential biomarkers of cancer. A recent study by Kischkel et al. calls for the need for more rigorous standardized methods of sampling, analysis and data processing including the effects of environmental contaminants [28]. This was further supported by a review of the volatiles from the healthy human body by de Lacy Costello et al., that identified 872 metabolites related to smoking, food consumption and medication in breath [53]. Also, it emphasises the fact that the compounds are typically present not only in cancer patients but also in healthy controls and that the clinical relevance is yet to be established for any candidate VOCs reported so far.

There were seventy-nine compounds identified as environmental and system contaminants in this study and these were present in all three independent experiments. Not considering these compounds in the analysis presented here was the most rigorous approach to avoiding reporting false positive compounds that do not derive from the lung cancer cells. It is noteworthy that a total of seventeen VOCs previously reported in the literature as produced by A549 cells were found as environmental and/or system contaminants in this study (**Table 5.2**). This is consistent with the need for minimising system contamination and the use of rigorous controls in *in vitro* studies of cancer metabolomics.

***Table 5.2*:** Environmental and system contaminants

No.	Compound	CAS No	References
1	Ethanol	64-17-5	[31, 54, 55]
2	n-Butanal	123-72-8	[55]
3	n-Pentanal	110-62-3	[55]
4	2,4-Dimethyl-1-heptene	19549-87-2	[31, 54]
5	Benzene	71-43-2	[55]
6	o-Xylene	95-47-6	[57]
7	p-Xylene	106-42-3	[55]
8	Styrene	100-42-5	[55]
9	Ethylbenzene	100-41-4	[55]
10	Acetone	67-64-1	[31]
11	2-Butanone	78-93-3	[55]
12	Ethyl acetate	141-78-6	[55]
13	**2-methyl-1-pentene**	763-29-1	[31, 54, 55]
14	**2-Ethyl Acrolein**	922-63-4	[31]
15	**3-methyl butanal**	590-86-3	[31, 54]
16	**n-Octane**	111-65-9	[31, 54, 55]
17	**n-Heptane**	142-82-5	[55]

Compounds are indicated in different coloured fonts to represent different groups as follows: Black = environmental and system contaminants in this study, Red = compounds found in this study in both controls and cells from both normoxic and hypoxic culture conditions but not incubator air, Blue = compounds that were found in this study only in controls from both the culture conditions and Green = compounds that were found in this study only in controls from normoxic conditions.

Differences in sampling methods and analyses will lead to different VOCs as system contaminants. Furthermore, some system contaminants may also be derived independently from endogenous origins. For example, ethanol, an alcohol that has been reported as produced by the tumour tissues, released by the A549 cell lines and exhaled by cancer patients [31] also can originate from food, beverages and is produced by gut bacteria such as *E coli* [54, 56] and it is also used as a disinfectant for aseptic technique in many cell culture laboratories. Also, aromatic hydrocarbons such as Benzene, o-Xylene, p-Xylene, Styrene and Ethylbenzene are known by-products of gasoline, air pollutants and related to cigarette smoking [57-62], and ketones such as 2-butanone and 2-heptanone are reported to be produced by lactic

acid bacteria [63]. The approach employed here whereby all system contaminants were eliminated from further study may have eliminated some VOCs that are relevant to disease, but it also ensures that the VOCs reported are in fact produced by the A549 cells, which may increase their utility as diagnostics.

This study demonstrates that culture conditions influence the VOC profile of A549. Controlled oxygen conditions in particular appear to be necessary to alter the cell metabolism to produce VOCs similar to those found on breath.

Conclusion

The results of this study indicate that the culture conditions play a vital role on the VOC profile and controlled oxygen conditions are required to better mimic the *in-vivo* tumour environment. Additionally, two of the compounds expressed in hypoxic culture, n-Pentane and 3-methyl hexane, were previously reported to be present on breath of lung cancer patients. This approach may help in creation of a panel of compounds which could be useful as biomarkers of lung cancer.

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Chapter 6 - **Discussion and Conclusions**

Overview

The initial hypothesis examined in this thesis is that hypoxia mediates changes in gene expression and altered cell metabolism in cancer cells which alters the VOCs produced, and may assist in identifying potential markers of the disease. This was tested with three studies. The first investigated the role of hypoxia on gene expression, identifying the ideal culture conditions to better mimic *in-vivo* conditions and where the metabolic switch from respiratory phenotype to glycolytic phenotype occurs. The second study examined free-radical production and lipid peroxidation in the hypoxic cultures, which may assist in identifying the possible biochemical origins of the VOCs. The third study assessed whether hypoxic cultures produced a distinctive VOC profile with the presence of an increased number of alkanes and methylated alkanes.

Hypoxia mediates the metabolic switch from respiratory phenotype to glycolytic phenotype.

The ideal oxygen percentage to culture the cells was determined Gene expression changes was analysed by qPCR on A549 cells cultured under various oxygen concentrations (1% O_2, 2% O_2 and 5% O_2) compared to standard cell culture conditions (21% O_2). This study showed statistically significant gene expression changes in the cells cultured under 2% O_2 and 1% O_2 and no significant changes were observed in the cells cultured under 5% O_2. Also, hypoxia caused increased *PDHK1* and *GLUT1* expression demonstrating a further shift towards glycolysis and away from OXHOS. These findings are consistent with other studies [1-3].

Hypoxia increases Lipid Peroxidation

The first stage of this study investigated the effect of hypoxia on auto-oxidation of lipids including co-localisation of peroxidation and the structure of mitochondria in A549 cells. It was hypothesised that hypoxia would result in an increase in oxidative degradation of lipids mainly localised to mitochondria. However, the data obtained shows that there is a significant increase in lipid peroxidation in hypoxic cultures that does not correspond to just mitochondria. Additionally, there were no visual significant changes in the structure of mitochondria in hypoxic cultures. This data is consistent with A549 cells having functional mitochondria in hypoxia.

Furthermore, the second stage of this study investigated the generation of ROS in A549 cells under hypoxia. The data obtained shows decreased ROS in hypoxic cultures of A549 compared to normoxic controls which is consistent with the reduction of ROS in hypoxia found previously by two other studies [1, 2]. In particular, the former study demonstrated that hypoxia ROS is attenuated by the overexpression of *PDHK1* – a HIF target gene [1] and we found *PDHK1* to be increased in expression in hypoxic cultures of A549. Additionally, there is evidence that there is altered lipid metabolism in various cancers *in-vitro* which may be a vital secondary energy source [4-8]. Wise et al. demonstrated altered citrate production in hypoxic cultures of glioblastoma cells [9]**.** Reduced ROS in hypoxic cultures may be due to the increase antioxidant defences or ROS scavengers such as NADPH that are produced to enhance cell survival. However, this needs further elucidation.

Hypoxia causes altered cell metabolism which may influence the VOCs produced by the cancer cells.

The third and final study demonstrated the influence of hypoxia on the VOC profile in A549 cells. Since altered cell metabolism was observed under hypoxia, we hypothesised that hypoxic cultures will produce VOCs more typical of those found in cancer breath. The data obtained shows a unique VOC profile of hypoxic cultures with 12 VOCs produced only by A549 cells in hypoxic conditions. These consisted mostly of branched hydrocarbons along with a smaller number of alkenes, ketones, alcohols and a cycloalkane. Of these, n-pentane and branched hydrocarbons are of particular interest as n-pentane has been identified as a volatile product of peroxidation of n-6 PUFAS [10-12]. Additionally, it was found in breath of patients with bronchial asthma [13] and breath of lung cancer patients [14, 15]. This finding further supports the outcome of the second study of this thesis that there is increased LPO under hypoxic conditions.

Detection of the branched hydrocarbons (3-methyl hexane, 2,3,4 - trimethyl decane, 3-ethylpentane and 3,6 - dimethyl undecane) in only hypoxic cultures was an important finding. Of them, 3-methyl hexane has been detected on cancer breath [14] and found significantly increased in the breath of children with chronic liver disease [16]. Also, it was found in the headspace of bile of patients with Cholangiocarcinoma [17].

It has been suggested by Vaupel et al. that the Warburg effect requires modification, that cancer cell metabolism is heterogeneous, and that the tumour microenvironment influences oxygen availability and that this contributes to glycolysis to lactate in tumours [18]. The data presented here are consistent with the view that A549 have intact mitochondria (Chapter 5 of this thesis), that hypoxia causes increased anaerobic metabolism associated with increased *PDHK1* and *GLUT1* expression (therefore HIF 1 activation), which results in a shift in cell metabolism as shown by changes in the VOCs being produced.

To identify the candidate VOCs obtained from cancer metabolomics studies as biomarkers, they have to be validated by breath analysis of the patients with cancer and healthy controls. To test whether or not the unique VOCs produced by the hypoxic cultures are present on lung cancer breath, an interim study involving the collection of breath from patients diagnosed with lung cancer and healthy controls (age and sex matched) is in progress. Breath is collected in 100 ml glass sampling tube and VOCs harvested and detected as described in this thesis. This project was approved by the Federation University Human Ethics Committee with the project number A15-180.

The preliminary results of this study (Myers MA, personal communication) are shown here (**Table 6.1**) with five cancer patients and five healthy controls matched for age and sex. Consistent with a previous study (Phillips et al.) 3-methyl hexane [14] was observed in the breath of two cancer patients, and 2,3-butanedione [19] was found on breath of four cancer patients. Both these compounds were not detected in the breath of healthy controls, while n-Pentane was detected in breath of one cancer patient and one healthy control. While the breath study outlined here is in its early stages and the sample size is currently insufficient for conclusions to be drawn, it does confirm that 3-methyl hexane can be detected on breath from lung cancer patients, so providing support for the use of *in vitro* cell culture under low oxygen for the discovery of potential biomarkers.

Diagnosis	Mean Age (Years)	Age range (Years)	Gender	Number (n)	3-methyl hexane	n-pentane	2,3-butanedione
Lung cancer	64.4	43 to 85	3M 2F	5	2	1	4
Healthy control	59.8	44 to 85	3M 2F	5	0	1	0

Table 6.1: *Preliminary results of the interim study*

VOCs found on the breath of the patients diagnosed with lung cancer vs healthy controls (M – Male and F – Female).

Conclusions

This thesis demonstrates the role of hypoxia in the regulation of the cell metabolism and VOC production in A549 cells. This data is consistent with our hypothesis that hypoxic cultures of A549 cells produce a unique VOC profile characteristic of cancer cells as they closely mimic the conditions of cancer cell growth *in vivo*. The relevance of this work to cancer diagnosis is outlined in Figure 6.1, which describes how micro physiological conditions in tumours could lead to altered cancer cell metabolism and VOC breath profiles.

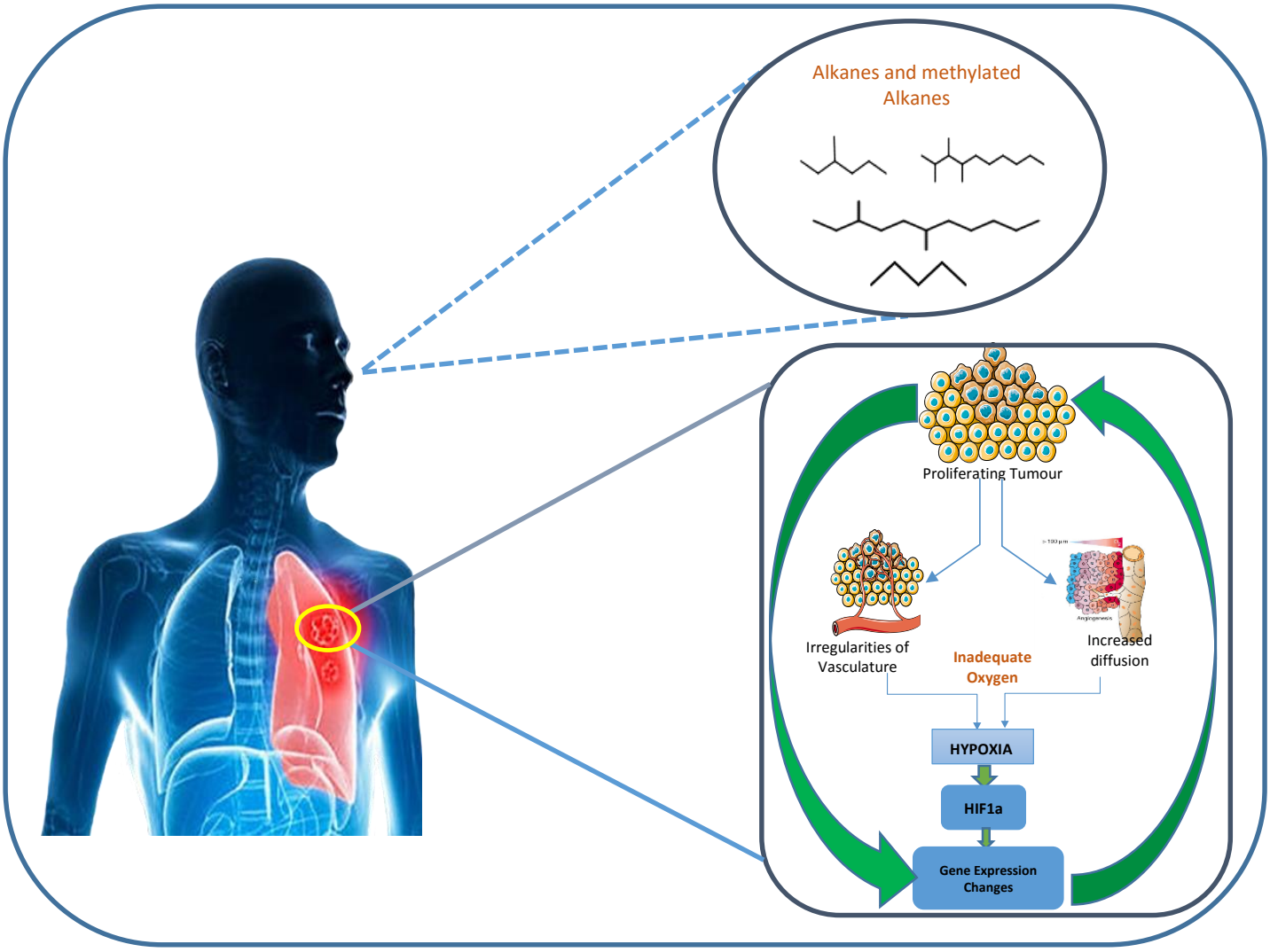

***Figure 6.1**: Influence of hypoxia on VOC output*

Micro-physiological conditions effect cell metabolism and may influence the VOCs produced in the breath, which may be useful for identifying markers for early diagnosis in lung cancer patients.

Future Directions

Cancer cell metabolomics in hypoxic conditions provides a great opportunity to test hypotheses around VOC profiles from different cancers and biochemical factors that may influence their production. Also, the cell culture metabolomics approach can be extended to other cancer cell lines such as liver cancer (HepG2), breast cancer (MCF7) to produce unique VOC profiles. This might provide more insight to the biochemical origins of VOCs. Another area for further exploration is antioxidant defence in cancer cells. It is hypothesised that under hypoxia, the antioxidant defences may be increased in cancer cells to protect the cell from cytotoxic effects of ROS and to aid cell survival and proliferation. This could be elucidated further by the treatment of A549 cells with inhibitors of SOD and/or NOX and measuring levels of ROS activity. Also, the above approach could help in clarification of the role of mitochondria in ROS production and the subcellular origins of VOCs. Furthermore, the breath of the cancer patients can be collected before and after the surgery (for example lung resection) or other treatment and analysed which may perhaps be useful to test the link between the cancer burden and its influence on VOC output, specifically related to LPO products, which could be implicated in disease prognosis.

Cell culture metabolomics under low oxygen may be exploited to discover biomarkers for the design of screening tests for early diagnosis of diseases such as Ischemia, Chronic Obstructive Pulmonary Disorder (COPD) and Diabetes. Technological advances are today making breath analysis as a point of care diagnostic possible. Identifying the most specific and useful disease markers should be aided by such insights in to how these VOCs are produced. These tests in the future will be a non-invasive method for cancer diagnosis and monitoring of disease progression.

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Appendix I

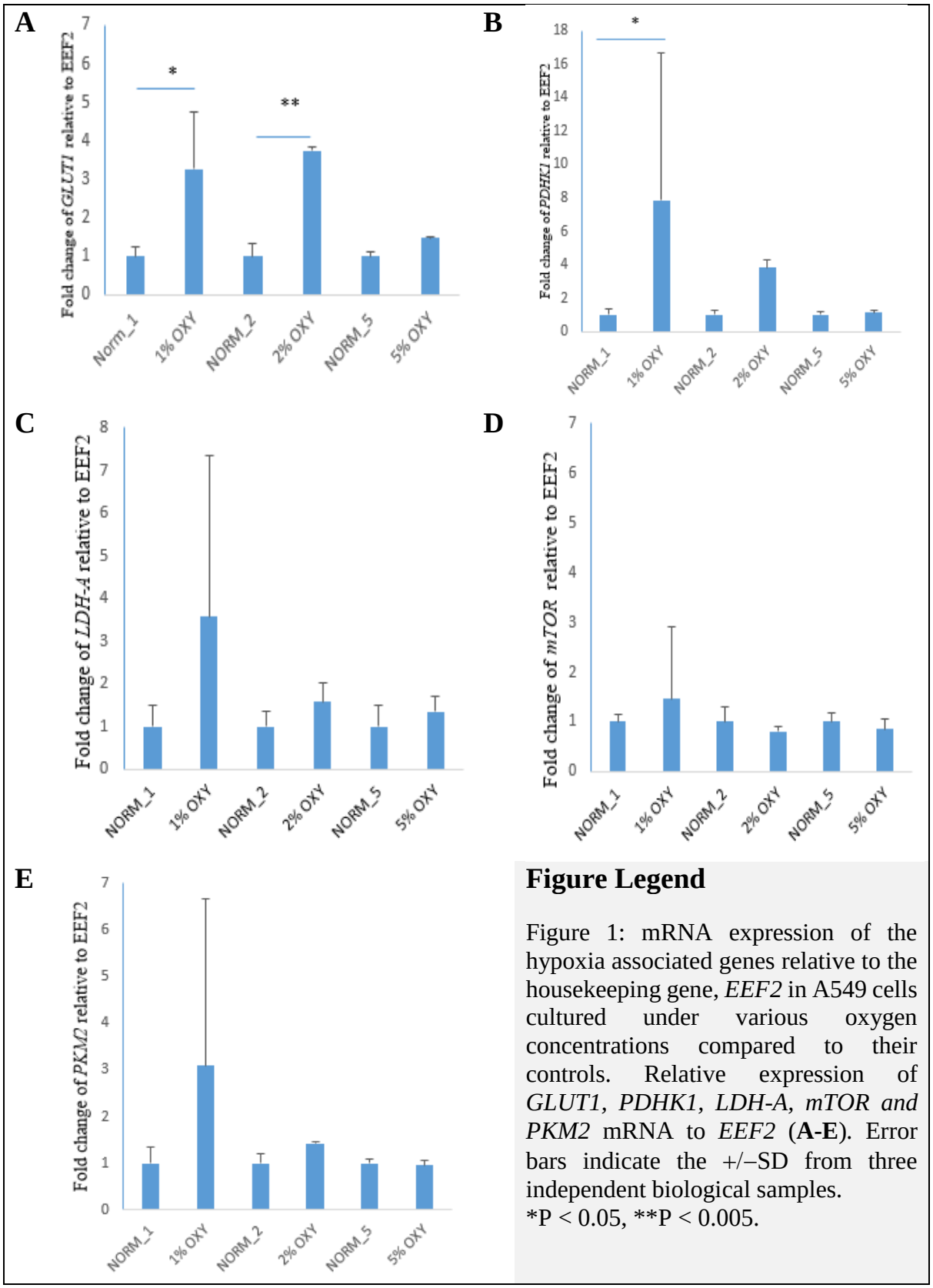

Figure Legend

Figure 1: mRNA expression of the hypoxia associated genes relative to the housekeeping gene, *EEF2* in A549 cells cultured under various oxygen concentrations compared to their controls. Relative expression of *GLUT1, PDHK1, LDH-A, mTOR and PKM2* mRNA to *EEF2* (**A-E**). Error bars indicate the +/−SD from three independent biological samples.
*P < 0.05, **P < 0.005.

Appendix II

ENVIRONMENTAL AND SYSTEM CONTAMINANTS			
CLASS	**NO.**	**COMPOUNDS**	**CAS. NO.**
ALKANES (ACYCLIC, METHYLATED AND SUBSTITUTED)	1	n-Hexane	110-54-3
	2	Tetradecane	629-59-4
	3	Octane, 4-methyl-	2216-34-4
	4	Decane, 3,6-dimethyl-	17312-53-7
	5	Decane, 1-iodo-	2050-77-3
	6	Undecane	1120-21-4
	7	Dodecane, 2-methyl-	1560-97-0
	8	Dodecane, 4,6-dimethyl-	61141-72-8
	9	Pentadecane, 7-methyl-	6165-40-8
	10	Tetracontane, 3,5,24-trimethyl-	55162-61-3
	11	Hexacosane	630-01-3
	12	1-Chloroeicosane	42217-02-7
	13	Heptane, 4-methyl-	589-53-7
	14	Pentane, 2,3,4-trimethyl-	565-75-3
	15	Trichloromethane	67-66-3
	16	Octane	111-65-9
	17	n-Heptane	142-82-5
	18	Heptane, 2,5,5-trimethyl-	1189-99-7
	19	Pentadecane	629-62-9
ALKENES			
	20	2-Heptene	592-77-8
	21	2-Octene, (Z)-	7642-04-8
	22	2,4-Dimethyl-1-heptene	19549-87-2
	23	1-Pentene, 2-methyl-	763-29-1
	24	2-Octene	111-67-1
	25	Cyclohexene	110-83-8
	26	3-Octene, (Z)-	14850-22-7
	27	1-Octene, 3,7-dimethyl-	4/01/4894
ALCOHOLS			
	28	Ethanol	64-17-5
	29	1-Butanol	71-36-3
	30	1-Hexanol, 5-methyl-2-(1-methylethyl)-	2051-33-4
	31	3-Heptanol	589-82-2
	32	Cyclohexanol	108-93-0
	33	1-Dodecanol, 2-hexyl-	110225-00-8
	34	2-Nonen-1-ol	22104-79-6
ALDEHYDES			
	35	Acetaldehyde	75-07-0
	36	Butanal	123-72-8
	37	Pentanal	110-62-3
	38	Hexanal	66-25-1
	39	Octanal	124-13-0
	40	Butanal, 3-methyl-	590-86-3
	41	2-Ethylacrolein	922-63-4
KETONES			
	42	Acetone	67-64-1
	43	2-Butanone	78-93-3

ENVIRONMENTAL AND SYSTEM CONTAMINANTS			
CLASS	**NO.**	**COMPOUNDS**	**CAS. NO.**
	44	2-Hexanone	591-78-6
	45	3-Heptanone	106-35-4
	46	2-Heptanone	110-43-0
	47	2-Octanone	111-13-7
	48	Methyl Isobutyl Ketone	108-10-1
	49	2,3-Butanedione	431-03-8
	50	4-Octanone	589-63-9
AROMATICS COMPOUNDS			
	51	Benzene	71-43-2
	52	Toluene	108-88-3
	53	Ethylbenzene	100-41-4
	54	Benzene, 1-ethyl-3-methyl-	620-14-4
	55	Benzene, 1-ethyl-4-methyl-	622-96-8
	56	Benzene, 1-ethyl-2-methyl-	611-14-3
	57	Mesitylene	108-67-8
	58	Benzene, 1,3-bis(1,1-dimethylethyl)-	1014-60-4
	59	Benzene, 1,4-diethyl-	105-05-5
	60	o-Cymene	527-84-4
	61	p-Cymene	99-87-6
	62	Styrene	100-42-5
	63	o-Xylene	95-47-6
	64	Phenol	108-95-2
	65	Acetophenone	98-86-2
	66	Benzaldehyde	100-52-7
HETEROCYCLIC COMPOUNDS			
	67	2,4-Dimethylfuran	3710-43-8
	68	Tricyclo[3.1.0.0(2,4)]hex-3-ene-3-carbonitrile	NA
	69	Hexanamide, N-methallyl-	NA
	70	Benzofuran	271-89-6
	71	Cyclotrisiloxane, hexamethyl-	541-05-9
ESTERS			
	72	2-Furancarboxylic acid, 2-tetrahydrofurylmethyl ester	NA
	73	Sulfurous acid, cyclohexylmethyl hexadecyl ester	NA
	74	Trifluoroacetic acid,n-tridecyl ester	NA
	75	Ethyl Acetate	141-78-6
	76	Formic acid, cis-4-methylcyclohexyl ester	NA
CYCLIC COMPOUNDS			
	77	Cyclohexane, 1-ethyl-1,3-dimethyl-, cis-	NA
	78	Bicyclo[4.2.0]octa-1,3,5-triene	694-87-1
OTHERS			
	79	Hydroperoxide, pentyl	74-80-6

Appendix III