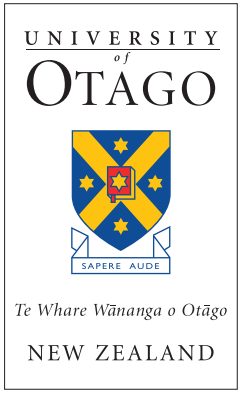
Oxygen Theme Meeting



Dunedin, 11th – 12th October 2012

Thursday 11th October

4:00-5:00 pm	Business Meeting	Staff Club
5:30-6:30 pm	Christine Winterbourn 2011 Rutherford Medal Recipient	

O1 *Life with Oxygen*

Hutton Lecture Theatre, Otago Museum, 419 Great King Street

7:30 pm	**Dinner: Etrusco**	**8A Moray Place**

Friday 12th October Arana College

Session 1 **Chair: Tony Kettle (CFRR Pathology UOC)**

8:30-8:35 am Opening remarks

8:35-8:50 am Andrew Das (CFRR Pathology UOC)

O2 *Conjugation of Glutathione to Oxidised Tyrosine Residues on Proteins*

8:50-9:05 am Nick Magon (CFRR Pathology UOC)

O3 *Oxidised Calprotectin as a Biomarker for Inflammatory Disease*

9:05-9:20 am Antonia Seidel (Chemistry UO)

O4 *Urate Oxidation by Lactoperoxidase*

9:20-9:35 am Andrew Bahn (Physiology UO)

O5 *Regulation of the major renal uric acid transporter GLUT9 by miRNAs*

9:35-9:50 am Tanya Flynn (Biochemistry UO)

O6 *Fine-Mapping of Two Urate Transporters (SLC22A11 and SLC22A12) Relevant to Gout*

9:50-10:05 am Simon Jackson (Biochemistry UO)

O7 *The Oxygen Evolving Centre of Photosystem II*

10:10-11:00 am **Morning tea & Poster Session**

Session 2 **Chair: Liz Ledgerwood (Biochemistry UO)**

11:00-11:20 am Mark Hampton (CFRR Pathology UOC)

O8 *Peroxiredoxin 3 as an Antioxidant, Sensor and Marker of Mitochondrial Oxidative Stress*

11:20-11:40 am Rebecca Poynton (CFRR Pathology UOC)

O9 *Kinetics of Peroxiredoxin 2 and 3 Hyperoxidation*

11:40-12:00 pm Trent Newman (Pathology UO)

O10 *A Developmental Role for Peroxiredoxin 3*

12:00-12:20 pm Tracy Josephs (Biochemistry UO)

O11 *Cytochrome c peroxidase activity and subsequent mitochondrial release during apoptosis*

12:20-12:40 pm Egor Petrovitch Tchesnokov (Chemistry UO)

O12 *Cysteine dioxygenase: rapid kinetics of enzyme-substrate complex formation*

12:40-2:00 pm **Lunch & Poster Session**

Session 3 **Chair: Guy Jameson (Chemistry UO)**

2:00-2:40 pm Roland Stocker

(Victor Chang Cardiac Research Institute Sydney)

O13 *Detection of reactive oxygen species*

2:40-3:00 pm Barrie Peake (Chemistry UO)

O14 *ROS –induced degradation of some β-blocker drugs in aqueous solution*

3:00-3:20 pm Sweta Kumari (Pharmacology & Toxicology UO)

O15 *Development of photoactivated nitric oxide donors as anticancer drugs*

3:20-3:40 pm Emma Spencer (CFRR Pathology UOC)

O16 *Exploring the anti-inflammatory and anti-cancer properties of novel isothiocyanates*

3:40-4:00 pm Bettina Zadehvakili (Pharmacy UO)

O17 *Synthesis, structure activity relationship studies and antioxidant activity evaluation of some organochalcogen catalysts as novel GPx mimics*

4:00-4:20 pm Helena Magrath-Cohen (Pathology UO)

O18 *Oxidative Stress Induced DNA Hemi-Hypomethylation and Maintenance Methylation*

4:20-4:30 **Concluding Remarks**

O1

Life with Oxygen

Winterbourn CC

Free Radical Research Group, University of Otago, Christchurch, NZ

Life depends on oxygen. We use it to "burn" food and extract the energy required to keep our body functioning. Harmful free radicals are by-products of this process. We survive in oxygen only through the body's antioxidant defences that protect against radical damage.

Professor Christine Winterbourn's work on free radicals began when it was becoming apparent that they are produced as part of normal metabolism, with radicals generated from oxygen in red blood cells as it is transported around the body. In this presentation Professor Winterbourn will discuss the development of knowledge since that time on the sources and consequences of free radical production, and on health problems that can arise when antioxidant defence is inadequate.

Professor Christine Winterbourn is a member of the Centre for Free Radical Research in the Pathology Department at the University of Otago, Christchurch. She is a Fellow of the Royal Society of New Zealand and a Companion of the New Zealand Order of Merit. In 2011 she won the prestigious Rutherford Medal, the first female scientist to do so.

O2

Conjugation of Glutathione to Oxidised Tyrosine Residues on Proteins

Das AB, Nagy P, Lechte T, Kettle AJ, Winterbourn CC
Free Radical Research Group, University of Otago, Christchurch, NZ

Tyrosine residues are sensitive to oxidation and can be converted to hydroperoxides by superoxide reacting with the Tyr radical. These hydroperoxides rearrange to bicyclic derivatives that are readily reduced to more stable hydroxides. The aromatic character of tyrosine is lost, but the product contains an unsaturated carbonyl group and is, therefore, an electrophile. Sperm whale myoglobin forms a hydroperoxide on Tyr-151 in a hydrogen peroxide/superoxide-dependent reaction. Using liquid chromatography/mass spectrometry, and western blotting analysis, we show that its hydroxide derivative reacts with GSH to form a conjugate. Unusual for GSH addition to an electrophile, the reaction is reversible, with a half-life of many hours for the reverse reaction. Detection of the conjugate required stabilization by reduction; otherwise, the reverse reaction occurred during tryptic digestion and analysis. Our findings represent a novel mechanism for peptide or protein glutathionylation involving a carbon-sulfur cross-link between oxidized Tyr and Cys. As with other electrophiles, the oxidized Tyr should undergo a similar reaction with Cys residues in proteins to give intramolecular or intermolecular protein cross-links. This mechanism could give rise to protein cross-linking in conditions of oxidative stress.

O3

Oxidised Calprotectin as a Biomarker for Inflammatory Disease

Magon NJ[1], Gearry RB[2], Hampton MB[1], Kettle AJ[1]

[1]Centre for Free Radical Research, Department of Pathology, University of Otago, Christchurch, NZ
[2]Department of Medicine, University of Otago, Christchurch, NZ

Accurate diagnostic and prognostic indicators are crucial for the management of inflammatory diseases. Stimulated neutrophils generate reactive oxidants that contribute to tissue damage during inflammation. These oxidants are short-lived species that oxidise proteins and lipids to generate stable products that may prove useful as biomarkers. Calprotectin, a complex of two proteins (S100A8 and S100A9), is present in the cytosol of neutrophils and commonly elevated in tissue and plasma in various inflammatory diseases. Previous reports indicate that calprotectin is susceptible to oxidative modification, especially by the neutrophil oxidant hypochlorous acid (HOCl). When calprotectin was treated with HOCl, stable heterodimer formation was observed by SDS-PAGE and anti-S100A8/S100A9 western blotting. The dimers were resistant to reduction by dithiothreitol indicating the presence of non-disulfide crosslinks. Analysis of these samples by tryptic digestion and mass spectrometry revealed the loss of peptides containing methionine and cysteine residues, and an increase in new peptides corresponding to methionine sulfoxide and cysteic acid formation. Stable intra- and inter-molecular sulfinamide crosslinks involving the cysteine residue of S100A9 were also observed. Interestingly, HOCl-treatment of calprotectin also resulted in the formation of dehydromethionine, a cyclic azasulfonium salt recently investigated by our group [1], on the N-terminal methionine of S100A8. Dehydromethionine formation was additionally observed on S100A8 in the cytosol and supernatant of stimulated neutrophils as well as in bronchoalveolar lavage fluid from patients with cystic fibrosis.

References

[1] Peskin, A.V. et al., *Biochemistry* **48**:10175-10182; 2009

O4

Urate Oxidation by Lactoperoxidase

Seidel A[1], Turner R[2], Wilbanks SM[3], Kettle AJ[2], Jameson GNL[1]

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Lactoperoxidase (LPO) is present in mammalian and human exocrine secretion liquids, including saliva, tears and milk, as well as in the mucosal surfaces of the airway system. It is considered to play a key role in the innate immune defence against invading organisms, since its main physiological substrate thiocyanate is oxidised by LPO to the potent bactericidal and fungicidal agent hypothiocyanate. Urate is the final product of human purine metabolism and is present not only in blood plasma, but also in respiratory lining fluids. It is believed to have a major antioxidative and neuroprotective function. Altered plasma urate levels have been associated with neurodegenerative diseases as well as with cardiovascular disease and gout. Our recent work shows that urate is a good substrate for bovine LPO. Analysis by mass spectrometry revealed that urate is oxidised by LPO to produce the intermediates dehydrourate and 5-hydroxyisourate, which finally yield allantoin as a stable product. We found that under physiological conditions, urate is capable of competing with the enzyme's main substrate thiocyanate for oxidation. Using stopped-flow spectroscopy, we investigated the kinetics of urate oxidation by LPO and determined the rate constants for the reaction of LPO compounds I and II with urate. Altogether, our findings suggest that urate is a physiological substrate for LPO and might influence the enzyme's function in the airway host defence system.

O5

Regulation of the major renal uric acid transporter GLUT9 by miRNAs

Rodrigues E, Johnstone R, Bahn A

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Urate is a metabolite of the purine metabolism and well known in the context of gout or as a scavenger neutralising reactive oxygen species equivalent to vitamin C. Active re-absorption of urate in the kidneys maintains a high plasma urate level, which if further elevated has been shown to be a risk factor for cardiovascular diseases, metabolic syndrome, type II diabetes or gout. Low levels of urate on the other hand have been connected to many neurodegenerative diseases such as Alzheimer's and Parkinson's disease or multiple sclerosis. Glucose transporter 9 (GLUT9) belongs to the family of glucose facilitators and was recently identified as a renal urate transporter. Single nucleotide polymorphisms (SNPs) in GLUT9 have already been connected to gout and Parkinson disease. In order to study the post-transcriptional regO5ulation of GLUT9 and the impact of GLUT9-SNPs we first analysed the 3' UTR region of GLUT9. Employing human primary renal epithelial and HepG2 cells, we identified a correlation of hsa-miR153 expression with the endogenous expression of GLUT9 in these two cell lines. Transfection of HepG2 cells with 153-anti-miRNAs resulted in an up-regulation of GLUT9 protein expression, suggesting miR-153 to be a regulator of GLUT9 translation. Further sequence analysis of a GLUT9-SNP, which results in an amino acid change, represents a possible binding site for a miRNA, namely hsa-miR4664. Incubation of GLUT9-transfected HEK293 cells with 500 µM urate reduced GLUT9 mRNA and hsa-miR4664 levels, but increased GLUT9 protein expression, indicating that miR4664 may be involved in GLUT9 translational regulation under high urate conditions. In summary, we have identified two miRNAs as possible regulators of GLUT9 expression acting via completely different pathways.

O6

Fine-Mapping of Two Urate Transporters (*SLC22A11* and *SLC22A12*) Relevant to Gout

Flynn TJ, Merriman TR

Biochemistry Department, University of Otago, Dunedin, NZ

Urate is the largest contributor to human antioxidant activity (35-65%), but raised serum urate levels are a primary risk factor for gout. GWAS studies have identified multiple single nucleotide polymorphisms (SNPs) involved in the natural fluctuations of serum urate levels among humans. Many of these relate to urate processing genes.

Among these GWAS results a region of chromosome 11 has been identified containing a pair of urate transporter genes, *SLC22A11* and *SLC22A12*. SNPs within both these genes have previously been associated with gout in different populations, *SLC22A11 (rs2078267)* in Caucasian, and *SLC22A12 (rs475688)* in Chinese and Solomon Islanders. *SLC22A12* has not been associated with gout in Caucasians; *SLC22A11* has not been studied in Non-Caucasians.

This research aims to investigate genetic variation within *SLC22A11* and *SLC22A12* in gout in New Zealand (NZ) Caucasian and Polynesian cohorts in an attempt to clarify the associations between these two genes and gout.

NZ Caucasian (421 cases, 638 controls), East Polynesian (315 cases, 439 controls) and West Polynesian (251 cases, 144 controls) individuals were recruited. Six variants (*SLC22A11; rs693591, rs17300741, rs2078267: SLC22A12; rs3825018, rs475688, rs476037*) were genotyped. Association analysis was conducted using PLINK and STATA software.

Association between *rs475688* (*SLC22A12*) and gout was observed in the Caucasian samples (OR=1.25, *P*=0.05) but no association was seen with *SLC22A11*. Gout in the Eastern Polynesian samples was associated with both *SLC22A11* (*rs2078267:* OR=1.46, *P*=0.04) and *SLC22A12* (*rs3825018*; OR=1.30, *P*=0.05). There were no significant associations in the Western Polynesian samples.

The first evidence for association between *SLC22A12* (*URAT1*) and gout in a Caucasian sample set is reported here, whilst this is the first study that has investigated *SLC22A11 (OAT4).* The association seen in the two genes was not independent in the Polynesian samples, but it was in the Caucasian samples.

O7

The Oxygen Evolving Centre of Photosystem II

Jackson SA, Eaton-Rye JJ

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Photosystem II (PS II) catalyses the light-driven oxidation of water to yield molecular oxygen, hydrogen ions and reduced plastoquinone molecules. The oxygen-evolving catalytic reaction centre of PS II, also known as the oxygen-evolving centre (OEC), is comprised of a Mn_4CaO_5 cluster. Oxidation of water within the OEC takes place over a series of four oxidation steps. Each step is driven by a separate electron extraction event. The hole for electron extraction is generated at the site of primary charge separation, a special chlorophyll cluster, also known as P680. The high electrochemical potential generated during primary charge separation (greater than 1100 mV) can result in promiscuous reactions between redox cofactors and their surrounding environments. These reactions can produce singlet oxygen, hydrogen peroxide and hydroxyl radicals which in turn react with and damage the protein structure of PS II. Oxygen formation and release can be detected using a bare platinum electrode constructed in-house. This highly sensitive oxygen electrode is capable of measuring the release of oxygen from PS II centres following a single turnover actinic flash of light. In addition an instrument to analyse thermally-stimulated radiative de-trapping of radical pairs (thermoluminescence) within the PS II complex has also been constructed. This has been used to investigate innate photoprotective mechanisms active during biogenesis of the OEC which may serve to protect the complex from oxidative damage.

O8

Peroxiredoxin 3 as a Mitochondrial Antioxidant and Sensor and Marker of Mitochondrial Oxidative Stress

Hampton M

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Mitochondria are a major site of oxidant generation, and oxidative stress within these organelles is associated with a variety of diseases. Peroxiredoxin 3 (Prx 3) is an abundant mitochondrial antioxidant protein that is extremely effective at removing hydroperoxides. Prxs also exhibit a number of intriguing properties that distinguish them from conventional antioxidants, including the ability to form complex oligomeric structures. It is speculated that Prx 3 can also function as a redox sensor that transmit signals as part of the cellular response to mitochondrial oxidative stress. In this presentation I will summarize the status of our investigations into the antioxidant and signalling properties of Prx 3, and the circumstances and consequences of Prx 3 oxidation.

O9

Kinetics of Peroxiredoxin 2 and 3 Hyperoxidation

Poynton R, Peskin A, Dickerhof N, Paton L, Winterbourn C, Hampton M

Free Radical Research Group, Department of Pathology, University of Otago, Christchurch, NZ

Mitochondria are a primary source of reactive oxygen species and a major antioxidant enzyme in this compartment is peroxiredoxin 3 (Prx 3). A unique characteristic of this antioxidant is its ability to be inactivated by its substrate H_2O_2. In the Prx 3 catalytic cycle the initial reaction of H_2O_2 with the peroxidatic cysteine results in a sulfenic acid. The sulfenylated cysteine subsequently condenses with the resolving cysteine on the opposing monomer. This dimerisation requires partial unfolding of the protein allowing time for H_2O_2 react with the sulfenylated cysteine to hyperoxidize the protein. Hyperoxidized Prxs are very stable in cells and this feature may make it useful as an indicator of oxidative stress. It has previously been shown Prx 3 is less susceptible to hyperoxidation relative to cytosolic Prx 2 using the semi-quantitative method of western blotting. We have further characterised this hyperoxidation using a liquid chromatography/mass spectrometry (LC/MS) method which allows us to quantitatively measure Prx redox states. Recombinant human Prx 3 was treated with increasing concentrations of H_2O_2 or used in a catalase competition assay. The samples were then reduced, denatured, alkylated and analysed by LC/MS. The measured reduced and hyperoxidized monomers were used for kinetic analysis. We found that the rate constants for hyperoxidation (k_2) of Prx 2 and 3 is 14,000 M^{-1} s^{-1}. The difference between these two proteins lies in the rate constant for dimerisation (k_3) where Prx 3 $k_3 = 26$ s^{-1} and Prx 2 $k_2 = 2$ s^{-1}. This faster dimerisation rate of Prx 3 reduces the time available for H_2O_2 to react with the sulfenylated cysteine making it less susceptible to hyperoxidation.

O10

A developmental role for Peroxiredoxin 3

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Peroxiredoxin 3 (Prx 3) is a protein that resides in the mitochondria and decomposes the hydrogen peroxide produced there. Prx 3 has biochemical and physical attributes that give it the potential to play a major role in mitochondrial redox signalling. A role for Prx3 in cellular signalling is currently only hypothetical. This study investigated the function of Prx3 in early zebrafish development using morpholino antisense oligonucleotide technology. Embryos injected with the Prx 3 morpholino before the 4-cell stage had a reduction in Prx 3 at the protein level. The antioxidant function of Prx 3 was tested using antimycin: an inhibitor of mitochondrial electron transport that causes increased ROS production. Treatment of Prx 3 morphants with antimycin resulted in developmental arrest that was more severe than in control fish. Interestingly, Prx 3 morphants had a build up of blood in the tail at 2 days post fertilization. At 24 hours post fertilization in situ hybridization showed that Prx 3 transcription was spatially aligned with the hematopoietic transcription factor gata1. Our results suggest that Prx3 functions as an antioxidant and has a regulatory role in zebrafish blood development.

O11

Cytochrome *c* peroxidase activity and mitochondrial release during apoptosis

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The only known naturally occurring mutation in cytochrome *c,* identified in a New Zealand family, is associated with mild thrombocytopenia due to dysregulation of platelet formation in the bone marrow. The mutation is from a glycine to serine at residue 41 (G41S). Cytochrome *c* has crucial roles in both cellular energy production and as a stimulus for programmed cell death. The ability of cytochrome *c* to activate apoptosis is dependent on two steps; the release of cytochrome *c* from mitochondria in response to apoptotic stimuli, and binding to apoptotic protease activating factor-1 (Apaf-1) in the cytosol to form a large caspase activating complex. We have previously shown that the G41S mutation enhances cytochrome *c*-induced caspase activation *in vitro*. In healthy cells cytochrome *c* is associated with cardiolipin, an anionic phospholipid found predominately in the inner mitochondrial membrane. It has been proposed that cytochrome *c* release is dependent on cytochrome *c*-dependent peroxidation of cardiolipin, although this remains controversial. Here we investigated the impact of mutations at residue 41 on cytochrome *c* peroxidase activity and release from mitochondria. Mutations of Gly41 to serine or threonine increased the peroxidase activity of cytochrome *c* 2- or 4-fold compared to wild type cytochrome *c*, respectively. We found that both G41S and G41T cytochrome *c* have an altered hydrogen bond network and a weakened axial Met80-Fe coordination that may contribute to the increased peroxidase activity. In addition, the increased peroxidase activity of G41S cytochrome *c* correlated with a more efficient release of cytochrome *c* from mitochondria isolated from livers of G41S knock-in mice. This result provides support for the hypothesis that the peroxidase activity of cytochrome *c* is necessary for its release from mitochondria in response to apoptotic stimuli.

O12

Cysteine dioxygenase: rapid kinetics of enzyme-substrate complex formation

Tchesnokov EP[1], Wilbanks SM[2], Jameson GNL[1]

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Oxidation of cysteine to cysteine sulfinic acid is the first step in cysteine catabolism and by implication is of immediate importance in human physiology and disease. Transfer of molecular oxygen to cysteinate is catalyzed by a non-heme mono iron enzyme cysteine dioxygenase (CDO) which chelates its Fe(II) metal cofactor by a three histidine motif in the active site. Although different reaction intermediates have been proposed for the reaction mechanism of CDO, none have been characterized *on pathway*. Importantly, characterization of the substrate dissociation constant, the on and off rates of enzyme-substrate complex formation and the rate of active site chemistry are yet to be determined. We have employed gamma ray absorption spectroscopy (Mössbauer) to monitor directly the CDO Fe (II) metal cofactor electronic environment. This has allowed us to carry out a substrate titration and directly follow substrate binding, thus ascertaining a substrate dissociation constant that indicates substrate is tightly bound in the active site. To measure the rate of substrate binding we have used the freeze-quench method in combination with Mössbauer spectroscopy to freeze the reaction on the millisecond timescale. Our preliminary data suggests an unexpectedly fast rate of substrate binding. The implications of these results will be discussed.

O13

Detection of reactive oxygen species

Stocker R

Victor Chang Cardiac Research Institute, Sydney, Australia

Roland is recognized internationally as an expert in redox biology, particularly for his research on antioxidants, and mechanisms and prevention of atherosclerotic vascular disease. Roland's work on bilirubin as a natural antioxidant is now referred to in biochemistry textbooks and it has contributed to a change in the threshold at which hyperbilirubinemia is treated in clinics. His work on vitamin E has changed the handling of parenteral nutrition in NICUs in New Zealand/Australia, and it provides a scientific rationale for the overall lack of benefit of vitamin E supplements on cardiovascular disease outcome.

O14

ROS-induced degradation of some β-blocker drugs in aqueous solution

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β-blocker drugs are widely prescribed for the control of blood pressure but are not easily removed from waste-water using the traditional primary and secondary treatment steps. In this talk we report the use of pulse radiolysis techniques to measure the rate of $^{\cdot}OH$ and e_{aq}^{-} induced degradation of atenolol, metoprolol and propanolol in aqueous solution. LC-MS analysis of the products arising from ^{60}Co irradiation provided information on the degradation pathways.

O15

Development of photoactivated nitric oxide donors as anticancer drugs

Kumari S, Giles NM, Giles GI

Department of Pharmacology and Toxicology, University of Otago, Dunedin, NZ

Nitric oxide (NO) is a small messenger molecule which plays multiple role in biological system. It has also vital functions in cancer biology, where it has been found that a high NO concentration (>1 μM) causes cell death. Currently available NO donor drugs are limited in therapeutic potential due to a lack of organ or tissue specificity and stability. These limitations have stimulated great interest in the development of compounds that can generate NO in a controlled and sustained manner. We therefore designed and characterised the photosensitive NO donor drug molecule tert-dodecane S-nitrosothiol (tDodSNO). Our data demonstrate that tDodSNO is highly stable in vitro in the absence of irradiation ($t_{1/2}$ > 3 h), while its rate of decomposition can be regulated by modulating the intensity or duration of the photostimulus. NO release kinetics were determined by direct real time electrochemical measurement using a NO sensing electrode. We found a steady state concentration of 8 μM ± 2 NO could be obtained upon photoactivation (350 W/m^2) of 100 μM tDodSNO for 30 minutes. Furthermore we also investigated the concentration dependent cytotoxicity of tDodSNO on the A549 lung cancer cell line. Our data revealed that irradiation (25 W/m^2) induced highly significant increases in cytotoxicity within a therapeutic drug concentration (1-100 μM). In conclusion, our study suggests that photosensitive tDodSNO has the potential to be a promising novel chemotherapeutic agent.

O16

Biological properties of isothiocyanates

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Isothiocyanates are a class of phytochemicals that have been shown to exhibit anti-cancer and anti-inflammatory activity. We are exploring their mechanisms of action at a molecular level. Isothiocyanates are able to inhibit the pro-inflammatory cytokine MIF (macrophage migration inhibitory factor) through covalent modification of the N-terminal proline residue. The inhibitory capacity of a selection of novel isothiocyanates is currently being investigated. We are also investigating the cytotoxicity of these compounds, with particular focus on their ability to trigger apoptosis in cell lines overexpressing the anti-apoptotic oncogene bcl-2.

O17

Synthesis, Structure Activity Relationship Studies and Antioxidant Activity Evaluation of some Organochalcogen Catalysts as Novel GPx Mimics

Zadehvakili B[1], Fawcett JP[1], Giles GI[2]

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[2]Department of Pharmacology and Toxicology, University of Otago, Dunedin, NZ

Organochalcogen compounds have interesting biological activities including glutathione peroxidase (GPx) mimic activity. GPx is an important mammalian selenoenzyme which is responsible for the reduction of toxic peroxides at the expense of glutathione (GSH), an endogenous thiol. GPx mimics have been designed and synthesized by incorporating a selenium or tellurium atom within an organic frame. Facile changes of oxidation state are a characteristic property of selenium and tellurium, which is catalytically favourable as they can be more easily oxidized and reduced between valance states. Their catalytic redox cycle plays an important role in biological systems, especially in sulphide/disulphide redox chemistry. It consists of the initial oxidation of the chalcogen atom (reduction of peroxide), followed by regeneration of the catalyst via oxidation of two equivalents of thiols. As a drug design criteria, modification of lipophilicity of GPx mimics may improve their cellular uptake. Structure-activity relationship studies determined beneficial modifications which lead to maximum activity. In this regard, we have synthesized a range of structurally related phenyl alkyl chalcogenides (butyl to heptyl) with increased lipophilicity (ClogP 5-9) and evaluated their GPx-like catalytic activity using electrochemical and spectrophotometric *in vitro* assays (K^{cat} values range from 4 to 0.4 min^{-1}) and compared with two well-known GPx mimics, ebselen and diphenyl ditelluride.

O18

Oxidative Stress Induced DNA Hemi-Hypomethilation and the Role of Maintenance Methylation

Magrath-Cohen H, Morison I, Weeks R
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Epigenetic modification, such as the addition of a methyl group to cytosine are involved in many important regulatory events. DNA methylation patterns are inherited through cell generations through the action of DNA methyltransferases (DNMTs). During DNA replication DNMTs recognise hemi-methylated DNA and, by the addition of methyl groups to the newly synthesised DNA strand, maintain methylation.
The source of methyl groups for the enzymatic reaction of DNMTs is the universal methyl donor S-adenosyl-L-methionine (SAM). The oxidative compound glycine chloramine depletes the cellular level of SAM. Additionally, experiments demonstrate that glycine chloramine prevents the irreversible capture of DNMT1 onto DNA, either directly by inhibition ofDNMT1, or by blocking DNMT1 binding to hemimethylated DNA during replication.
The PCDHGA12 gene promoter is heavily methylated in Jurkat cells (immortalised T acute lymphoblastic leukaemia cell line).
In our experiments, Jurkat cells are treated with glycine chloramine to inhibit DNMT1 and hairpin-bisulfite PCR can then be performed to determine the methylation status of both DNA strands of the PCDHGA12 promoter.
The aims of the project are to investigate the role of hypoxia/oxidative stress in DNA hypomethylation and to understand the mechanisms of cancer hypo- and hypermethylation.

P1

Modulation of HOCl production in neutrophil phagosomes

Albrett AM, Kettle AJ, Winterbourn CC

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Neutrophils eliminate microbes by ingesting them into an intracellular vacuole called a phagosome. This process is an integral part of innate immunity. During this process, a number of reactive oxygen species are generated including hypochlorous acid (HOCl), the bactericidal properties of which are sufficient to kill the ingested microbe. HOCl is produced from H_2O_2 and Cl^- by the green haem enzyme myeloperoxidase. Though HOCl is the most bactericidal oxidant produced by neutrophils, it is not certain how neutrophils use reactive oxidants to kill microbes. Our aim is to resolve this issue, providing a sound understanding of host defence and a platform for developing strategies to overcome bacterial resistance. Historically, investigation of HOCl production in neutrophil phagosomes has been limited by a lack of an adequate HOCl detection method. However, recently highly selective and specific fluorescent probes derived from rhodamine have been developed by Yoon and Lee which allow for the specific detection of physiological HOCl production. These live cell compatible probes allow visualisation of HOCl production in the phagosome of human neutrophils. Utilising these probes we have investigated the kinetics of HOCl production in neutrophil phagosomes as they ingest microbes. Factors that modulate the production of HOCl have also been explored by employing inhibitors of MPO and NADPH-oxidase and a scavenger of HOCl. We have shown that HOCl formation is dependent on an active NADPH-oxidase. Inhibition reduced both intra- and extracellular HOCl production. Inhibition of extracellular MPO partially reduced the amount of extracellular HOCl produced. HOCl scavenger methionine reduced essentially all extracellular HOCl. Selectivity studies with chloramines have shown the probe to be responsive to taurine chloramine and ammonia chloramines, in addition to HOCl. We are in the process of developing methods to effectively probe the intracellular HOCl production and visualise this using fluorescence microscopy.

P2

Seeking Evidence for a Halogenating Intermediate of Myeloperoxidase

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Recently, it was proposed that myeloperoxidase has an enzyme bound chlorinating intermediate in its catalytic cycle and that the enzyme can chlorinate substances either directly or via the release of free hypochlorite. The existence of a chlorinating intermediate suggests that certain substrates may be specifically chlorinated. Thus chlorination by myeloperoxidase could rely on favourable binding of substrates to the enzyme rather than kinetically favourable reactions with free hypochlorite. In this investigation we have sought additional evidence of a chlorinating intermediate in the catalytic cycle of myeloperoxidase. We used NADH as a detector of free hypohalous acids. It reacts rapidly with hypohalous acids but should complete poorly with small molecules that can enter the active site of myeloperoxidase and be directly halogenated by the enzyme. The reaction of NADH with hypohalous acids at pH 7.4 resulted in the stoichiometric generation of NADH-halohydrins, which were identified by mass spectroscopy. Chlorination and bromination of NADH produced several isomers. The chlorohydrins were more stable than the bromohydrins. The strong UV absorbance of the halohydrins allowed us to monitor the kinetics of their formation by myeloperoxidase. We found that taurine, a small substrate compared to NADH, inhibited chlorination of NADH by myeloperoxidase or reagent hypochlorous acid equally well. The ability of taurine to inhibit chlorination by myeloperoxidase was independent of the concentration of chloride between 10 and 100 mM. Together these results suggest that under physiologically relevant conditions myeloperoxidase releases predominantly free hypochlourous acid and there is little involvement of a chlorinating intermediate that reacts preferentially with small substrates such as taurine and chloride.

P3

Review of Methods for reactive oxygen species (ROS) detection in aqueous environments

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A comprehensive review of these methods has just been published online in Aquatic Sciences 2012 (doi 10.1007/s00027-012-0251-x) accompanied by a comprehensive, computer-searchable Excel database (http://neon.otago.ac.nz/research/bmp/data/ros_database.xlsx) of these methods. This review summarizes direct and indirect analytical methods for the detection and quantification of the reactive oxygen species (ROS): $^1O_2$, $O_2^{\cdot-}$/$HOO^{\cdot}$, H_2O_2, $HO^{\cdot}$, and $CO_3^{\cdot-}$ in aqueous solution. Each section briefly describes the chemical properties of a specific ROS followed by a table (organized alphabetically by detection method, i.e., absorbance, chemiluminescence, etc.) summarizing the nature of the observable (associated analytical signal) for each method, limit of detection, application notes, and reaction of the probe molecule with the particular ROS.

P4

The Kinetics of Oxidation of 3-Mercaptopropionic Acid in the Presence of Iron

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Thiol oxidation is important yet remarkably complicated due to a wide variety of different processes that can occur. Because of its pivotal role in Biology, cysteine has received particular attention, but although 3-mercaptopropionic acid (3-mpa) has a similar structure to cysteine, it only differs by the absence of the alpha-amino group, the oxidation kinetics have not been as thoroughly studied as cysteine. The aim of this work is to investigate the oxidation kinetics of 3-mpa in the presence of Fe(II) and Fe(III) in both aerobic and anaerobic conditions. Oxygen electrode results have shown that the oxidation of 3-mpa by dioxygen in the presence of Fe(II) is stoichiometric with respect to iron, which is in stark contrast to cysteine where iron is catalytic. To investigate this, a combination of freeze-quench Mössbauer spectroscopy and stopped-flow kinetics have been utilised. Initial results using these techniques have suggested that aqueous Fe(II) either forms a $[Fe(II)(3\text{-mpa})_2]$ bis-complex or decays to a ferric and diferric-oxo species where the ferric species can oxidise 3-mpa to form the disulfide and reform the bis-complex. The causes and the implications of this different reactivity compared to cysteine will be discussed.

P5

Complex roles of reactive oxygen species in the formation and antimicrobial action of neutrophil NETs

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Background. Various inflammatory stimuli induce neutrophils to release extracellular traps (NETs). These consist of a network of chromatin strands associated with predominantly granule proteins, including myeloperoxidase (MPO). NETs are proposed to contribute to host defence by trapping and killing micro-organisms. They may also be detrimental to the host in chronic inflammation or severe sepsis.
Aim. To determine whether reactive oxygen species produced by the neutrophil NADPH oxidase and MPO are required for NET formation with a variety of stimuli, and whether MPO is involved in bacterial killing by released NETs.
Results. Phorbol myristate acetate (PMA) and several bacterial species induced NETs. This was prevented by inhibition of the NADPH oxidase. However, the calcium ionophore, ionomycin, efficiently induced NETS independently of oxidase activity. MPO involvement depended on the stimulus. With PMA, NET formation was reduced when MPO was inhibited or MPO-deficient neutrophils were used. In contrast, with the more physiological bacterial stimuli, NET formation was not reduced when MPO was absent or inhibited. With normal neutrophils and PMA, more than 30% of the total cellular MPO was released with NETs. The majority was NET-associated and bound enzyme was active. In our hands, isolated NETs trapped but did not kill *Staphylococcus aureus*. However, killing was observed when hydrogen peroxide was added, and this was prevented by MPO inhibitors.
Conclusions. The involvement of oxidants in NET formation is complex and the mechanism unknown. With most inducers, NADPH oxidase activity is required. MPO activity, while important with PMA, is is not required with phagocytic stimuli. However, the enzymatic activity of bound MPO could provide antimicrobial activity of released NETs.

P6

Detection of Pro-Myeloperoxidase in Circulation

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Myeloperoxidase (MPO), a member of the heme peroxidise family, has emerged as a potential mediator of chronic inflammation. MPO has been identified as an important potential participant of acute and chronic cardiovascular disease. A few studies indicate that MPO is synthesized as precursors that undergo a complex series of proteolytic processing events to achieve the final native protein. During biosynthesis, the majority undergoes proteolytic processing to generate mature MPO, whilst the remainder (90kD proMPO with one heme) is constitutively secreted from myeloid. The function and fate of proMPO during inflammation is unknown. The aim of this work was to determine whether proMPO is present in circulation of healthy controls and individuals with cardiovascular disease. MPO was purified using an affinity column with anti-MPO antibodies attached to NHS-activated Sepharose 4 Fast Flow beads. Preliminary experiments were performed for recovery by spiking plasma with purified standards as MPO, proMPO, and HL60 cell lysates. ProMPO was purified and detected in plasma from 24 healthy controls, 18 patients with myocardial infarctions (MI) and 11 patients with chronic stable angina (CSA) plasma samples. ProMPO was assessed by 10 % SDS-PAGE under reducing conditions and then by immunoblot. ProMPO band was elevated in plasma samples from patients, compared with healthy controls. Furthermore, an LC/MS method was developed for the detection of a proMPO specific tryptic peptide. This method enabled us to confirm that the elevated band described above indeed contained proMPO. We also used this method to analyse plasma samples that were purified by affinity chromatography. We were able to detect proMPO in one out of six (1/6) analysed samples from healthy controls, 4/5 samples from patients with MI and 1/3 with CSA. Thus, these results demonstrate that proMPO obviously is present in circulation, and it is elevated in plasma from patients with cardiovascular disease.

P7

The RyR2 Binding Protein Triadin Decreases the Propensity for Store Overload Induced Ca^{2+} Release

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Arrhythmia occurs in a number of types of heart disease including heart failure (HF) and Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT). It has been shown that store overload induced calcium release (SOICR) is a common mechanism underlying many types of arrhythmia. It is also known that SOICR occurs due to the inappropriate opening of the cardiac ryanodine receptor (RyR2), as a result of increased sensitivity to sarcoplasmic reticulum (SR) luminal Ca2+. RyR2 forms a large macromolecular complex with other proteins which have been proposed to regulate RyR2 activity. One such protein is the luminal protein triadin which is known to be lost from the RyR2 complex in HF and CPVT. In the present study, we investigated whether the interaction of triadin with RyR2 is important for the luminal Ca2+ sensitivity of RyR2 and therefore the propensity for SOICR. Single cell cytosolic Ca2+ imaging, using the high affinity Ca2+ indicator Fluo4-AM, showed that the presence of triadin delayed the initiation of SOICR events, suggesting an inhibitory role for triadin. This was confirmed by single cell luminal Ca2+ imaging, using the ER targeted Ca2+ indicator protein (D1ER), in cells expressing a hereditary arrhythmogenic mutant form of RyR2 (V4653F). In these cells the addition of triadin significantly increased the luminal Ca2+ level required for SOICR, restoring it to near RyR2wt levels. These results provide the first evidence that triadin directly de-sensitises RyR2 to luminal Ca2+ and reduces the propensity for SOICR. Therefore this suggests that the loss of triadin in HF and CPVT is arrhymogenic and that stabilising the interaction of triadin with RyR2 is likely to be therapeutic.

P8

Cysteine Dioxygenase unfaithful – and why we are excited about that

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The enzyme *cysteine dioxygenase* is linked to various diseases including rheumatoid arthritis and several neurological diseases, such as Alzheimer's and Parkinson's diseases. Here we present a bacterial form of the enzyme of the organism *Pseudomonas aeruginosa* which shows reactivity for two different thiol substrates at the same active site, which is unique within in the realm of thiol dioxygenases. We are investigating this intriguing reactivity using a number of different kinetic and spectroscopic approaches. HPLC and NMR analyses show that the enzyme can not only utilize cysteine to produce cysteine sulfinic acid but also turns 3-mercaptopropionic acid into 3-sulfinopropionic acid. Preliminary studies of the kinetic parameters suggest a higher affinity for 3-mercaptopropionic acid as a substrate and a higher turnover rate. However, other substrate homologs, specifically homocysteine and cysteamine, could not be utilized but weakly inhibit cysteine turnover. This broader but still very specific substrate profile gives further insight into the function of cysteine dioxygenases in general and may lead to a better understanding of its mammalian counterpart.

P9

Hydrogen peroxide-dependent interaction of peroxiredoxin 2 with ERp46

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Introduction Peroxiredoxin 2 (Prx2) is a mainly cytosolic ubiquitously expressed antioxidant enzyme. Its absence has been associated with oxidative stress, apoptosis and cancers. Prx2 has a high reactivity with H_2O_2, forming a sulfenic acid on the Prx2 peroxidative cysteine (Cys^P), which then reacts with the resolving cysteine (Cys^R) of another Prx2 molecule, creating disulfide linked dimers. If Cys^P is further oxidised by H_2O_2 before it can dimerise, it becomes hyperoxidised to a sulfinic acid, preventing further catalytic removal of H_2O_2. Prx2 has been implicated in H_2O_2-mediated cellular signalling. This could be facilitated by two possible mechanisms. Reduced Prx2 could remove H_2O_2, preventing its reaction with other targets. Alternatively, overoxidation of Prx2 could alter binding affinities and enable thiol-disulfide exchanges with interacting proteins and thus transmit the signal. Our objective has been to identify H_2O_2-dependent protein interactors of Prx2 that are specific to its hyperoxidised state.

Methods Immunoprecipitations from lysates of untreated or H_2O_2 -treated Jurkat cell lysates, size-exclusion chromatography, SDS-PAGE, immunoblotting, and recombinant proteins were used to characterise hyperoxidised Prx2 interactions.

Results Immunoprecipitations of Prx2 revealed a novel interaction that occurred only with H_2O_2 treatment. Prx2 was hyperoxidised under these conditions and the interaction was reversed by dithiothreitol. The interacting protein was identified as ERp46, a member of the protein disulfide isomerase family, which is mainly endoplasmic reticulum resident and important for secretory protein folding. Addition of mutant Prx2 proteins to cell lysates showed that Cys^R but not Cys^P is required for the interaction. This interaction was disrupted by alkylation and denaturation, implicating a non-covalent affinity between Prx2 and ERp46 that relies on internal disulfide linkages.

Discussion These findings characterise a novel interaction of hyperoxidised Prx2 with ERp46 that possibly links protein folding pathways to H_2O_2 regulation, and may represent a wider mechanism for signalling roles of hyperoxidised peroxiredoxins.

P10

Allantoin as a biomarker of severity of heart disease and its relationship with myeloperoxidase

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Introduction Myeloperoxidase is elevated in patients with cardiovascular disease. Allantoin is the major oxidation product of urate in humans and has been shown to be produced when urate is exposed to myeloperoxidase *in vitro*. We investigated whether allantoin levels were increased in patients with cardiovascular disease. Its relationship to myeloperoxidase activity and to established markers of heart disease was determined.
Methods Myeloperoxidase activity/protein and allantoin levels in plasma from femoral arteries of healthy controls (n=31) and patients with ST elevated myocardial infarcts (n=31), stable angina (n=12) and 4 months post myocardial infarctions (n=25) were measured using an ELISA and LCMS/MS respectively. Differences between groups and for correlations between biomarkers were examined. We also measured the troponin, creatinine kinase and magnitude of ST elevation in ST-elevated myocardial infarcts (STEMI) and determined their relationship with femoral plasma allantoin concentrations.
Results Allantoin was significantly elevated ($p<0.001$) in patients with ST-elevated myocardial infarcts (median 6.2 μM; IQR 5.1-7.8 μM) as compared to healthy controls (2.4; 1.4-3.7), patients who had angina (4.0; 2.3-5.4) and patients 4 months post-myocardial infarction (3.1; 2.0-4.2). It correlated with markers of severity of STEMI disease such as creatinine kinase MB, peak troponin I levels and magnitude of ST elevation ($P<0.05$).The allantoin concentration was correlated with myeloperoxidase activity (CC= 0.504; $p=0.0047$) and MPO protein (CC = 0.452; $p=0.01$). In patients with STEMI, allantoin was also found to be inversely correlated with neutrophil intracellular myeloperoxidase mean fluorescence intensity (CC = -0.450; $p=0.001$), a measure of neutrophil degranulation.
Discussion We propose that higher plasma allantoin levels indicate increased oxidative stress in patients with STEMI and allantoin is good biomarker of severity of heart disease. The correlation of myeloperoxidase with plasma allantoin suggests that it is partially responsible for oxidation of urate to allantoin *in vivo.*

P11

Protein Expression of SERCA2a and PLB in Diabetes

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Type 2 diabetes mellitus (T2DM) is characterised by impaired relaxation of the heart that can eventually lead to heart failure. The cardiac relaxation pathway is governed by the combined activity of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2a) and phospholamban (PLB). SERCA2a actively resequesters free cytosolic Ca^{2+} to the sarcoplasmic reticulum, promoting cardiac relaxation, whereas PLB inhibits this SERCA2a activity, thereby reducing cardiac relaxation. Several type 1 diabetes mellitus (T1DM) animal and human studies and a few T2DM animal studies have found decreased SERCA2a activity and increased PLB expression in the left ventricle of the heart, indicating reduced cardiac relaxation. However, mechanisms underlying reduced SERCA2a activity in humans with T2DM, remain undefined. To examine SERCA2a and PLB expression, we carried out a western blotting analysis in human atrial appendages from T2DM and non-diabetic patients with preserved ejection fraction, undergoing coronary artery bypass surgery (CABG). Blot analysis revealed similar total SERCA2a protein levels between T2DM and non-diabetic patients. Unexpectedly, we found a decrease in PLB protein levels in T2DM patients compared to non-diabetic patients, resulting in a decreased PLB:SERCA2a ratio in T2DM patients compared to non-diabetic patients. Thus, in contrast to previous studies in diabetic animals (T1DM and T2DM) and humans (T1DM), our current data in T2DM patients, who do not yet have systolic dysfunction, indicate that they have an increased SERCA2a activity, suggesting improved atrial relaxation. This may suggest that in the pre-failing diabetic heart SERCA2A activity is increased as a compensatory mechanism prior to the development of overt systolic dysfunction.

P12

Hypoxia Extends Neutrophil Survival but does not Necessarily Retain Cell Function

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An adequate supply of oxygen is essential for the proper functioning of neutrophils. Inflammatory sites are often hypoxic, however, and this can affect neutrophil function. Previous studies have shown that neutrophil apoptosis is inhibited by hypoxia but the eventual fate of the cells is not clear.
The aims of this project are: (1) to characterize the mechanism of extended survival of neutrophils under hypoxic conditions; (2) to determine whether, in the absence of apoptosis, neutrophils eventually die by necrosis and (3) to monitor neutrophils function under extended hypoxia.
We have investigated neutrophil survival, measured the release of cytotoxic granule proteins in to the extracellular medium, followed the activation of apoptotic pathways (caspase 3 activation, morphology changes and Phosphatidylserine exposure) and monitored neutrophil function (superoxide production, Neutrophil Extracellular Trap-NET formation, bacterial killing) under conditions of anoxia or hypoxia.
In conditions of severe hypoxia, neutrophils were still viable after 48hr. Apoptosis was inhibited, but these same cells showed considerable levels of caspase 3 activation. The later is considered a classic hallmark of apoptosis, but in this case was not accompanied by any other signs of cell death. Superoxide production by neutrophils was low when the cells had been exposed to severe hypoxia for 4 hours and no radical production occurred after 24 hours. The amount of NET formation was diminished in cells exposed to hypoxia.
These results show that extended neutrophils survival under hypoxic conditions may not necessarily translate to prolonged activity and this may impact on the effectiveness and resolution of the inflammatory response.

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