Report on the Winterbourn Festschrift and 7th Joint Meeting of the Australasian and Japanese Societies of Free Radical Research

December 7-10 December 2015

University of Otago Christchurch

Four days of excellent science was started by a Festschrift that celebrated the career -so far - of Christine Winterbourn. This was a great day of celebration and brought together many people who had worked with Christine either in her lab or as international collaborators. Noted international speakers included Professors Wim Koppenol (Switzerland), Roland Stocker (Australia), Mike Davies (Denmark), Peter Nagy (Hungary) and Victor Darley-Usmar (USA). The Vice Chancellor of the University of Otago, Professor Harlene Hayne, spoke of Christine's huge contribution to the University and the wider research community in New Zealand and internationally. She also presented Christine with a Festschrift book (attached). About 100 people attended the day's celebration that included 15 oral presentations. The last presentation was given by Christine Winterbourn where she typically focused on what needs to be done to strengthen the field of free radical research. The day was preceded by a BBQ at Tony Kettle's home in which many previous collaborators attended.

The Joint Meeting of the Australasia and Japan Societies of Free Radical Research started directly after the Festschrift when Prof Raman Kalyanaraman (USA) gave a plenary talk. That evening all invited speakers were taken out to dinner at a local restaurant where the friendly spirit and tone of the meeting was established. The SFRR meeting was then held over the following three days. There were 105 registered delegates, 48 oral presentations, and 50 posters (program attached). It was a pleasure to have Professor Rafael Radi, President of the International Society of Free Radical Research, give the first talk on the first morning of the meeting. The support of ISFRR to bring Professor Radi to the meeting was gratefully acknowledged.

The meeting concluded with a conference dinner which was held in the Christchurch Botanical Gardens. Overall the meeting was a big success. It was a pleasure to celebrate Christine Winterbourn's career as she has given much to the field through her research and administrative work. It was also a pleasure to bring many international colleagues to Christchurch and return the hospitality we have enjoyed from them in various parts of the world including Australia, Japan and the USA. Also, it was an excellent way to introduce young New Zealand, Australian, and Japanese scientists to the high quality of research on free radicals in chemistry, biology and medicine.

This meeting was possible because of the generous support from the University of Otago, the Society for Free Radical Research-International, The Society for Free Radical Research Japan, the Maurice and Phyllis Paykel Trust, Biocell Corporation, Taylor & Francis, and illumine.

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Peter Nagy
Budapest

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Roland
Stocker
Sydney

The Society for Free Radical Research
AUSTRALASIA & JAPAN

Seventh Joint Meeting

Incorporating the University of Otago Oxygen Theme Meeting 2015

Programme and Abstracts

7-10 December 2015
Christchurch, New Zealand

Haere Mai, Welcome

Kia ora koutou katoa & welcome to you all.

He aha te kai ō te rangatira? He Kōrero, he kōrero, he kōrero.
(What is the food of the leader? It is knowledge. It is communication.)

On behalf of the organizing committee of this joint Australasia and Japan meeting of the Society for Free Radical Research, I would like to welcome you to Christchurch. Thanks for making the effort either to cross the ditch or venture down-under, and contributing to the success of our meeting.

We have approximately 100 delegates from all over the globe. Over the next four days, we are going to be busy with 50 talks and about 60 posters. There is plenty of novelty for everyone. On top of this, we have the pleasure of celebrating Christine Winterbourn's wonderful career by hearing talks from her former students and collaborators, plus the presentation of a Festschrift book by Professor Harlene Hayne, the Vice Chancellor of the University of Otago.

I would like to acknowledge the support given to the meeting by our sponsors. The chief sponsor is the University of Otago. Through their generous support we have been able to attract several excellent international speakers. We have also had generous support from the International Society for Free Radical Research, the Japanese Society for Free Radical Research, the Paykel Trust, BioCell, Taylor & Francis and Illumina.

I would also like to thank the other members of the organizing committee for the considerable amount of work they have put into organizing the meeting. If you require any assistance during the meeting, please ask for help at the registration desk or talk with members of the local Centre for Free Radical Research. They will be more than happy to help you.

Please challenge each other scientifically and enjoy each other's company throughout the week. I hope many strong collaborations and friendships will result from this meeting.

Na mihi and all the very best

Tony Kettle
Chair of the Organising Committee

Sponsors

We greatly appreciate the support of our sponsors:

Conference Venue

All sessions will be held in the Rolleston Lecture Theatre, Ground Floor, University of Otago, Christchurch building.

Oral Presentations

All presentations will be by Powerpoint. Presenters should bring their talks on a USB stick to the audio visual assistants at the lectern in the Rolleston Lecture Theatre no later than the break before their session begins.

Please keep to the allocated time of your talk. Session chairs will be instructed to stop speakers if time is exceeded.

Poster Sessions

There will be two poster sessions in the Rolleston Foyer. We ask that presenters are present to answer questions during their session.

Poster Session A is from 5.30-6.30pm on Tuesday evening. The presenters in this session should hang their posters as early as possible on Tuesday. They should be removed by 2.00pm on Wednesday.

Poster Session B is from 5.10-6.10pm on Wednesday. The presenters in this session should hang their posters on Wednesday afternoon before 5.00pm and leave them up until Thursday.

Velcro dots will be available to attach posters to the boards. Please place your poster on the numbered board that corresponds to the number assigned to your abstract in this book.

Catering

Morning and afternoon teas will be provided. Light lunches will also be available to registered delegates. All catering will be available in the Ground Floor Seminar Room.

Weather permitting, delegates may like to take a stroll through Hagley Park and the Christchurch Botanical Gardens during lunch breaks.

AGM

The Annual General Meeting of the Society for Free Radical Research Australasia will be held on Thursday, 10 December at 12.30pm in the Rolleston Lecture Theatre. Society subscription costs are included in the registration fee so all Australasian delegates will become members of the society for 2016.

A business meeting between the Executive Committees of the Societies for Free Radical Research Australasia and Japan will be held on Wednesday, 9 December at 12.30pm in Room 712 on Level 7 of the UOC building.

Conference Dinner

The conference dinner will be held at the ilex in the Christchurch Botanical Gardens starting at 7.00pm on Thursday, 10 December. See map of the botanical gardens on page 10 for directions.

Please note that the Hagley Park Armagh Street Car Park closes at 10.30pm.

Organising Committee

Tony Kettle
Mark Hampton
Yuji Naito
Louisa Forbes
Paul Pace
Alice Milnes

Scientific Advisory Committee

Tony Kettle
Mark Hampton
Yuji Naito
Paul Witting
Steven Gieseg

Administration and website support

Alice Milnes
Joie Deng
Linda Kerr
Anna Young
Hamed Hawwari

Map of Central Christchurch

Conference Programme

Monday 7 December 2015	
10.00am	Registration for SFRR A+J Conference Delegates
10.30am	Welcome
	Chair: Tony Kettle
10.35am	Diana Averill-Bates Professor of Biochemistry, University of Quebec, Montreal
10.55am	John French Director of Cardiovascular Research, Liverpool Hospital, Sydney
11.10am	Margreet Vissers Professor, Centre for Free Radical Research, Christchurch
11.30am	Tony Kettle Professor, Centre for Free Radical Research, Christchurch
11.45am	Glenn Vile General Manager, NZ Extracts Ltd, Blenheim
12.00pm	Mark Hampton Professor, Centre for Free Radical Research, Christchurch
12.20pm	Anitra Carr Senior Research Fellow, Centre for Free Radical Research, Christchurch
12.35pm	Lunch
	Chair: Mark Hampton
1.30pm	Maurice Owen Scientific Director, Canterbury Scientific, Christchurch
1.45pm	Wim Koppenol Professor of Biochemistry, ETH Zurich
2.05pm	John Windsor Professor of Surgery, Auckland University
2.20pm	Roland Stocker Professor of Vascular Biology, Victor Chang Cardiac Research Institute, Sydney
2.40pm	Mike Davies Professor of Biochemistry, University of Copenhagen
2.55pm	Recorded tributes
3.05pm	Peter Nagy Professor, National Institute of Oncology, Budapest
3.25pm	Victor Darley-Usmar Professor of Redox Biology, University of Alabama
3.40pm	Afternoon tea
	Chair: Margreet Vissers
4.00pm	Harlene Hayne Vice Chancellor of the University of Otago
4.10pm	Christine Winterbourn Professor, Centre for Free Radical Research, Christchurch & Guest of Honour
5.00pm	Refreshments Registration for SFRR A+J Conference Delegates
Opening of the SFRR A+ J Seventh Joint Meeting	Chair: Tony Kettle
6.00pm	Welcome
6.05pm	Plenary Lecture: Raman Kalyanaraman | Medical College of Wisconsin, USA *Identification of NADPH oxidase 2 isoform inhibitors from screening a small library of drug-like molecules: Strategies for discovering novel inhibitors of peroxynitrite*

Tuesday 8 December 2015	
8.30am	Registration
8.55am	Welcome
Session 1	Chair: Tony Kettle
9.05am	Rafael Radi | University of the Republic, Uruguay *Biochemistry of Mn-superoxide dismutase nitration and inactivation*
9.45am	Yorihiro Yamamoto | Tokyo University of Technology, Japan *Increased oxidative stress in patients with amyotrophic lateral sclerosis and the effect of edaravone administration*
10.10am	Stewart Cordwell | University of Sydney, Australia *Proteomic-scale approaches for identifying reversible and irreversible cysteine redox post-translational modifications in myocardial ischemia/reperfusion*
10.30am	Morning tea
Session 2	Chair: Paul Witting
11.00am	Jeffrey Erickson | University of Otago, New Zealand *Diabetes mellitus enhances apoptosis in the heart following myocardial infarction*
11.25am	Gemma Figtree | Royal North Shore Hospital & University of Sydney, Australia *Redox modification of caveolar proteins as important mediators in cardiovascular disease*
11.50pm	Sally McCormick | University of Otago, New Zealand *Ribose-cysteine increases glutathione-based antioxidant status in mouse models of hyperlipidaemia*
12.15pm	Christopher Stanley | Victor Chang Cardiac Research Institute, Australia *The tryptophan metabolite cis 3-hydroperoxypyrroloindole causes arterial relaxation in part via oxidative dimerization of protein kinase G1α*
12.35pm	SFRR Australasia and Japan Conference Photo
12.45pm	Lunch
Session 3	Chair: Margreet Vissers
1.35pm	Mike Davies | University of Copenhagen, Denmark *Peroxynitrous acid modifies the structure and function of the extracellular matrix in vitro and in vivo: implications for atherosclerosis*
2.15pm	Shinya Toyokuni | Nagoya University Graduate School of Medicine, Japan *Novel fluorescent probe for detecting catalytic ferrous iron*
2.40pm	Greg Giles | University of Otago, New Zealand *Development of the photoactivated nitric oxide donor drug tDodSNO*
3.00pm	Afternoon tea
Session 4	Chair: Christine Winterbourn
3.30pm	Annemarie Grindel | University of Vienna, Austria *Chromosomal damage in female patients with type 2 diabetes depends on HbA1c, diabetes duration and medication*
3.45pm	Naoko Suga | University of Hyogo, Japan *Modification of cellular proteins and induction of self-defense genes expressions by tryptamine-4,5-dione, a serotonin oxidation product*
4.00pm	Lanfeng Dong | Griffith University, Australia *Mitochondrial targeting of tamoxifen enhances its activity against Her2^{high} breast cancer via inhibiting mitochondrial complex I function*
4.15pm	Karina O'Connor | University of Otago, Christchurch, New Zealand *Mitochondrial redox changes during TNF-mediated necroptosis*
4.30pm	Belal Chami | University of Sydney, Australia

	4-Methoxy TEMPO attenuates murine experimental colitis
4.45pm	Amandeep Kaur | University of Sydney, Australia *A toolbox of reversible and ratiometric fluorescent probes for imaging cellular oxidative stress*
5.00pm	David Cheng | Victor Chang Cardiac Research Institute, Australia *Effects of pharmacological inhibition of myeloperoxidase on endothelial dysfunction in mouse models of inflammation*
5.15pm	Jereme Spiers | University of Queensland, Australia *Repeated psychological stress exposure causes inflammation and nitrosative stress in addition to antioxidant gene upregulation in the rat hippocampus*
5.30-6.30pm	Poster session A + Refreshments and nibbles

Wednesday 9 December 2015

Session 5	Chair: Jiri Neuzil
8.50am	Wim Koppenol | Swiss Federal Institute of Technology, Switzerland *Kinetics and energetics of cytochrome P450-mediated hydroxylation*
9.30am	Ghassan Maghzal | Victor Chang Cardiac Research Institute, Australia *Generation of singlet oxygen by indoleamine 2,3 dioxygenase and hydrogen peroxide – A novel path for the metabolism of tryptophan to N-formyl-kynurenine*
9.55am	Tomohisa Takagi | Kyoto Prefectural University of Medicine, Japan *The role of heme oxygenase-1 in intestinal inflammation using a murine colitis model*
10.15am	Hideo Utsumi | Kyushu University, Japan *Development of a free radical imaging system for clinical research*
10.35am	Morning tea
Session 6	Chair: Jan Gebicki
11.05am	Peter Nagy | National Institute of Oncology, Hungary *Protein persulfides: Insights into the molecular mechanisms of H_2S signaling*
11.45am	Takaaki Akaike | Tohoku University Graduate School of Medicine, Japan *Antioxidant and redox signaling functions of reactive persulfide species translationally formed on various proteins*
12.10pm	Nina Dickerhof | University of Otago, Christchurch, New Zealand *Evaluation of glutathione sulphonamide and allantoin as blood and urine based indicators of exacerbations in children with cystic fibrosis*
12.30pm	Lunch Business meeting of the SFFR Australasia and Japan Executive Committee
Session 7	Chair: Mark Hampton
1.20pm	Leslie Poole | Wake Forest School of Medicine, USA *Experimentally dissecting the structural origins of peroxiredoxin catalytic prowess*
2.00pm	Liz Ledgerwood | University of Otago, New Zealand *Examining the role of peroxiredoxin 1 in redox signalling*
2.25pm	Paul Pace | University of Otago, Christchurch, New Zealand *Interaction of peroxiredoxin 2 with collapsing response mediator protein 2*
2:45pm	Markus Dagnell | Karolinska Institute, Sweden & University of Otago, Christchurch, New Zealand *TrxR1 protects PTP1B from oxidative inactivation by hydrogen peroxide*
3.05pm	Afternoon tea

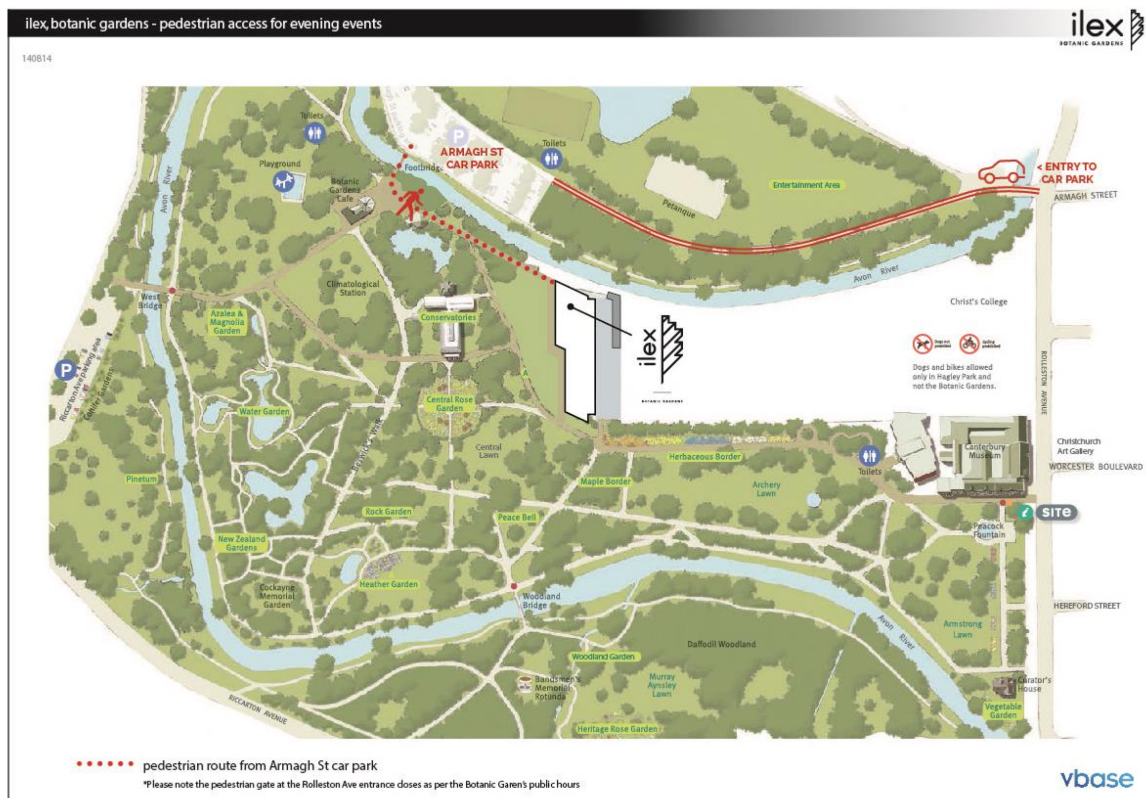
Session 8	Chair: Ghassan Maghzal
3.35pm	Clare Hawkins | Heart Research Institute & University of Sydney, Australia *Novel pathways of macrophage dysfunction and inflammation induced by myeloperoxidase-derived oxidants*
3.50pm	Yoji Kato | University of Hyogo, Japan *Myeloperoxidase-derived modification of biomolecules and its prevention*
4.25pm	Guy Jameson | University of Otago, New Zealand *Ascorbic acid as an essential cofactor in the production of hypothiocyanite by lactoperoxidase*
4.50pm	Louisa Forbes | University of Otago, Christchurch, New Zealand *Strategies for inhibiting myeloperoxidase and limiting oxidative stress during inflammation*
5.10-6.10pm	Poster session B + Refreshments and nibbles

Thursday 10 December 2015

Session 9	Chair: Alfons Lawen
9.00 am	Stavros Selemidis | Monash University, Australia *Endosomal NOX2 oxidase exacerbates virus pathogenicity*
9.25 am	Mike Berridge | Malaghan Institute of Medical Research, New Zealand *Is mitochondrial transfer between cells a normal physiological process? Implications for cellular stress responses and free radical generation*
9.45 am	Jiri Neuzil | Griffith University, Australia & Czech Academy of Sciences, Czech Republic *Acquisition of mitochondrial DNA by cancer cells devoid of mitochondrial genome is a prerequisite for tumor initiation*
10.05 am	Jianhua Zhang | University of Alabama at Birmingham, USA *Expected and unexpected impact of Nrf2 knockout and endurance exercise on autophagy and mitochondria in aging mouse brain*
10.25am	Morning tea
Session 10	Chair: Andrew Bulmer
11.05am	Yuji Naito | Kyoto Prefectural University of Medicine, Japan *Multiple targets of carbon monoxide gas in the intestinal inflammation*
11.30am	Alison Heather | University of Otago, New Zealand *3β-Hydroxysteroid-24-reductase (DHCR24): a key protein involved in protecting human coronary artery endothelial cells from inflammatory stress*
11.50pm	Andrew Bahn | University of Otago, New Zealand *GLUT9 alleviates the development of type 2 diabetes mellitus under hyperuricemic conditions*
12.10pm	Kazuhiko Uchiyama | Kyoto Prefectural University of Medicine, Japan *Heme oxygenase-1 prevents intestinal ischemia-reperfusion injury via the regulation of the inflammasome*
12.30pm	SFRR Australasia AGM Lunch
Session 11	Chair: Anitra Carr
1.30pm	Alan Crozier | University of California, Davis, USA *New insights into the bioavailability of dietary flavonoids*
1.50pm	Kevin Croft | University of Western Australia, Australia *The acute effect of quercetin-3-O-glucoside on blood pressure, endothelial function and no production in healthy men and women*
2.10pm	Nicola Brasch | Auckland University of Technology, New Zealand

	Pulse radiolysis and ultra-high performance liquid chromatography/high resolution mass spectrometry (UHPLC/HRMS) studies on the reactions of the carbonate radical with vitamin B_{12} complexes
2.30pm	Steven Gieseg |University of Canterbury, New Zealand *Is plasma neopterin the product of intracellular oxidant scavenging by 7,8-dihydroneopterin?*
2.50pm	Afternoon Tea
Session 12	Chair: Tony Kettle
3.20pm	Victor Darley-Usmar |University of Alabama at Birmingham, USA *The interface between bioenergetics and redox biology; from bench to bedside*
4.00pm	Roland Stocker | Victor Chang Cardiac Research Institute, Australia *Heme oxygenase-1 and metabolic reprogramming in response to ischemia*
4.25pm	Margreet Vissers | University of Otago, Christchurch, New Zealand *Intracellular ascorbate availability in health and disease and effects on the regulation of the HIF hydroxylases*
4.45pm	Summary
Dinner	
7.00pm	Conference Dinner at ilex, Christchurch Botanical Gardens

Directions to the conference dinner venue



Oral Abstracts

Identification of NADPH oxidase 2 isoform inhibitors from screening a small library of drug-like molecules: Strategies for discovering novel inhibitors of peroxynitrite

Jacek Zielonka, Monika Zielonka, Lynn VerPlank, Gang Cheng, Micael Hardy, Olivier Ouari, Mehmet Menaf Ayhan, Radoslaw Podsiadly, Adam Sikora, J David Lambeth, Balaraman Kalyanaraman

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Using a high-throughput screening (HTS)-compatible reactive oxygen and nitrogen species (ROS/RNS) assay, we identified potential inhibitors of NADPH oxidase (Nox2) isoform from a small library of FDA-approved bioactive compounds. By using multiple probes (hydroethidine, hydropropidine, Amplex Red, and coumarin boronate) with well-defined redox chemistry that form highly diagnostic marker products upon reaction with superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$), the number of false positives was greatly decreased. Selected hits for Nox2 were further screened for their ability to inhibit $ONOO^-$ formation in activated macrophages. New diagnostic marker product for $ONOO^-$ is reported. In this talk, the concept that newly developed HTS/ROS assays could also be used to identify potential inhibitors of $ONOO^-$ formed from Nox2-derived $O_2^{\bullet-}$ and nitric oxide synthase (NOS)-derived nitric oxide, is discussed.

Biochemistry of Mn-superoxide dismutase nitration and inactivation

Rafael Radi

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Mn-containing superoxide dismutase (MnSOD) is an essential antioxidant enzyme of the mitochondrial matrix in mammalian cells. Under inflammatory conditions that enhance nitric oxide ($^{\bullet}NO$) formation, MnSOD is tyrosine nitrated and inactivated *in vitro* and *in vivo*; this process facilitates a vicious cycle of alterations in mitochondrial redox homeostasis. The inactivation of MnSOD via tyrosine nitration requires the exclusive modification of only one out of nine tyrosine residues present in mammalian MnSOD, namely Tyr34. This tyrosine is located in the entrance channel to the active site, only 5 A away from the Mn atom. The selective nitration of Tyr34 is caused as a consequence of the fast reaction of peroxynitrite with the Mn center (ca. 10^5 $M^{-1}s^{-1}$), which generates nitrating species at the active site. In the presentation, recent experimental and computational (QM/MM) data regarding the kinetics, reaction mechanism at the atomic level and the thermodynamics of peroxynitrite reactions with MnSOD will be analyzed. As a consequence of the reaction, the incorporation of the $–NO_2$ group in Tyr34 creates steric and electrostatic restrictions that impede the diffusion of superoxide radical anion ($O_2^{\bullet-}$) to the active site Mn atom, therefore leading to enzyme inactivation. Overall, nitration and inactivation of MnSOD reflect reactions mediated by peroxynitrite in mitochondria and underscores that the reaction of $O_2^{\bullet-}$ with $^{\bullet}NO$ can outcompete MnSOD-catalyzed $O_2^{\bullet-}$ dismutation. Peroxynitrite-mediated MnSOD inactivation represents a contributory molecular mechanism in mitochondrial dysfunction and apoptotic signaling in a variety of pathological processes.

Increased oxidative stress in patients with amyotrophic lateral sclerosis and the effect of edaravone administration

Yorihiro Yamamoto[1], Midori Nagase[1], Yusuke Miyazaki[1], Hiide Yoshino[2]

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Compared to age-matched healthy controls (n=55), patients with amyotrophic lateral sclerosis (ALS) (n=26) showed increased oxidative stress as indicated by a significantly increased percentage of oxidized coenzyme Q10 (%CoQ10) in total plasma coenzyme Q10, a significantly decreased level of plasma uric acid, and a significantly decreased percentage of polyunsaturated fatty acids in total plasma free fatty acids (FFA). Therefore, the efficacy of edaravone, a radical scavenger, in these ALS patients was examined. Among 26 ALS patients, 17 received edaravone (30 mg/day, 1-4 times a week) for at least 3 months, and 13 continued for 6 months. Changes in revised ALS functional rating scale (ALSFRS-R) were significantly smaller in these patients than in edaravone-untreated ALS patients (n=19). Edaravone administration significantly reduced excursions of more than one standard deviation from the mean for plasma FFA levels and the contents of palmitoleic and oleic acids, plasma markers of tissue oxidative damage, in the satisfactory progress group (ΔALSFRS-R ≥ 0) as compared to the ingravescent group (ΔALSFRS-R < -5). Edaravone treatment increased plasma uric acid, suggesting that it is an effective scavenger of peroxynitrite. However, edaravone administration did not decrease %CoQ10. Therefore, combined treatment with agents such as coenzyme Q10 may further reduce oxidative stress in ALS patients.

Proteomic-scale approaches for identifying reversible and irreversible cysteine redox post-translational modifications in myocardial ischemia/ reperfusion

Jana Paulech[1], Melanie Y White[1,2,3], Stuart J Cordwell[1,2,3]

[1]School of Molecular Bioscience, School of Medical Sciences, University of Sydney, Australia; [2]Discipline of Pathology, School of Medical Sciences, University of Sydney, Australia; [3]Charles Perkins Centre, University of Sydney, Australia

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Redox post-translational modifications (PTM) are emerging as important regulatory mechanisms in signaling and pathogenesis. Cysteine (Cys) is the most redox active amino acid and is a target for these PTM, some of which are biologically reversible (e.g. disulfides, sulfenic acid) while others (sulfinic [Cys-SO_2H] and sulfonic [Cys-SO_3H] acids) are considered irreversible. We have developed enrichment methods to examine these PTM on a proteome-wide scale. Rapid and specific alkylation of free Cys, followed by thiol-based reduction and resin capture by thiol-disulfide exchange chemistry was applied to isolate reversibly modified Cys-containing peptides. The method was applied to a complex protein lysate generated from rat myocardial tissue and 6559 unique Cys-containing peptides from 2694 proteins were identified by tandem mass spectrometry (MS/MS). We next developed an enrichment method to isolate Cys-SO_2H/SO_3H-containing peptides from complex tissue lysates. The method is based on electrostatic repulsion of Cys-SO_2H/SO_3H-containing peptides from cationic resins (i.e. 'negative' selection) followed by 'positive' selection using hydrophilic interaction liquid chromatography (HILIC). We identified 181 Cys-SO_2H/SO_3H sites from rat myocardial tissue subjected to physiologically relevant concentrations of H_2O_2 (<100 µM) or to ischemia / reperfusion (I/R) injury *via* Langendorff perfusion. I/R significantly increased Cys-SO_2H/SO_3H-modified peptides from proteins involved in energy utilization and contractility, as well as those involved in oxidative damage and repair. Finally, we have combined these methods to enable multiplexed quantitative analysis of reversible/irreversible Cys redox PTM in response to I/R and in the presence of a broad-spectrum antioxidant (*N*-2-mercaptopropionyl glycine, MPG). We quantified >1350 Cys sites that are reversibly and/or irreversibly oxidized by I/R, including many sites that are protected by MPG. This technique allows for the quantitative profiling of reversible/irreversible Cys PTMs in response to oxidant / antioxidant stimulus, and their delineation within the context of protein abundance, during I/R injury and cardioprotection.

Diabetes mellitus enhances apoptosis in the heart following myocardial infarction

Rachel S Wallace, Jeffrey R Erickson

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Excessive oxidative stress in the heart is associated with pathological remodelling and cell death, particularly following myocardial infarction. Diabetes mellitus, a metabolic disorder characterized by impaired insulin signaling and hyperglycemia, is known to enhance cardiac oxidative stress. As there are more than 250,000 people currently diagnosed with diabetes in New Zealand alone, it is critical that we elucidate the connection between diabetes-induced oxidative stress and cardiac pathology so that new therapies can be developed to prevent heart disease. Here, we show that diabetic patients are more susceptible to apoptotic cell death after myocardial infarction than age-matched non-diabetic patients. Moreover, we show that diabetic patients have higher levels of myocardial oxidative stress than non-diabetic patients, despite the observation that expression and activity of at least one key protective enzyme, methionine sulfoxide reductase A, is increased in diabetic patients. These data suggest that increased production of oxidative stress may underlie pathological signaling in the diabetic heart and point to the potential for anti-oxidant therapies to improve cardiac health following myocardial infarction.

Redox modification of caveolar proteins as important mediators in cardiovascular disease

Gemma A Figtree[1,2], Keyvan Karimi[1,2], Owen Tang[2], Elena Arystarkhova[3], Kathleen Sweadner[3]

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Despite the well-recognized role of dysregulated oxidative signalling in cardiovascular pathophysiology, attempts at reducing cardiovascular events using antioxidant scavenger approaches have failed in large clinical trials. The caveolae represent a microdomain enriched in reactive oxygen species (ROS)-sensitive signalling molecules, as well as ROS producing enzymes. In disease states, this can create an effective furnace that is notoriously difficult to penetrate with scavenging strategies. Understanding the regulation of redox signalling in the caveolae may identify novel therapeutic targets with broad clinical relevance.

Endothelial nitric oxide synthase (eNOS) is regulated by glutathionylation, the reversible addition of a 205 Da, negatively charged tripeptide adduct. This mediates uncoupling of the enzyme, and a switch from NO production to O2.-. We have shown that both Ang II and diabetes results in eNOS glutathionylation in both the endothelium as well as the myocardium. FXYD1, a membrane protein with highly conserved reactive cysteine, acts as an endogenous protector against this, with manipulation of its expression having important functional effects. Protection against eNOS glutathionylation can also be achieved by β3-adrenergic receptor agonists. We have also characterized redox regulation of the Na+-K+ ATPase as a representative and functionally important caveolar protein. Ang II, diabetes, and heart failure result in glutathionylation of its β1 subunit, subsequent shift in E1/E2 conformational state, and inhibition of its pump activity. In addition, redox-induced conformational change also activates Na+-K+ pump-coupled Src activity, and downstream proliferative and fibrotic pathways. Therapeutic approaches to upregulate FXYD1 expression in the caveolae have been shown to protect against β1 subunit glutathionylation, and receptor-coupled methods have proven to be an additional effective approach.

Improved characterisation of redox regulation of caveolar signalling proteins provides novel therapeutic approaches relevant to patients with cardiovascular disease.

Ribose-cysteine increases glutathione-based antioxidant status in mouse models of hyperlipidaemia

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Ribose-cysteine is a cysteine analogue designed to increase the synthesis of the cellular antioxidant, glutathione (GSH). Glutathione peroxidase (GPx) requires GSH as a cofactor for the reduction of lipid peroxides. A low GPx activity and increased lipid peroxides are associated with an increased risk of developing cardiovascular disease (CVD). We investigated the effect of ribose-cysteine on GSH, GPx, lipid peroxides and plasma lipids in two different mouse models of hyperlipidaemia that are prone to developing atherosclerosis. Human lipoprotein(a) transgenic mice [Lp(a) mice] and apolipoprotein E knockout mice (apoE-/- mice) (n=9) were treated with 4 mg/day ribose-cysteine for 8 weeks. Blood, livers and aortae were harvested from treated and untreated controls and GSH, GPx activity, F2-isoprostanes and plasma lipid concentrations measured. Ribose-cysteine treatment significantly increased GSH concentrations in the liver and plasma of Lp(a) and in the livers of apoE-/- mice. Ribose-cysteine increased GPx activity in the liver and erythrocytes of both lines of mice. F2-isoprostane levels were significantly reduced in the livers and arteries of both Lp(a) and apoE-/- mice. Ribose-cysteine treatment was associated with a significant decrease in low density lipoprotein (LDL) and apolipoprotein B concentrations in Lp(a) mice. Analysis of plasma lipids levels and atherosclerotic lesions in the apoE-/-mice is currently underway. In conclusion, ribose-cysteine exerts an antioxidant effect by increasing GSH-based antioxidant status and lowering oxidised lipids. This combined with its LDL-lowering property, suggest that it might be an ideal supplementary intervention to increase protection against CVD.

The tryptophan metabolite *cis* 3-hydroperoxypyrroloindole causes arterial relaxation in part via oxidative dimerization of protein kinase G1α

CP Stanley[1], GJ Maghzal[1], PC Chadha[1], S Prysyazhna[2], P Eaton[2], R Stocker[1]

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Severe sepsis is associated with widespread inflammation, hypotension and multiple organ failure that can lead to fatality within hours. Clinical treatment of sepsis relies on the maintenance of blood pressure and fluid levels while the causes of the hemodynamic complications remain largely unknown. We have previously identified a role for the metabolism of L-tryptophan (Trp) to L-kynurenine (Kyn) by the heme enzyme indoleamine 2,3-dioxygenase 1 (Ido1) in producing hemodynamic changes (decreased blood pressure, vasorelaxation) in experimental models of sepsis (*Nat Med* 2010;16:279-285). Furthermore, we reported that in human sepsis Ido1 is expressed in resistance vessels, and that systemic Ido1 activity correlates with the need of vasopressors by septic patients in ICU (*Crit Care Med* 2011;39:2678).

Here we describe *cis* 3-hydroperoxypyrroloindole (*cis* 3HPPI), an intermediate generated from Trp by Ido1 in the presence of H_2O_2, to relax conduit arteries and resistance arterioles in mice and rats in a dose-dependent manner. *cis* 3HPPI-mediated relaxation of mouse abdominal aortas was attenuated in the presence of inhibitors of protein kinase G (PKG) and A, high potassium Krebs solution, and blockers to certain ion channels. *In vitro* treatment of recombinant PKG1α with *cis* 3HPPI caused dimerization of the protein, a process previously shown to be associated with activation of this kinase. Such dimerization was not seen with C42S mutant PKG1α and it was more pronounced with *cis* 3HPPI compared with equimolar amounts of *trans* 3HPPI. Moreover, we observed that relaxation induced by *cis* 3HPPI was attenuated significantly in mesenteric arterioles isolated from redox dead PKG1α-C42S knock-in compared with wild-type mice. Together with our previous studies, these results identify novel pathways by which endothelial expression of Ido1 may contribute to arterial relaxation and hypotension in inflammatory conditions known to be stimulated during severe sepsis.

Peroxynitrous acid modifies the structure and function of the extracellular matrix *in vitro* and *in vivo*: implications for atherosclerosis

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The extracellular matrix (ECM) of the vascular basement membrane is critical to the functional and mechanical properties of arteries, with matrix proteins interacting with growth factors and enzymes to regulate endothelial and smooth muscle cell function. These interactions are perturbed in atherosclerosis, where activated leukocytes generate oxidants that induce endothelial cell dysfunction, modify ECM composition and alter smooth muscle cell proliferation, resulting in ECM remodelling, arterial stiffening and plaque formation.

The aim of this study was to investigate the effect of peroxynitrous acid, an oxidant generated by activated macrophages, on the structure and function of the ECM laid down by human coronary artery endothelial cells (HCAECs) *in vitro* and in human atherosclerotic lesions.

It is has been shown that exposure of HCAEC-derived native matrix components to peroxynitrous acid (but not decomposed oxidant) at concentrations > 1 µM results in a loss of antibody recognition of perlecan, collagen IV and cell binding sites on laminin and fibronectin. Loss of recognition was accompanied by decreased HCAEC adhesion. Real time PCR showed upregulation of inflammation-associated genes, including matrix metalloproteinases (MMP) 7 and 13 as well as down-regulation of laminin-α2 chain, in HCAECs cultured on peroxynitrous acid-treated-matrix compared to native. Immunohistochemical studies provided evidence of co-localisation of laminin with 3-nitrotyrosine, a biomarker of peroxynitrous acid damage, in multiple grades of human atherosclerotic lesions (Types II to IV), consistent with matrix damage occurring during disease development *in vivo*. These data suggest a mechanism through which peroxynitrous acid modifies EC-derived native ECM proteins of the arterial basement membrane in atherosclerotic lesions. These changes to ECM and particularly perlecan and laminin may be important in inducing cellular dysfunction and weakening of the structure of the artery wall, thereby contributing to atherogenesis and eventual rupture of lesions.

Novel fluorescent probe detecting catalytic ferrous iron

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Iron overload of a chronic nature has been associated with various human diseases, including infection, carcinogenesis, and atherosclerosis. Recently, a highly specific turn-on fluorescent probe (RhoNox-1; RhoNox-M) specific to labile ferrous iron Fe(II), but not to labile ferric iron Fe(III), was developed. The evaluation of Fe(II) is more important than Fe(III) in vivo in that Fe(II) is an initiating component of the Fenton reaction. Here we applied this probe to frozen sections of an established Fenton reaction-based rat renal carcinogenesis model with an iron chelate, ferric nitrilotriacetate (Fe-NTA). Catalytic iron induces the Fenton reaction specifically in the renal proximal tubules that finally leads to a high incidence of renal carcinogenesis. Notably, this probe reacted with Fe(II) but with neither Fe(II)-NTA, Fe(III) nor Fe(III)-NTA in vitro. Prominent red fluorescent color was explicitly observed in and around the lumina of renal proximal tubules 1 h after an intraperitoneal injection of 10-35 mg iron/kg Fe-NTA, which was dose-dependent, according to semiquantitative analysis. The RhoNox-1 signal colocalized with the generation of hydroxyl radicals, as detected by hydroxyphenyl fluorescein (HPF). The results demonstrate the transformation of Fe(III)-NTA to Fe(II) in vivo in the Fe-NTA-induced renal carcinogenesis model. Therefore, this probe would be useful for localizing catalytic Fe(II) in a variety of studies. Diverse applications of this novel probe to biological samples such as tissues, human amniotic fluids and endometriosis would be presented and discussed.

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Development of the photoactivated nitric oxide donor drug tDodSNO

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The molecule tDodSNO is a recently developed photoactivated nitric oxide (NO) donor drug. It exhibits markedly superior controlled NO release characteristics in comparison to the known agents s-nitrosoglutathione (GSNO) and S-nitroso-N-acetylpenicillamine (SNAP) [1] and is currently being developed as an anti-cancer therapeutic [2]. However little is known about its mechanism of action. We therefore used single cell infrared spectroscopy to investigate the effects of NO release on MDA-MB-231 triple negative breast cancer cells. Spectra were acquired using a Hyperion microscope coupled to a FTIR spectrometer with an aperture of 5 µm and a resolution of 8 cm^{-1}. Analysis of the initial effects of NO upon the cells (0-100 µM tDodSNO, 18 W/m^2 light intensity, 10 min exposure) revealed that most drug-induced effects were associated with either protein modification (as evidenced by variance within the amide I and II regions of the spectrum) or changes to lipids, which may be a result of NO-initiated peroxidation (OH stretching region 3200-3300 cm^{-1}). A clear distinction in tDodSNO's mechanism was observed as a result of light activation, with dose dependent changes identifiable in the infrared spectra of photoactivated cells. These mechanistic insights reinforce the potential use of tDodSNO as a targeted NO donor molecule for cancer chemotherapy.

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Chromosomal damage in female patients with type 2 diabetes depends on HbA1c, diabetes duration and medication

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Diabetes mellitus type 2 (T2DM) is a growing global health problem with a prevalence of 8.3 % in 2014. In addition to the well-known comorbidities such as micro- and macro vascular complications, T2DM is also associated to a higher cancer risk. The Cytokinesis-block micronucleus cytome (CBMN) assay is one main tool to assess chromosomal damage and genome instabilities and therefore serves as a preclinical cancer biomarker. In order to link glycemic control measured by glycated haemoglobin (HbA1c), the diabetes duration (DD) and medication on chromosomal damage, the MIKRODIAB study was performed.

A total of 142 female T2DM patients from the local Diabetes Outpatient Clinic donated blood samples during the annual health check. Additionally to the CBMN assay analyses from isolated mononuclear cells (PBMC), anthropometric and biochemical parameters were assessed.

A higher amount of bridges in binucleated cells were found in patients with a high HbA1c (> 7.5 %, n = 67) vs. a low HbA1c (≤ 7.5 %, n = 71) ($p < 0.01$). However, other parameters of the CBMN assay did not differ between the HbA1c groups. A significant higher occurrence of micronuclei was detected in patients suffering longer from T2DM (DD-3 = 23.1 years, n = 45) compared to patients with shorter DD (DD-2 = 13.4 years, n = 48; DD-1 = 6.9 years, n = 49; $p < 0.05$). Patients with insulin treatment (n = 57) have significantly more micronuclei and bridges compared to patients with alternative medical treatment ($p < 0.05$).

Our results indicate higher chromosomal damage in patients with higher HbA1c, longer DD and insulin treatment. Therefore, an early detection, an adequate medical treatment and long-term lifestyle improvements in T2DM are necessary to lower the risk of developing cancer.

Modification of cellular proteins and induction of self-defense genes expressions by tryptamine-4,5-dione, a serotonin oxidation product

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Myeloperoxidase oxidizes serotonin (5-hydroxytryptamine, 5HT) to a reactive quinone, tryptamine-4,5-dione (TD) (Ximenes et al., Biochem J., 2009). We have found that TD reacted with specific thiol residues in a model thiol protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Kato et al., Chem. Res. Toxicol., 2012). Moreover, some cytoskeletal proteins, such as tubulins, vimentin and neurofilament-L, were preferentially modified in SH-SY5Y neuroblastoma cells exposed to TD (Kato et al., Redox Biol., 2014). However, the fate of generated TD-modified proteins and the profiles of TD-responsive gene expression have not so far been reported. In this study, we first analyzed TD-modified proteins in SH-SY5Y cells. Western blot analyses using a monoclonal antibody specific to quinone-modified proteins showed that the modified proteins gradually disappeared in a time-dependent manner. When TD-modified bovine serum albumin (BSA) was incubated with native GAPDH in a phosphate buffer, the immunoreactivity against TD modification was partly shifted from BSA to GAPDH, and this transfer reaction was disrupted by *N*-acetyl-L-cysteine, suggesting that TD modification changed from BSA to GAPDH. Next, we investigated the effect of TD on the expression of cytoprotective genes, including heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), functional subunit of the cystine/glutamate transporter xc-system (xCT), and peroxiredoxin 1 (PRX1) by using RT-PCR. Some of the genes examined were up-regulated by TD exposure in dose-dependent manners. The results suggest that TD increase self-defense capacity by adapting to oxidative stress derived from thiol modifications.

Mitochondrial targeting of tamoxifen enhances its activity against Her2^{high} breast cancer via inhibiting mitochondrial complex I function

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Tamoxifen (TAM) is an established anti-cancer agent used primarily against hormone-dependent breast cancer. We have synthesized its mitochondrially targeted version, MitoTamoxifen (MitoTAM), generated by the addition of the triphenylphosphonium group. We found that MitoTAM was more efficient than TAM in killing breast cancer cell lines, in particular those with high level of HER2, causing their demise more efficiently than that of the HER2^{low} cells, and the agent suppressed HER2^{high} breast cancer growth more efficiently than the parental compound. Intriguingly, we found a large amount of HER2 protein present in mitochondria. Cells with level of HER2 in mitochondria in also featured higher levels of mitochondrial complex I (CI) as well as the respirasome composed of CI, CIII and CIV. This likely explains the high efficacy of MitoTAM in killing HER2^{high} cells, since CI is its molecular target, as indicated by inhibition of specifically CI-dependent respiration by the agent. This advocates that MitoTAM can be potentially used as an efficient anti-cancer agent, especially against the recalcitrant HER2^{high} breast carcinomas that constitute almost 30% of all breast cancers.

Mitochondrial redox changes during TNF-mediated necroptosis

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Necroptosis is a form of programmed cell death that was first observed in cells treated with tumour necrosis factor (TNF). A number of other triggers have been identified, and necroptosis is now implicated in the pathology of a range of human diseases. The molecular machinery of necroptosis and its regulatory mechanisms are poorly understood. The key effector protein is the pseudokinase mixed lineage kinase-domain like (MLKL), which associates with cellular membranes upon activation. The mechanism by which MLKL triggers cell death is controversial. The aim of this study was to explore the role of mitochondria and reactive oxygen species in necroptosis signalling. Wild type and MLKL knockout murine dermal fibroblasts (MEF) and murine embryonic fibroblasts (MEF), and human colorectal HT29 cells, were treated with TNF in combination with apoptosis and survival pathway inhibitors to initiate necroptosis. Cell death and mitochondrial membrane potential was monitored by flow cytometry and live-cell imaging, respiratory function was measured with a Seahorse Extracellular Flux Analyzer, and the redox state of mitochondrial and cytoplasmic peroxiredoxins (Prxs) was measured by western blotting. In all cell types MLKL-dependent loss of cell viability was detected following treatment with TNF, and this was preceded by dissipation of mitochondrial membrane potential. The timing of these events varied between cell types. A rapid burst in mitochondrial oxygen consumption was detected following TNF stimulation, followed by a decline in mitochondrial respiration. This decline was associated with oxidation of mitochondrial and cytoplasmic Prxs in MEF and MDF cells, but not in HT29 cells. We conclude that MLKL disrupts mitochondrial function early during necroptosis signalling, and this leads to disruption of redox homeostasis. The significance of these intracellular events for cell death is not yet clear.

4-Methoxy TEMPO attenuates murine experimental colitis

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Inflammatory bowel disease (IBD) is a chronic condition characterised by leukocyte recruitment to the gut mucosa. MPO-mediated oxidative damage may play an important role in exacerbating tissue injury in IBD and in attracting proinflammtory cells to the affected gut.

Aim: To test whether the MPO-inhibotor and anti-inflammatory compounbd 4-Methoxy TEMPO (MetT), (Witting, Jeong, Chami unpublished) can ameliorate the damaging affects of IBD.

Methods: Experimental colitis was induced in C57Bl/6 mice by administering 3% v/v dextran-sodium-sulphate (DSS) in drinking water *ad libitum* over 9 days with mice also administered (*i.p.* injection) MetT (15mg/Kg) or vehicle control (10% DMSO+90% PBS) twice daily during DSS challenge.

Results: MetT attenuated bodyweight loss (50%, $p<0.05$, n=6) and improved clinical score (53%, $p<0.05$, n=6) at day 9 of DSS challenge and inhibited the DSS-mediated increase in serum lipid hydroperoxide levels. Histopathological analysis of the inflamed colon revealed that damage to gut tissues and several inflammatory markers were markedly decreased in MetT-treated mice, as judged by maintenance of crypt integrity, goblet cell density and decreased cellular infiltrate. Specifically, levels of $Ly6C^+$ monocytes and $Ly6G^+$ neutrophils decreased markedly in the gut of MetT-treated mice. Also, constitutively expressed IL-10 (an anti-inflammatory cytokine) was significantly reduced in the colon of DSS-challenged mice; MetT-treated mice showed a trend to increased IL-10 expression. Levels of MPO were decreased in MetT-treated mice with DSS-induced colitis; likely a consequence of reduced neutrophilmotility. Notably, crypt integrity was negatively correlated to MPO^+ labelling (p=0.011, n=24), suggesting that increased MPO levels were associated with increased severity of colitis. MetT protected against MPO-producing neutrophil oxidative damage in a myocytes in an *in vitro* co-culture system.

Conclusion: MetT significantly reduced the severity of experimental colitis, though it remains unclear whether MetT actions were mediated via inhibition of MPO or anti-inflammatory pathways, or both.

A toolbox of reversible and ratiometric fluorescent probes for imaging cellular oxidative stress

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Understanding the physiological and pathological consequences of oxidative events in the cell is a long-standing challenge. Existing fluorescent probes are capable of reporting on individual reactive oxygen species and redox couples, but such reaction-based probes are not reversible, and therefore cannot be used to visualise redox dynamics, nor can they distinguish transient physiological oxidative events from chronic and pathological oxidative stress.

To contribute tools to complement existing selective ROS probes, we are developing reversible fluorescent probes that can measure oxidative stress. Our first-generation probe, NpFR1,[1] is based on a flavin scaffold, and therefore has an appropriately-tuned reduction potential. NpFR1 undergoes a 110-fold increase in fluorescence upon oxidation. NpFR1 localises in the cytoplasm, and has been applied study changes in oxidative capacity that accompany glucose treatment of adipocytes, and PGRMC1 mutation status in Mia PaCa-2 cell lines. Our subsequent research has built on these results, in two main directions.

Firstly, we have modified the structure of NpFR1 to give NpFR2,[2] which localises in the mitochondria, enabling delineation of oxidative events in mitochondria and cytoplasm. NpFR2 was used to report on mitochondrial oxidation status in mouse haematopoietic cells.

Secondly, we have developed probes with colour-based (ratiometric) rather than intensity-based output. For example, FCR1[3] emits blue fluorescence in its reduced form and green upon oxidation. FCR2 can report on the redox state in a variety of biological systems including cultured cells, 3D tumour spheroids and nematodes. Furthermore, the probe response can be monitored using various modalities in addition to confocal microscopy, including fluorescence lifetime imaging microscopy (FLIM) and flow cytometry. Based on this principle, we have also developed a ratiometric, mitochondrially-localised redox probe.[4]

These new generation probes have been tuned to operate at biologically-relevant redox potentials and have excellent potential to aid in deciphering the role of oxidative stress in pathogenesis.

Effects of pharmacological inhibition of myeloperoxidase on endothelial dysfunction in mouse models of inflammation

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Inflammation-driven endothelial dysfunction is an initiating event of atherosclerosis, leading to plaque formation and eventual rupture that causes major clinical complications associated with the disease. A potential culprit, myeloperoxidase (MPO) is released by neutrophils into the vascular endothelium where it utilizes hydrogen peroxide to generate a number of oxidants including the very reactive hypochlorous acid (HOCl). MPO and its oxidants are thought to be involved in the early and late stages of atherosclerosis and are abundant in human atherosclerotic plaques. Here, we evaluated the effects MPO inhibition by a 2-thioxanthine, AZM198, on endothelial dysfunction using three models of inflammation. In all three models of inflammation (C57BL/6 mice fed a high fat high sucrose diet for 1 week, femoral cuff in apolipoprotein E-deficient [*Apoe*$^{-/-}$] mice, tandem stenosis [TS] model in *Apoe*$^{-/-}$ mice) endothelial function was impaired, as assessed by relaxation of pre-constricted abdominal aortic rings in response to acetylcholine and sodium nitroprusside. Diet supplementation with AZM198 (500 µmol/kg) significantly prevented endothelial dysfunction in the aortas of mice in the femoral cuff and TS mouse models, but not in the acute model of diet-induced insulin resistance. AZM198 inhibited arterial MPO activity in regions of interest in the femoral cuff and TS models, as assessed by the conversion of hydroethidine to 2-chloroethidium determined by LC-MS/MS. These beneficial effects of AZM198 were observed with plasma concentration of the drug of ~2 µM, and without changes to circulating leukocytes and cytokines, demonstrating the specificity of AZM198 for MPO. Further work is currently in progress to confirm the mechanisms of MPO inhibition and improvement in endothelial function. Our results suggest that inhibition of MPO is a potential therapeutic target for a range of diseases where vascular function is compromised, including atherosclerosis.

Repeated psychological stress exposure causes inflammation and nitrosative stress in addition to antioxidant gene upregulation in the rat hippocampus

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Neuronal nitric oxide synthase (NOS) derived-nitric oxide, a free radical with both physiological and pathological functions, has recently been shown to mediate chronic stress-induced depressive-like behaviour in rodents. To determine the origins of this pathway, we previously assessed the expression of neuronal NOS following an acute stress in order to associate increased nitric oxide production with increased oxidative/nitrosative status in the hippocampus. However, neuronal NOS gene expression was significantly downregulated following acute stress. One potential candidate responsible for this acute downregulation is the enzyme, heme oxygenase (HO), known to produce endogenous carbon monoxide which utilises the same receptor, soluble guanylyl cyclase, as nitric oxide. Therefore, in the present study we have utilised indicators of oxidative/nitrosative stress and members of the antioxidant HO pathway to observe the changes that occur in the hippocampus following exposure to repeated psychological stress. Male Wistar rats were subject to control conditions or 6 hours of restraint stress applied for 1, 2, or 3 days (n=8 per group) after which the hippocampus was isolated for nitrosative assays and relative gene expression. The stress treatment effectively elevated the circulating stress hormone, corticosterone, for all stress groups compared to controls. The hippocampus showed increased fluorescence of 4-amino-5-methylamino-2', 7'-difluorofluorescein triazole (DAF-FM T) formation, indicative of higher nitric oxide, in addition to decreased availability of reduced glutathione. The increase in DAF-FM T was observed with an increase in inducible NOS mRNA and other markers of neuroinflammation including interleukin-6, while neuronal NOS mRNA was decreased over all stress treatment groups. Stress transiently increased expression of HO-1 and nuclear factor (erythroid-derived 2)-like 2, commonly known as Nrf2. Together, these results demonstrate that early changes in hippocampal nitric oxide are likely produced by inducible NOS which, together with the soluble guanylyl cyclase-signalling HO-1, may modulate expression of neuronal NOS.

Kinetics and energetics of cytochrome P450-mediated hydroxylation

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Cytochrome P450 from *Pseudomonas putida* (CYP101) hydroxylates camphor stereospecifically. Although the protein is well characterized, the nature of the hydroxylating intermediate remains a topic of discussion. We used pulse radiolysis to supply the second electron in the cytochrome P450 cycle, thereby circumventing the slow enzymatic reduction. At 4°C, we delivered a hydrated electron to the oxy-form, CYP101$[FeO_2]^{2+}$, with camphor present. CYP101$[FeOOH]^{2+}$, or, Cpd 0 accumulates within 5 µs with a distinct absorption at 440 nm and decays by 1st–order decay reactions $k_{440\text{-}I}$ = 9.5×10^{4} s^{-1}. Another absorbance at 410 nm, assigned to CYP101$[FeO]^{2+}$, TyrO$^{\bullet}$, follows a biphasic decay, with rate constants $k_{410\text{-}I}$ = 4.2 × 10^{4} s^{-1} and $k_{410\text{-}II}$ = 2.5 × 10^{3} s^{-1}. Within 1 ms, the spectrum of CYP101Fe^{3+} is observed. Importantly, no transient absorptions were observed that can be assigned to Cpd I, CYP101$[FeO^{2+}por^{\bullet+}]$, or Cpd II, CYP101$[FeO^{2+}]$, which implies that these transients do not accumulate. Hydroxylated camphor was found in good yield. Given the rate of decay of Cpd 0 of 9.5×10^{4} s^{-1}, that no Cpd I or Cpd II is observed, and the binding dissociation energy of hydrogen in the 5-position, it can be estimated that the reactions from Cpd 0 to Cpd II are isoenergetics with small activation energies. The hydroxylation is driven by the favourable rebound step, Cpd II to CYP101Fe^{3+}.

Generation of singlet oxygen by indoleamine 2,3 dioxygenase and hydrogen peroxide – A novel path for the metabolism of tryptophan to N-formyl-kynurenine

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Indoleamine 2,3-dioxygenase 1 (IDO1) is a heme enzyme that catalyzes the oxidative metabolism of tryptophan (Trp) to *N*-formyl-kynurenine (NFK), which is then converted to kynurenine and other biologically active metabolites. IDO1 has numerous biological roles, including participation in the regulation of the innate immune response and vascular relaxation. Although much is known about its function, the mechanism of IDO1-mediated conversion of Trp to NFK is not fully elucidated. We now report that in the presence of hydrogen peroxide (H_2O_2), recombinant human IDO1 converts Trp to seven products including tricyclic hydroperoxides that decay to NFK. Of these products, only the tricyclic *cis* 3a hydroperoxide (*cis* 3a-TrpOOH) has vascular relaxing activity. The formation of *cis* 3a-TrpOOH is mediated by the generation of singlet molecular oxygen, O_2 ($^1\Delta_g$), as evidenced by the decrease in yield of *cis* 3a-TrpOOH in the presence of O_2 ($^1\Delta_g$)-scavengers. We also provide evidence that addition of H_2O_2 to recombinant IDO1 generates O_2 ($^1\Delta_g$) measured by light emission at 1270 nm and by detection of specific O_2 ($^1\Delta_g$)-probes. This reaction does not require reductants such as superoxide, ascorbate/methylene blue or cytochrome b_5, thought previously to be essential for IDO1 dioxygenase activity, and is also distinct from the peroxidase activity of IDO1, *e.g.*, in that it requires the presence of molecular oxygen. Additionally, our preliminary data suggests that excited carbonyl species and possibly a Russell mechanism may be involved in the generation of O_2 ($^1\Delta_g$) by IDO1. Our results indicate that IDO1/H_2O_2 generate light in a dark reaction via formation of O_2 ($^1\Delta_g$), and that the latter oxidizes Trp to *cis* 3a-TrpOOH that is a vasorelaxant and a precursor of NFK.

The role of heme oxygenase-1 in intestinal inflammation using murine colitis model

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Background: Heme oxygenases (HOs) are the rate-limiting enzymes in heme degradation, catalysing the cleavage of the heme ring to form carbon monoxide (CO), ferrous iron and biliverdin. Although HO-1 is up-regulated in response to various stimuli including oxidative stress and its transcriptional induction provides an important defence mechanism, the role of HO-1 in the intestinal inflammation remains unclear. The present study investigated the possible role of HO-1 in the regulation of inflammatory process using a dextran sodium sulfate (DSS)-induced colitis in mice.

Materials and Methods: Acute colitis was induced with dextran sodium sulfate (DSS) in male C57BL/6 (wild-type). We evaluated the enhancement by treatment of an HO-1 inhibitor, zinc protoporphyrin IX (ZnPP 25mg/kg i.p., daily). A disease activity index (DAI) was determined on a daily basis for each animal, and consists of a calculated score based on changes in body weight, stool consistency, and intestinal bleeding. The myeloperoxidase (MPO) activity and inflammatory cytokines in the intestinal mucosa were evaluated. In addition, we evaluated the anti-inflammatory effect of HO-1 in intestinal inflammation using the deficient mice of Bach1, which is a physiological repressor of HO-1 and plays an important role in the feedback regulation of HO-1 expression.

Results: After DSS administration, DAI score was increased in a time-dependent manner and Co-administration with ZnPP enhanced the increase in DAI score. On the other hand, DAI score was significantly inhibited in Bach1(-/-) mice compared to wild type mice. Administration of DSS enhanced MPO activity and the expression of pro-inflammatory cytokines, such as KC, TNF-alpha and IFN-gamma, which was increased in the mice treated with ZnPP and was significantly suppressed in Bach1(-/-) mice.

Conclusion: These results indicate that HO-1 plays a protective role in the intestinal inflammation through the regulation of cytokine expression.

Development of free radical imaging system for clinical research

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The generation of reactive oxygen species and the redox status are reportedly believed to be the causes and modulated factors for oxidative diseases. Free radical imaging using ESR (electron spin resonance) have been utilized in life science but not so widely as MRI (magnetic resonance imaging) due to its low spatiotemporal resolution. DNP (dynamic nuclear polarization)-MRI, a new imaging method for free radical species *in vivo,* was first reported by Lurie, et al. (1988). We developed new-sequence for DNP-MRI and succeeded in simultaneous and spectroscopic imaging of plural free radicals by changing the condition of ESR resonance (1, 2). The images of DNP-MRI demonstrated the pharmaco-dynamics of the probes (3) and the redox status of animal models (4).

The large difference of gyromagnetic ratio between electron and proton spins restricted ESR excitation and the proton detection fields. In this paper, first DNP-MRI for clinical research was developed by rotating two magnets, 5mT for ESR and 0.3T for MRI in the jacket with high stability and safety. MR Imaging were carried out with the gradient echo sequence, 2-10sec/cycle of magnets rotation, with/without 1-6sec of ESR excitation. Using this system, the clear MRI and free radical images of the phantoms were obtained simultaneously by changing the sequence with and without ESR irradiation. This novel imaging technology, DNP-MRI would have a significant advantage for imaging *in vivo* redox status.

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Protein persulfides: Insights into the molecular mechanisms of H_2S signaling

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Hydrogen sulfide is a small messenger molecule that has been shown to mediate a plethora of physiological and pathophysiological processes. The most widely studied pathways of sulfide signaling is persulfide formation on regulatory or functional protein Cys residues. In my presentation I will summarize our mechanistic chemical investigations (which were conducted in collaborations with a number of research groups) that were designed to provide deeper insights into the kinetics, mechanisms and potential physiological implications of protein persulfidation processes. I will discuss proposed persulfide formation and reduction pathways and their impacts on protein functions.

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Antioxidant and redox signaling functions of reactive persulfide species translationally formed on various proteins

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Cysteine persulfide is now known as a physiological metabolite formed ubiquitously in various organisms including mammals. Our recent study revealed that the biosynthesis of cysteine persulfide is catalyzed mostly by two enzymes such as cystathionine γ-lyase (cystathionase, CSE) and cystathionine β-synthase (CBS) by use of cystine (CysSSCys) as a major substrate. We also verified generation of appreciable amount of cysteine persulfide, especially its major derivative glutathione persulfide (GSSH) in cultured cells and tissues in vivo. These reactive sulfur derivatives were potent scavengers for reactive oxygen species, specifically hydrogen peroxide. Indeed, the hydrogen peroxide scavenging property of persulfides was much greater than that of H_2S, GSH, and other Cys-related compounds. Intriguingly, persulfides and related species (e.g., hydropolysulfides) showed a strong redox signaling regulatory function via electrophile thiolation. A prime example is a chemical and biological interaction of persulfide species with the endogenously generated electrophile 8-nitroguanosine 3′,5′-cyclic monophosphate. Apart from its high output of biosynthesis and potential antioxidant redox signaling function, the biological relevance of cysteine persulfides/polysulfides is now increasingly recognized as essential structural residues or prosthetic components of several proteins and enzymes, which may include metal ligands most typically observed with iron sulfur clusters. Surprisingly, a clear translation-coupled Cys polysulfuration was revealed herein, directly mediated via translational CysSSH biosynthesis and its incorporation into proteins ubiquitously occurring among different organisms. Therefore, exploring such unique biosynthesis and multiple functions of polysulfur proteins may potentially develop a new paradigm shifted from the central dogma of molecular biology, which may then open up to an era of innovation in the redox biology.

Evaluation of glutathione sulfonamide and allantoin as blood and urine based indicators of exacerbations in children with cystic fibrosis

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Cystic fibrosis (CF) is the most common lethal hereditary disease in Caucasian populations. Lung disease, which begins during early childhood, eventually results in the patient's death. The major risk factors for disease progression are infection, primarily with *Pseudomonas aeruginosa*, and neutrophilic inflammation. Bronchalveolar lavage (BAL), an invasive procedure, is currently used to detect infection and inflammation in the lung. Hence, there is an urgent need for earlier diagnosis via blood and urine based biomarkers.

We have previously shown that glutathione sulfonamide (GSA), an oxidation product of glutathione specific to the neutrophil-derived oxidant hypochlorous acid, is elevated in the lungs of children with CF. We now investigated whether blood and urine levels of GSA reflect infection and inflammation status of the lung using matched samples of BAL, serum and urine. We also measured allantoin, a stable oxidation product of urate and a potential biomarker of oxidative stress in these samples.

Both, GSA and allantoin were elevated in the lungs of children with pulmonary infections compared to those without detectable infections ($n=55$, $p<0.05$). Pulmonary GSA and allantoin correlated well with other markers of neutrophilic inflammation and oxidative stress, such as myeloperoxidase ($r=0.54$, $p<0.0001$ and $r=0.50$, $p<0.0001$, respectively), 3-chloro- tyrosine ($r=0.5$, $p<0.01$ and $r=0.40$, $p<0.05$, respectively) and methionine sulfoxide ($r=0.4$, $p<0.05$ and $r=0.3$, $p=0.15$, respectively). Plasma GSA was significantly elevated in children with infections ($n=51$, $p<0.01$) and was weakly correlated with pulmonary GSA ($r=0.26$, $p=0.07$). GSA in urine was not elevated in children with infections, but was correlated with pulmonary GSA ($r=0.42$, $p<0.05$). Neither plasma nor urinary allantoin was significantly elevated in infected compared to uninfected children.

This study demonstrates that pulmonary GSA and allantoin reflect airway inflammation and infection and they may have potential to be used as blood based biomarkers.

Experimentally dissecting the structural origins of peroxiredoxin catalytic prowess

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From their delayed recognition as dominant peroxidases across species to the remarkable redox-sensitive oligomeric properties of prominent members, peroxiredoxins (Prx) have been the focus of numerous studies which have been paradigm shifting. As emphasized in this presentation, recent advances have enabled refinement of the kinetic and molecular properties and associated reaction and transition state models in key representatives of the broad Prx family. In rapid reaction kinetic studies, data collected with the bacterial Prx AhpC have allowed for the resolution of three steps associated with (i) peroxide binding, (ii) reduction of the peroxide, and (iii) disulfide bond formation. In studies of mutant versions of AhpC with substitutions of select active site residues, separable roles for conserved active site residues in stabilization of the thiolate as well as the developing negatively-charged transition state, as well as in binding and activating the substrate, have been elucidated. Such mechanistic studies are important as the evolved chemical and physical features of Prxs are fundamental to the multiple modes through which their sensitivity and regulation are linked to cellular signaling responses, in addition to their more traditionally appreciated antidoxidant defense roles.

Examining the role of peroxiredoxin 1 in redox signalling

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Peroxiredoxins are among the most efficient enzymes in mammalian cells for reduction of peroxides. In addition, changes in peroxiredoxin expression have been observed in numerous disease states. For this reason there is much interest in understanding the relationship between the peroxidase activity of peroxiredoxins and redox signalling in cells.

It is generally accepted that H_2O_2 can act as a signalling molecule within cells, however the mechanism(s) by which this can occur in the presence of high concentrations of peroxiredoxins remains uncertain. We and others have proposed that one function of mammalian peroxiredoxins is to "translate" H_2O_2 into a useful cellular signal. In this model peroxiredoxins catalyse disulfide bond formation in target proteins, resulting in a change in protein function which is reversed by the action of thioredoxins.

In this presentation I will describe our recent work, both in cell culture and using recombinant proteins *in vitro*, investigating the mechanism of this proposed function of peroxiredoxin 1 in redox signalling.

Interaction of peroxiredoxin 2 with collapsin response mediator protein 2

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Peroxiredoxin 2 (Prx2) is a ubiquitously expressed thiol peroxidase that has a high reactivity with H_2O_2 and has been implicated in H_2O_2-mediated cellular signalling. Various mechanisms have been proposed for Prx2 participation in redox signalling, including thiol exchange with interacting proteins. In this study Prx2 was immunoprecipitated from Jurkat T-lymphoma cells and binding partners identified both before and after the exposure of cells to H_2O_2. Collapsin response mediator protein 2 (CRMP2) was identified as a major interaction partner regardless of the redox state of Prx2. CRMP2 promotes both the formation and collapse of microtubules in Jurkat cell migration and neuronal development. Crucially, CRMP2 is modified by an oxidation step that leads to its inactivation by phosphorylation, causing the collapse of microtubules. We show that exposure of cells to H_2O_2 caused simultaneous oxidation of both Prx2 and CRMP2. Furthermore, knockdown of Prx2 expression resulted in decreased CRMP2 oxidation, supporting the possibility that Prx2 facilitates the oxidation and regulation of CRMP2.

TrxR1 protects PTP1B from oxidative inactivation by hydrogen peroxide

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Dysregulation of receptor tyrosine kinase signaling is a major feature of many pathological conditions, including cancer and cardiovascular disease. Redox control of the active site cysteine in protein tyrosine phosphatases (PTPs) is a key factor in the regulation of tyrosine signaling cascades during growth factor signaling. The activity of PTPs is redox dependent with growth-factor-mediated generation of reactive oxidants, mainly from NADPH oxidases, leading to inactivation of these thiol proteins. This is counterbalanced by re-activating antioxidant enzymes such as the thioredoxin system or GSH. Previous literature has highlighted the low reactivity of hydrogen peroxide with most thiol proteins, including PTPs, and suggested the possibility of highly reactive sensor proteins, such as peroxiredoxins, acting as mediators of oxidation. To understand further the mechanisms of PTP inactivation and reactivation we have investigated the effects of components of the peroxiredoxin/thioredoxin system on the catalytic activity of purified recombinant PTP1B.

We found that peroxiredoxins 1 and 2 did not transfer oxidative equivalents to the active site cysteine of PTP1B during exposure to hydrogen peroxide, but instead protected against inactivation. We and others have previously shown that a thioredoxin system, comprised of thioredoxin reductase (TrxR1), thioredoxin (Trx) and NADPH, is able to reactivate oxidized PTP1B. Here we have characterized the influence of different components of the Trx system on hydrogen peroxide-mediated PTP inactivation. We found that TrxR1 and NADPH (without Trx) was able to protect reduced PTP1B if present during exposure to hydrogen peroxide. The protective effect was dependent of TrxR1 concentration and was blocked by the TrxR1 inhibitor auranofin. This unexpected mode of protection has led us to speculate on a new model for PTP regulation in which diversion of TrxR1 to other activities could limit its ability to protect PTP against oxidation.

Novel pathways of macrophage dysfunction and inflammation induced by myeloperoxidase-derived oxidants

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Chronic inflammation is the major driving force of atherosclerosis, with the infiltration of leukocytes, particularly neutrophils and macrophages, to the vessel wall intricately linked with lesion development. Myeloperoxidase (MPO) is released by activated neutrophils and plays a key role in the pathogenesis of atherosclerosis, which has been linked its ability to produce potent oxidants. The major MPO oxidant, hypochlorous acid (HOCl), reacts rapidly with biological materials and can trigger the release of extracellular traps (ETs) from neutrophils. ETs consist of a mesh of DNA, histones, MPO and proteolytic enzymes, and have been implicated in lesion formation in atherosclerosis, but the pathways involved are poorly defined. In this study, we show that exposure of human monocyte-derived macrophages (HMDM) to pathophysiological levels of HOCl results in the extrusion of histones and DNA into the cellular supernatant. ET release was supported by results obtained on staining HMDM with the cell impermeable fluorescent DNA stain Sytox Green and scanning electron microscopy imaging. In contrast to neutrophils, the HOCl-induced ET release was independent of MAP kinase signaling, NADPH oxidase activity or peptidylarginine deiminase (PAD)-mediated citrullination. HOCl reacts rapidly with DNA to form a series of chlorinated nucleoside products, which may be relevant to ETs, given the localised production of HOCl by MPO bound to the DNA. We show that chlorinated nucleosides become incorporated into the RNA and DNA of macrophages. This promotes increased DNA strand breaks and the formation of modified bases. Chlorinated nucleosides also increased pro-inflammatory cytokine expression in macrophages, particularly interleukin 1β (IL-1β), and interleukin 18 (IL-18), which in some cases, was associated with nuclear translocation of the NF-κB sub-unit p65. These data indicate a novel role for macrophages and HOCl in mediating ET formation and a role of chlorinated nucleosides in the propagation of the inflammatory response evident in atherosclerosis.

Myeloperoxidase-derived modification of biomolecules and its prevention

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Myeloperoxidase generates reactive hypochlorous or hypobromous acids in the presence of hydrogen peroxide and chloride or bromide ions. These species play important roles in host defense against the invasion of microorganisms. On the other hand, these enzyme products modify biomolecules in hosts during excess inflammation.

Myeloperoxidase uses other endogenous compounds, such as serotonin, urate, and L-tyrosine, as substrates. This broad-range specificity may have some biological implications. We have focused on peroxidase activity of myeloperoxidase. Serotonin is a substrate of the enzyme and, as a product, quinone (tryptamine 4,5-dione, TD) is generated. A metabolite of serotonin, 5-hydroxyindoleacetic acid, is also oxidized by the enzyme and a corresponding reactive quinone is formed. These quinones rapidly react with protein thiols and might modulate function of proteins. We successfully established specific monoclonal antibodies which recognize quinone-modified proteins. Using the antibodies, quinone-modifications were found in human atherosclerotic lesion. Recently we have investigated serotonin oxidation during the formation of neutrophils extracellular traps (NETs) by differentiated HL60. We would like to show some preliminary data.

Moderate inhibition of this enzyme might be critical for prevention/modulation of excess, uncontrolled inflammatory events. Some phytochemicals inhibit myeloperoxidase, which might explain the reductive effect caused by intakes of vegetables and fruits on cardiovascular diseases. We have found that some phytochemicals such as quercetin and curcumin inhibit the enzyme in vitro. Manuka honey is a unique honey from flowers of *Leptospermum scoparium* in New Zealand and has some biological activities including well-known strong antibacterial activity. We identified a novel glycoside, leptosperin, on the basis of inhibitory effect on myeloperoxidase activity. Because leptosperin is exclusively found in honeys from *Leptospermum* species, the measurement of leptosperin is one of certifications for genuine manuka honey.

Ascorbic acid as an essential cofactor in the production of hypothiocyanite by lactoperoxidase

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Lactoperoxidase catalyzed production of hypothiocyanate plays a central role in the initial anti-bacterial defense mechanism in the mucous of the airway of mammals. The two major antioxidants in mucous are urate and ascorbate. Pre-steady state and steady state kinetics show that ascorbate reacts with all three major reactive intermediates of LPO, compounds I, II and III. The kinetic parameters of the peroxidase reaction determined *in vitro* suggest that reaction with compound II is the rate-determining step (k = 4.6 × 10^3 $M^{-1}s^{-1}$). Previous work[1] has shown that urate impairs LPO anti-bacterial activity through a remarkably fast reaction with compound I followed by hydrogen peroxide-dependent conversion to compound III which is comparatively unreactive and stable. Strikingly, reaction with ascorbate rescues LPO activity by conversion of compound III back to ferric LPO. This ability to rescue LPO and prevent diversion of urate radicals from bactericidal byproducts to compound II conversion, means ascorbate is an essential component of the lactoperoxidase/thiocyanate system.

Strategies for inhibiting myeloperoxidase and limiting oxidative stress during inflammation

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Myeloperoxidase – a major neutrophil protein - is a valuable weapon in the immune system's armoury for fighting infection. It is released into sealed neutrophil phagosomes to aid killing and destruction of ingested pathogens. If not confined to the phagosome, however, myeloperoxidase can cause indiscriminate damage to healthy tissue by producing destructive oxidants including chlorine bleach and reactive free radicals. This is evident in many inflammatory diseases, where myeloperoxidase leaves characteristic footprints of oxidative stress. Due to the damage myeloperoxidase can propagate during inflammation, it has long been recognised as a pharmacological target. Currently, there are no therapeutic drugs for inhibiting myeloperoxidase. Although several are under investigation, the identification of a stable compound that can selectively and efficiently inhibit myeloperoxidase in a physiological setting remains elusive. We believe it is vital to take a rational approach in order to discover inhibitors of the enzyme. With this strategy in mind, we will outline the accrued knowledge of how this complex enzyme behaves, and current proven mechanisms for its inhibition - including substantive advances in understanding how several inhibitors target the enzyme. We will also review methods that are suitable for finding inhibitors and illuminating their mechanism of action, and methods for demonstrating their efficacy in models of inflammation. This knowledge is essential if we are to find new inhibitors that will control this powerful enzyme in inflammatory diseases and limit its destructive potential.

Endosomal NOX2 oxidase exacerbates virus pathogenicity

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Viruses cause global devastation and with looming epidemics, pandemics, emerging anti-viral resistance and long lag times for vaccine development, there is an urgent need for novel pharmacological approaches that ideally target the resultant pathology regardless of the infecting strain. The innate immune system utilizes reactive oxygen species (ROS) for the elimination of pathogenic bacteria. However, ROS actually promote viral pathogenicity by a mechanism that is yet to be clearly defined. Here we show that NOX2 oxidase, the primary enzymatic source of ROS production in phagocytic cells, is expressed and activated within endosomes; providing a platform for ROS production that operates within minutes after the internalization of single stranded RNA and DNA viruses into endocytic compartments. The assembly and activation of NOX2 oxidase was critically dependent on endosomal acidification and the engagement of either the pattern recognition receptor TLR7 for ssRNA viruses or TLR9 for DNA viruses. Importantly, the virus-dependent activation of NOX2 oxidase and endosomal ROS generation was distinct from the classical ROS generation pathways that are triggered by bacteria. The production of endosomal H_2O_2 caused modification of crucial cysteine residues on the ectodomain of TLR7, resulting in the suppression of innate and humoral anti-viral signaling networks. We concluded that a previously unrecognized endosomal signaling platform was activated by viruses, which suppresses fundamental components of RNA immunity, and this has major implications for the control and treatment of viral infections.

Is mitochondrial transfer between cells a normal physiological process? Implications for cellular stress responses and free radical generation

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Mitochondrial transfer between cells has been demonstrated in a variety of mammalian cell culture systems *in vitro* and in mouse models on acute lung injury and inflammation, and phylogenetic evidence supports repeated mitochondrial acquisition in a canine transmissible venereal tumour that has been passed on for 11,000 years. We have recently demonstrated that melanoma and breast cancer cells lacking mitochondrial DNA (mtDNA) will not grow as tumours until they have acquired mtDNA from the host mouse. Stable cell lines from these tumour cells contain host mtDNA polymorphisms and recovery of mitochondrial respiration is observed. In the 4T1 breast cancer model, full recovery of mitochodnrial respiration following mitochondrial acquisition requires prolonged environmental exposure, whereas B16 melanoma cells recover rapidly. Preliminary results suggest critical differences in the control of mitochondriral fusion and fission.

In other projects we have shown mitochondrial transfer between neonatal mouse astrocytes and neurons in culture, suggesting that brain function in long-lived neurons, particularly at synaptic junctions, may be maintained by local intercellular mitochondrial transfer processes, rather than by classical axon trafficking. In this context, it has recently been shown that mitochondria from the optic nerve are packaged into exosomes that are taken up and degraded by adjacent astrocytes. We are developing a range of models to determine whether intercellular mitochondrial transfer is involved in embryonic development and in bone marrow transplantation where conditioning regimens such as irradiation and anti-cancer drug treatment damage mtDNA.

Acquisition of mitochondrial DNA by cancer cells devoid of mitochondrial genome is a prerequisite for tumor initiation

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We have recently shown that cancer cells devoid of mitochondrial (mt) DNA (rho^0 cells) form syngeneic tumours with a delay, and the tumour cells contain the host mtDNA. Here we studied what happens during the lag phase before a tumour initiates its growth. We show that several days after grafting, tumour cells start to acquire mtDNA from host cells with host polymorphism, and its level increases until the tumour appears and starts to progress. This is accompanied by mtDNA replication and transcription, which is promoted by retrograde signaling from the recovering mitochondrion to the nucleus, resulting in the assembly of mitochondrial complexes and recovery of respiration. We conclude that cancer cells devoid of mtDNA ought to recover respiration to a threshold level, which is a prerequisite for the cells to initiate tumour formation.

Expected and unexpected impact of Nrf2 knockout and endurance exercise on autophagy and mitochondria in aging mouse brain

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Age-associated neurodegeneration is connected to inefficient autophagic removal of damaged or toxic proteins and organelles, resulting in impairment of proteostasis and accumulation of mitochondrial damage. Previous studies have shown that exercise and NRF2 redox regulatory pathway play important roles in regulating proteastasis in peripheral tissues in young adults. However, how NRF2 interplays with exercise and regulate protein and mitochondrial homeostasis in the brains of aging mice is unknown. We performed studies in wildtype and *Nrf2* knockout mice at >23 months of age, with and without endurance exercise, We found that antioxidant G6PD and SOD2 proteins are decreased by both endurance exercise and Nrf2 knockout in brain cortex. NQO1 protein is decreased by *Nrf2* knockout but not by endurance exercise. At mRNA level *G6pd* expression is down-regulated in *Nrf2* knockout mouse brains. Interestingly, autophagy protein LC3II and its mRNA *Lc3b* are up-regulated by both endurance exercise and Nrf2 knockout. There are also upregulation of BECN1 protein by Nrf2 knockout and upregulation of *Vps34* mRNA by endurance exercise. The apparent upregulation of autophagy proteins did not impact the steady state levels of autophagy substrates sequestosome1/p62, aggregation-prone alpha-synuclein, or ubiquitinated proteins. However, Nrf2 knockout and endurance exercise both decreased mtDNA damage, indicating an improvement of mitochondrial quality control. While endurance exercise increased *Pgc-1α* mRNA, MFN-1 and PGC-1α proteins, Nrf2 knockout decreased DRP-1 protein. These observations demonstrate that in aging mouse brains Nrf2 knockout and endurance exercise have both common and distinct effects on antioxidants and autophagy pathways, a subset of these proteins are regulated at either transcriptional or post-transcriptional levels. Furthermore, neither endurance exercise nor Nrf2 knockout seem to have significant impact on steady state levels of autophagy substrate proteins, but both impact mitochondrial dynamics and decreased mtDNA damage, likely through mechanisms involving mitochondrial biogenesis, mitochondrial dynamics and mitophagy.

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Multiple targets of carbon monoxide gas in the intestinal inflammation

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Inflammatory bowel diseases (IBDs) such as ulcerative colitis and Crohn's disease are chronic relapsing and remitting inflammatory disorders of the intestinal tract. It is important to investigate the precise pathogenesis of IBD, to evaluate new anti-inflammatory agents, and to develop novel drugs. Carbon monoxide (CO) has emerged as an important regulator of acute and chronic inflammation of the gastrointestinal tract. The mechanism underlying its anti-inflammatory effects is only partially understood. CO-derived from hemeoxygenase-1 plays a crucial role in the intestine, affecting the critical immune system, such as maintenance of mucosal integrity, fibroblast activation, T cell cytokine expression, differentiation of T cells, macrophage activation, and ATP release from bacteria. Although several lipid- or water-soluble CORMs have shown efficacy in animal models of disease, none have been tested in humans because their safety for human use has not been resolved. We have reported that oligosaccharides from agar, which is a natural compound and is already being used in humans as a supplement, significantly inhibited murine intestinal inflammation through the induction of HO-1 expression in intestinal macrophages. To overcome the high toxicity of CO during *in vivo* CO inhalation treatment, we prepared CO-saturated saline and administered the solution to rats intrarectally. Recent study addressed the challenge by developing a CO-releasing tablet for oral use, called the oral carbon monoxide release system (OCORS), which is designed to shuttle CO to the site of gastrointestinal lesion or inflammation through controlled delivery. Finally, we have discussed the direction of translational research with respect to launching a novel agent for releasing CO in the intestine.

3β-Hydroxysteroid-24-reductase (DHCR24): a key protein involved in protecting human coronary artery endothelial cells from inflammatory stress

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DHCR24 is increasingly recognised to possess important regulatory properties beyond that of catalysing the final step in cholesterol biosynthesis. In some cell types, it is tightly involved in physiological as well as pathological processes, such as cytoprotection, apoptosis, and inflammation. The role of DHCR24 in human coronary artery endothelial cells is not known. We suspected it may have an important role in protecting against a TNFα-induced inflammatory response after discovering that it is a key enzyme involved in mediating the anti-inflammatory effects of high density lipoproteins. We now show that DHCR24 localizes to the endoplasmic reticulum in HCAECs. When DHCR24 is overexpressed in cells, It abrogates TNFα-induced expression of vascular cell adhesion molecule-1 (VCAM-1) and subsequent monocyte adhesion. Moreover, we show that the underlying protective mechanism of DHCR24 is via the blockade of TNFα-induced NF-κB signaling throught the suppression of TNFα-induced reactive oxygen species (ROS) generation. DHCR24 catalyses cholesterol formation via an oxidoreductase site in the N-terminal, and using site-directed mutagenesis to alter this crucial region of the enzyme, we demonstrate that this oxidoreductase site is central for the capcity of DHCR24 to protect against TNFα insult. Together, the results suggest that DHCR24 is a moonlighting protein in HCAECs: it catalyzes cholesterol formation however also has a central protective role evidenced by its ability to decrease ROS levels, decrease NF-κB activation and inhibit the inflammatory response.

GLUT9 alleviates the development of type 2 diabetes mellitus under hyperuricemic conditions

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Besides the inability to maintain blood glucose levels, additional hallmarks of type 2 diabetes mellitus (T2DM) are insulin resistance and a loss of pancreatic β-cell mass. A common observation in T2DM is an elevated urate plasma level or hyperuricemia, a condition known in the context of gout.

Aim: To identify the role of GLUT9 in pancreatic β-cell function and consequently the development of T2DM under hyperuricemic conditions.

Methods: Mouse and human pancreatic β-cells (MIN6 and 1.1B4 cells) were cultured under varying levels of urate and glucose reflecting normal and diabetic conditions. In addition, GLUT9 was knocked down via siRNAs. q-PCR and western blot analysis were performed to examine the levels of GLUT2, GLUT9, glucokinase, AMP-kinase, LC-III, INS1 and INS2, miR-153 and miR-34a. Cell proliferation assays to monitor survival of β-cells were also performed.

Results: Hyperuricemia did not alter the glucose sensing machinery. However, GLUT9 expression, a known urate transporter, is glucose dependent. Expression of miR-34a and miR-153, which we identified as regulator of GLUT9, are urate and GLUT9 dependent. Most interestingly, hyperuricemic conditions showed an increase in AMPK phosphorylation and a decrease in INS1 and INS2 expression. Cell viability assays, caspase 3/7 assays and western blot analysis of LC-III revealed that hyperuricemia significantly increased β-cell death facilitated by autophagy, which could be reverted by benzbromarone and GLUT9-knock-down.

Discussion: Our findings suggest an important role of GLUT9 in β-cell function under hyperuricemic conditions. The significant increase in AMPK activity induced by hyperuricemia may ultimately cause inhibition of insulin synthesis and secretion. The urate-dependent increase in miR-34a and LC-III expression indicates pancreatic β-cell death is facilitated by autophagy in a GLUT9-dependent manner. In summary, we have identified a new mechanism that may regulate insulin release and β-cell survival leading to the development of T2DM.

Heme oxygease-1 prevents intestinal ischemia-reperfusion injury via the regulation of inflammasome

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Introduction Small intestine ischemia-reperfusion injury is caused by such as mesenteric artery occlusion and cardiovascular surgery. If adequate treatment is carried out, it causes serious conditions such as multiple organ failure. Ischemia-reperfusion injury (IRI) is characterized by inflammation due to oxidative stress and leukocyte recruitment. The Nlrp3 inflammasome plays a central role in the induction of inflammatory responses through the secretion of IL-1b. On the other hand, it has been reported that the antioxidant enzymes Heme oxygease-1 (HO-1) and carbon monoxide (CO) which is a by-product controls the various inflammatory conditions. However, the role of the inflammasome and the mechanisms whereby HO-1 controls the inflammasome in intestinal IRI remain unclear.

Methods To investigate the role of Bach1 in the control of the Nlrp3 inflammasome and disease, IRI was inducted in Bach1 KO mice that is highly expressed HO-1 by occluding the superior mesenteric artery for 45 min. Inflammatory responses in the small intestine were assessed 4h following reperfusion by measuring mucosal IL-1β, Caspase1, Nlrp3 mRNA and protein expression by RT PCR and Western blotting respectively.

Results IRI was decreased significantly in the absence of Bach1, and this decreased effect was abolished by the administration of the HO-1 inhibitor. IL-1b mRNA were increased significantly during IRI in the intestinal mucosa of WT mice but not in Bach1 KO mice (66.5% decrease $p<0.05$). IL-1b and caspase-1 protein were expressed by IRI in the intestinal mucosa of WT mice but not in Bach1 KO mice. The inhibitory effect of IL-1b mRNA in Bach1 KO mice was abolished by the administration of the HO-1 inhibitor.

Conclusions These findings suggest that HO-1 modulates the small intestinal IRI through its ability to regulate the Nlrp3 inflammasome.

New insights into the bioavailability of dietary flavonoids

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Although dietary flavonoids have protective effects against chronic diseases including cancer, cardiovascular disease and cognitive decline, there is only limited information on the mechanisms underlying these effects, in part due to limited information on the bioavailability of flavonoids within the body following ingestion. There is a belief that flavonoids are poorly bioavailability with anthocyanins, for instance, occurring transiently in the bloodstream in low nM concentrations and being excreted in urine, and hence having passed through the circulatory system, in amounts equivalent to <1% of intake[1,2]. Recent studies have involved feeds with healthy volunteers and ileostomists as well as in vitro incubations with fecal material. This has provided information on the absorption and phase II metabolism of flavonoids in the small intestine and the impact of the colonic microbiota on the catabolism of flavonoids which pass from the small to the large intestine. Elucidation of events occurring in the proximal and distal gastrointestinal tract following the ingestion of raspberry anthocyanins[3], orange juice flavanones[4] and [^{14}C]-(–)epicatechin have established that when their metabolites and colonic catabolites are taken into account these flavonoids are much more bioavailable than previously envisaged. It is also important to appreciate that it is flavonoid metabolites and catabolites which are absorbed into the circulatory system. It is, therefore, these compounds, and not their ingested parent compounds that should be used with in vitro models designed to investigate the mode of action through which the protective effects of dietary flavonoids are mediated.

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The acute effect of quercetin-3-O-glucoside on blood pressure, endothelial function and no production in healthy men and women

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Background: Epidemiological studies suggest an inverse association between flavonoid consumption and cardiovascular disease. Results from randomised, controlled trials indicate that this protection could occur through effects on endothelial function and blood pressure (BP). Few human trials, however, have been performed with isolated flavonoids. Quercetin is thought to be a key flavonoid in the cardioprotective effects of fruit and vegetables.

Aim: Assess dose-related effects of dietary derived quercetin-3-O-glucoside (Q3G) on blood vessel function, BP and plasma nitric oxide production in healthy human volunteers.

Methods: In a double blind, randomised, placebo-controlled, cross-over trial the acute effects of Q3G (0, 50, 100, 200 and 400mg) were compared in healthy men and women (60.8 ± 9.3 years, n=15). Endpoints included plasma nitric oxide production (determined by measuring S-nitrosothiols and other nitroso species (RXNO), nitrate and nitrite), BP, flow-mediated dilatation (FMD) and plasma quercetin metabolites (measured as total quercetin/isorhamnetin by LC/MS/MS). These were measured at baseline and one hour post intervention.

Results: A linear dose-response to Q3G intake was observed with the quercetin metabolites, in plasma ($p<0.001$) 1 hour post intervention. No Q3G was detected in plasma pre or post intervention. Q3G intake did not alter BP, FMD or plasma nitric oxide production 1 hour post intervention.

Conclusion: Despite observed increases in plasma quercetin metabolites, no changes in FMD, BP or plasma nitrate, nitrite or nitric oxide were observed for any dose of Q3G one hour after ingestion. These results support a recent randomized intervention trial with 160mg/day Q3G for 4 weeks which found no significant change in FMD or BP. The results with pure Q3G contrast with that of whole fruit extract which have shown significant acute changes in FMD.

Pulse radiolysis and ultra high performance liquid chromatography/high resolution mass spectrometry (UHPLC/HRMS) studies on the reactions of the carbonate radical with vitamin B_{12} complexes

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Vitamin B_{12} complexes, also known as cobalamins (Cbls), are essential micronutrients in all living organisms, and are required as cofactors for mammalian methionine synthase (MS), and mitochondrial L-methylmalonyl-CoA mutase (MM-CoA mutase). It is now recognized that up to 20% of individuals over the age of 65 are Cbl-deficient, and severe cobalamin deficiency is associated with oxidative stress. Furthermore, vitamin B_{12} supplementation has been used for decades to treat chronic inflammatory diseases, with no real understanding as to why it might be beneficial. Cell studies show that vitamin B_{12} derivatives protect against superoxide and hydrogen peroxide - induced intracellular oxidative stress, suggesting a potential role of cobalamin as a ROS/RNS scavenger for at least some of these ROS/RNS. An important consideration is whether ROS/RNS damage the corrin ring of the cobalamin in addition to simply oxidizing the metal center. In this study pulse radiolysis has been utilized to investigate the reactions of one of the most potent ROS, the carbonate radical anion, with Cbls. Ultra-high-performance liquid chromatography combined with high-resolution mass spectrometry analysis has been successfully applied for the first time to analyse the complex mixture of corrinoid isomers formed. It is anticipated that this methodology will also be useful for the closely related porphyrin systems.

Is plasma neopterin the product of intracellular oxidant scavenging by 7,8-dihydroneopterin?

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Neopterin has been extensively used as a clinical marker of immune cell activation in a variety of conditions including cardiovascular disease (CVD)[1]. Though cited often as the oxidation product of 7,8-dihydroneopterin, the mechanism and location of neopterin generation during cardiovascular disease is unknown. As 7,8-dihydroneopterin is a potent inhibitor of oxidant dependent oxLDL induced macrophage death, we suggest that plasma neopterin is the product of intracellular scavenging of oxidants during plaque inflammation[1].

The addition of γ-interferon to cultured human plaque (from endarterectomy surgery) resulted in firstly the generation of 7,8-dihydroneopterin, then neopterin[2]. The addition of oxLDL to either U937 cells or human macrophages in the presence of 7,8-dihydroneopterin caused a time dependent increase in intracellular neopterin. Inhibition of 7,8-dihydroneopterin uptake by monocytes using nucleotide-transporter inhibitors, blocked the protection from AAPH derived peroxyl radicals. This suggests neopterin is generated by oxidant/radical scavenging by 7,8-dihydroneopterin within macrophage cells.

We have previously shown 7,8-dihydroneopterin inhibits of AAPH, H_2O_2, Fe^{2+}, and HOCl induced cell death[3], yet only the oxidant HOCl has been observed to generate neopterin. All the other oxidants generate xanthopterin. Inhibition of HOCl generation using the myeloperoxidase inhibitor ABAH failed to protect macrophage cells suggesting another oxidant is involved in oxLDL induced macrophage death. 7,8-Dihydroneopterin inhibits DHE staining following oxLDL exposure to macrophages[4] suggesting superoxide is a key oxidant generated in response to oxLDL. Superoxide generated by electron beam radiolysis caused the oxidation of 7,8-dihydroneopterin to neopterin with 30% of the lost 7,8-dihydroneopterin appearing as neopterin.

These results support the hypothesis that elevated plasma neopterin observed in CVD patients is generated in part by superoxide scavenging by 7,8-dihydroneopterin within plaques.

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The interface between bioenergetics and redox biology; from bench to bedside

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The redox biology has long recognized that mitochondrial function is particularly susceptible to oxidative stress and pathological processes involving inflammation result in the controlled generation of reactive oxygen species such as HOCl and hydrogen peroxide. These findings have led to the concept that the mitochondrion can act as the "canary in the coal mine" by serving as an early warning of bioenergetic crisis in patient populations. Furthermore, cellular mitochondrial function is known to vary between populations due to differences in genetic background and in response to lifestyle changes including diet and exercise. Bioenergetics is now at the forefront of our understanding of pathological mechanisms, new therapies and as a biomarker for the susceptibility of disease progression in metabolic diseases, neurodegeneration, cancer and cardiovascular disease. We have developed high-throughput assays to measure cellular energetic function in the small numbers of cells that can be isolated from human blood and we show how they can act as a particularly sensitive sensor of oxidative stress. The sequential addition of well characterized inhibitors of oxidative phosphorylation allows a bioenergetic profile to be measured in cells isolated from normal or pathological samples. This profile can define the extent to which these cells utilize mitochondrial oxygen consumption to produce ATP, are using protons for other processes or leak and the maximal respiration and are sensitive to oxidative stress. We therefore propose the development of the Bioenergetic Health Index (BHI), which is a single value that defines bioenergetic health based upon the analysis of cellular mitochondrial profiles in cells isolated from human subjects. Ultimately, BHI has the potential to be a new biomarker for assessing patient health of (or for) both prognostic and diagnostic value particularly those in which oxidative stress is a contributory component.

Heme oxygenase-1 and metabolic reprogramming in response to ischemia

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Heme oxygenase-1 (Hmox1) is a stress protein induced by hypoxia and glucose deprivation. As Hmox1 is increasingly recognized as a regulator of cellular homeostasis, we are investigating its potential role in metabolic reprogramming in ischemia after vascular occlusion, a key problem associated with cardiovascular diseases. Using yeast as a model revealed that the absence of Hmox1 results in altered expression of genes, including those involved in cellular redox control, glucose transport, coenzyme Q synthesis and mitochondrial respiration. Similarly, studies with isolated mouse skin fibroblasts showed that Hmox1 deficiency was associated with a decrease in cellular coenzyme Q and mitochondrial respiration. Hmox1-deficient fibroblasts had an abnormal metabolic adaptative response to hypoxia, with decreases in the accumulation of reactive oxygen species and the stabilization of HIF1α. Using littermate *Hmox1*$^{-/-}$, *Hmox1*$^{+/-}$ and *Hmox1*$^{+/+}$ mice and a unilateral hind limb model of ischemia, we observed *Hmox1*$^{-/-}$ mice to have impaired blood flow recovery and neovascularization, and to suffer from severe tissue necrosis and auto-amputation that preceded blood flow recovery. Bone marrow transfer experiments showed that the Hmox1 deficiency in bone marrow cells did not explain the observed auto-amputation. Compared with *Hmox1*$^{+/+}$ mice, skeletal muscle fibers of *Hmox1*$^{-/-}$ mice had decreased oxidative phosphorylation capacity and elevated steady state glucose uptake. Ischemia was associated with induction of Hmox1 transcript, protein and enzyme activity. Similar to the situation in fibroblasts, this induction of Hmox1 in skeletal muscle preceded both the stabilization of HIF1α and the induction of HIF1α target genes, such as *glucose transporter-1* and *vascular endothelial growth factor*. We propose that Hmox1 is both downstream and upstream of HIF1α, and that Hmox1 regulates cellular energy reprogramming in response to hypoxia, in part via regulation of the cellular redox state.

Intracellular ascorbate availability in health and disease and effects on the regulation of the HIF hydroxylases

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Vitamin C (ascorbate) acts as a co-factor for a variety of metal-containing enzymes in the body. Among these are the Fe-containing 2-oxoglutarate-dependent dioxygenases (2-OGDDs) that include the hydroxylases that regulate hypoxia-inducible factor (HIF)-1, a major driver of cancer growth and progression. The 2-OGDDs depend on oxygen, iron and 2-oxoglutarate availability and their activity is compromised when ascorbate is limiting. The HIF hydroxylases down-regulate HIF-1 activity and we found that under *in vitro* metabolic stress conditions, low intracellular ascorbate levels exacerbated HIF-1 alpha protein accumulation and its transcriptional activity. HIF-1 is an important driver of tumour development, and in a retrospective observational analysis of colorectal or endometrial tumour tissue, we found that HIF-1 activity was inversely related to tumour ascorbate content. These results suggest that sub-optimal intracellular ascorbate can up-regulate HIF-1 activity by modulating the HIF hydroxylases, thereby providing cancer cells with a metabolic and survival advantage.

Ascorbate is delivered to cells via the vasculature, and intracellular levels are compromised when plasma levels are low. In addition, the ability of ascorbate to penetrate into tissues remote from blood vessels is unknown. This may be important in cancer, as solid tumours often contain regions with dysfunctional vasculature that results in impaired oxygen and nutrient delivery. Using a 3-dimensional pharmacokinetic model, we have measured ascorbate diffusion and transport parameters through dense tumour cell layers *in vitro*. The data demonstrated heterogeneous distribution of ascorbate in tumour tissue at physiological blood levels and provide insight into the range of plasma ascorbate concentrations and exposure times needed to saturate all regions of a tumour. The predictions suggest that saturating plasma ascorbate levels (up to 100 µM) are required to ensure normal tissue saturation, whereas only supra-physiological plasma ascorbate concentrations (>100 µM) will result in effective delivery of ascorbate to poorly vascularised tumour tissue.

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Poster Abstracts
Session A

A1: Substrate specificity in a thiol dioxygenase from *Pseudomonas aeruginosa* is altered by a glutamine to arginine substitution

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Thiol dioxygenases are non-heme mononuclear iron enzymes which catalyze the oxidation of thiols to their respective sulfinic acids by addition of two oxygen atoms from molecular oxygen. So far four types of thiol dioxygenases have been identified – cysteine dioxygenase, aminoethanethiol dioxygenase, 3-mercaptopropionate dioxygenase and mercaptosuccinate dioxygenase – each of which is highly specific for its substrate. A recent study[1] using sequence alignment and crystallographic data suggested that thiol dioxygenases can be split into "arg-type", those that react with cysteine, and "gln-type" those that react with 3-mercaptopropionate. However, we[2] have shown that the thiol dioxygenase from *Pseudomonas aeruginosa* (p3MDO), although formally a "gln-type", is able to react with both cysteine and 3-mercaptoprionate but with a preference for the latter. Comparison of the sequence of this enzyme with that of mercaptosuccinate dioxygenase (MSDO) made us hypothesize that substituting glutamine with an arginine in p3MDO should confer MSDO activity. To explore this, site-directed mutagenesis was used to generate a Gln-62-Arg variant of p3MDO. Dioxygenase activity was measured with NMR, mass spectrometry and Ellman's assay. The variant converts cysteine and 3-MPA to their corresponding sulfinic acids similar to the wild type, albeit with lower activity. Importantly however, the variant has additional reactivity over p3MDO, mirroring MSDO activity by converting (R)-mercaptosuccinate to (R)-sulfinosuccinate. The (S)-enantiomer is converted only to its disulfide. These data suggest the key components that define substrate specificity in thiol dioxygenases, and the significance of this will be discussed.

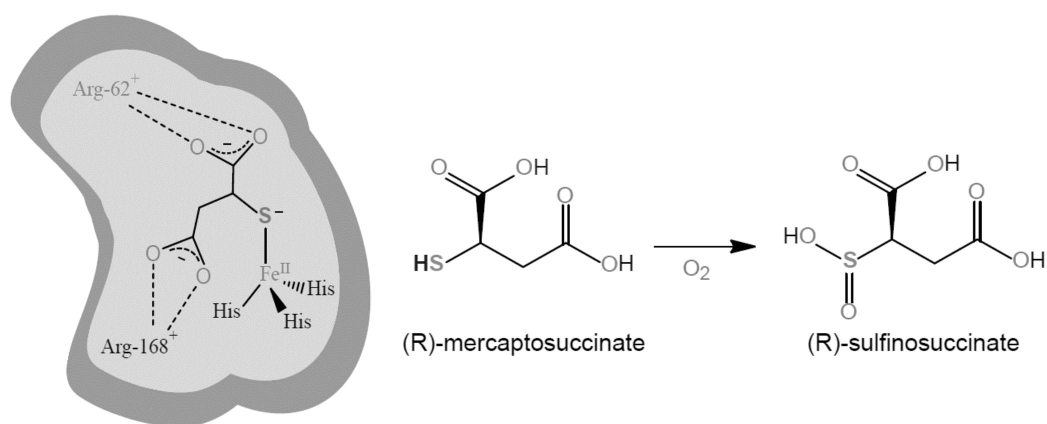

Figure: Left: A schematic representation of the active-site of p3MDO, with the native Gln-62 residue replaced with an Arg, showing the proposed binding mode of mercaptosuccinate. Right: The additional reaction that is catalyzed by the variant.

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A2: Efficiency of singlet oxygen production from oxidized platinum nanoparticle and hydrogen peroxide

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Pt nanoparticle is a strong reductant and has been used as an antioxidant in cosmetics and medicine. It was reported to have catalase-like activity which converts hydrogen peroxide to water and oxygen. However, freshly prepared Pt nanoparticle was almost inert towards decomposing hydrogen peroxide. The catalase-like activity of Pt nanoparticle increased when the Pt nanoparticle was exposed to air, indicating that oxidized Pt nanoparticle has catalase-like activity. Formation of singlet oxygen from hydrogen peroxide and oxidized Pt nanoparticle was confirmed by the following experiments: i) addition of 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one hydrochloride (MCLA) produced chemiluminescence; ii) addition of uric acid reduced the intensity of MCLA chemiluminescence, while that of superoxide dismutase remained unchanged; and iii) addition of uric acid resulted in the formation of its singlet oxygen-specific product, parabanic acid. Efficiency of singlet oxygen production from oxidized platinum nanoparticle and hydrogen peroxide was estimated as follows. i) counted photons from MCLA per mole of singlet oxygen produced from naphthalene endoperoxide; ii) counted photons from MCLA in the presence of hydrogen peroxide and oxidized Pt nanoparticle, and iii) measured the decay of hydrogen peroxide in the presence of oxidized Pt nanoparticle. The efficiency was calculated to be approximately 2% and this number is under careful inspection.

A3: Inhibition of autophagy sensitizes cells to hydrogen peroxide-induced apoptosis: protective effect of mild thermotolerance induced at 40°C

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The exposure of cells to lower doses of different stresses (e.g. oxidative stress, heat shock, radiation) can activate adaptive survival responses, whereas higher levels of stress generally trigger cell death. Adaptive survival responses allow cells and organisms to continue their normal functions in the face of an adverse stimulus. These responses appear to be mediated by multiple factors, including anti-apoptotic proteins, heat shock proteins (Hsps), antioxidants and autophagy, which can protect cells against damage caused by diverse environmental insults. Mild heat shock induced at a non-lethal temperature such as 40°C (thermotolerance) is a well-known inducer of the heat shock response and involves increased accumulation of Hsps. If the adaptive survival response cannot protect the cell against an adverse stimulus, then the damaged cell will likely be eliminated by cell death processes such as apoptosis or necrosis. This study aims to determine whether low doses of pro-oxidant stress can increase induction of autophagy as a survival response, the cellular outcome when autophagy is inhibited, and whether mild thermotolerance induced at 40°C can guard against cell demise when autophagy is compromised. Lower doses of hydrogen peroxide caused an increase in markers of autophagy in HeLa cells, whereas higher doses triggered cell death by apoptosis. Mild thermotolerance protected cells against hydrogen peroxide-induced apoptosis. In addition to being a survival response, autophagy can also act as a mediator of cell death by mechanisms that are not entirely clear. When autophagy was inhibited using 3-methyladenine and bafilomycin A1, hydrogen peroxide induced cell death by apoptosis. Caspases from multiple signaling pathways were activated: mitochondria, death receptors and the endoplasmic reticulum. Mild thermotolerance partially protected cells against peroxide-induced caspase activation when autophagy was inhibited. These findings indicate that thermotolerance and autophagy appear to act as complementary survival responses to protect against peroxide-induced cell death.

A4: Effect of peroxidase substrates and inhibitors on the peroxidasin-catalysed sulfilimine cross-link in collagen IV

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Mammalian heme peroxidases catalyse the conversion of hydrogen peroxide (H_2O_2) and halides to their respective hypohalous acids. Peroxidasin oxidises bromide to hypobromous acid (HOBr). Peroxidasin and HOBr were recently shown to play a role in cross-linking of collagen IV. The crosslink is characterized as sulfilimine bond that forms between juxtaposed methionine and lysine residues joining triple-helical protomers of collagen IV end to end. This intermolecular bond is crucial for the integrity of the extracellular matrix (ECM). The exact mechanism of sulfilimine crosslink formation in collagen IV is not completely understood. To elucidate this mechanism, we investigated the effect of several substrates and inhibitors of other heme peroxidases on the activity of peroxidasin and the cross-linking of collagen IV. We cultured endothelial cells to deposit ECM and added different treatments either directly to cells in culture during formation of ECM, or to the isolated ECM. Our results show that the sulfilimine crosslink formed when we treated isolated uncross-linked ECM with both H_2O_2 and bromide, or with HOBr. The crosslink did not form when we treated the ECM with H_2O_2 and chloride, or with hypochlorous acid. Cross-linking of collagen IV was not affected by treatment with inhibitors of myeloperoxidase, such as thioxanthine and ABAH in cell culture or isolated ECM. Interestingly, cross-linking was inhibited when treated with physiological concentration of thiocyanate or therapeutic concentration of acetaminophen (paracetamol) both in cell culture and isolated ECM. This data indicate that a resident peroxidase within the ECM uses bromide as a substrate to catalyse the formation of the sulfilimine crosslink. The mechanism of peroxidasin catalysis appears to be analogous to the classical mechanism of peroxidases, however different to the specific mechanism of myeloperoxidase. Our results indicate that efficiency of cross-linking of collagen IV is susceptible to modulation by endogenous and exogenous substances.

A5: Redox-independent interaction between peroxiredoxin 2, hemichromes and the red cell membrane

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Background: It is still unclear how Peroxiredoxin 2 (Prx2), an abundant antioxidant protein in erythrocytes, is able to protect erythrocytes from Heinz body anemia. Additionally, while it is known that Prx2 binds to the red blood cell membrane where it might be involved in red blood cell ageing, the mechanism it is still not clear. We set out to establish how Prx2 interacts with the red cell membrane, hemoglobin and hemichromes, and if these interactions are dependent on Prx2 redox state.

Study design and methods: To determine redox-dependent interactions of Prx2 with the red cell membrane, we used erythrocytes, purified Prx2, isolated hemoglobin free ghosts and hemichromes prepared from hemolysate. We also used hemolysate, the known Heinz body inducers phenylhydrazine and acetylphenylhydrazine, as well as recombinant Prx2 to examine interactions between hemoglobin derivatives and Prx2 using spectral analysis and western blotting.

Results: Our results indicate that membrane binding of Prx2 in red blood cells is independent of its redox state, but at least partially dependent on the presence of calcium. Oxidized Prx2 is also able to limit the precipitation of denatured hemoglobin induced by phenylhydrazine.

Conclusion: Prx2 might be able to reduce Heinz body precipitation and premature erythrocyte removal by acting as a redox-independent holdase in erythrocytes.

A6: New aspects of assembly and functions of mitochondrial respiratory complex II in tumour formation

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Newly emerging evidence shows that the mitochondrial respiratory function is essential for neoplasia. Mitochondrial respiratory complex II (CII), also called succinate dehydrogenase (SDH), contains subunits SDHA, SDHB, SDHC and SDHD, which are nuclearly encoded and post-translationally imported into mitochondria to form the respiratory complex. A number of diseases associated with CII dysfunction have been reported, and SDH subunits are categorised as tumour-suppressor proteins. We have discovered that SDHA exists in multiple hetro-oligomeric 'forms'. To date SDHA is known to only form ~140 kDa hetero-oligomeric complex with the SDHB, SDHC and SDHD subunits (i.e. fully assembled CII); the second state of SDHA, migrating as ~100 kDa band on blue native (BN)-PAGE has no known biological function. We knocked out/depleted subunits of CII using genomic editing and RNAi, and studied their functional significance in relation to bioenergetics and tumour formation using cutting-edge methods, including SWATH-MS proteomic and *in vivo* tumour models. We found that the ~100 kDa SDHA form is more prevalent in adenocarcinoma cells 4T1 cells depleted of their mtDNA (4T1ρ^0 cells). MDA-MB-231 cells with low level of ~100 kDa SDHA lost their ability to control energy- and nucleotide-demanding G1-S cell cycle progression. Further, mice with SDHB$^{-/-}$ cells formed tumours with low rate, while tumour formation was completely blocked in mice grafted with SDHB$^{-/-}$SDHA^{low} breast cancer cells, in this the ~100 kDa SDHA form in missing. Taken together, our results point to a new role of SDHA independent of the other subunits of complex II, which is likely to have importance for the (patho)physiology of a cell, e.g in the context of tumour initiation and progression.

A7: Fetal oxidative stress prevention during artificial placentation

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Premature birth is the early and often sudden delivery of a baby before 37 weeks of a normal 9 month (40 week) term and is now the second highest cause of infant deaths across the world. Babies born under 6 months (22-25 weeks) are pre-viable. These extremely preterm infants or "micro-preemies" often die because their hearts, kidneys and lungs have not developed to the point that they can function on their own. In addition, extremely preterm infants are vulnerable to infection and have limited defenses to oxidative stress. Many of these infants could be saved if they could remain in the womb.

The scale of the problem is concerning, with approximately 270,000 extremely preterm infants born each year around the developed world and 70% only surviving the first few hours of life. Survivors are at significant risk of developing severe sort and long term health issues.

Medical technology can help these babies by preventing the physiological changes that occur during the perinatal transition, thereby keeping the infant in an artificial womb-like environment, nourished and oxygenated by an artificial placenta. This allows a pre-viable infant to continue on its normal developmental and growth trajectory.

Dr Bird will discuss the artificial placenta and womb project with emphasis on strategies to prevent fetal oxidative stress during artificial placentation.

A8: Radical pairs or intramolecular electron conduction? The mechanism of cryptochrome magnetoreception

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Extremely low frequency magnetic fields (ELF MF) are classified as possibly carcinogenic to humans, but the biophysical mechanisms of a causal relationship remain unclear. A cryptochrome-based radical pair mechanism (RPM) has been invoked as the primary MF sensor in animal magnetoreception to explain effects from MF strengths in the nT range. Model studies of the RPM performed in aprotic solution at cryogenic temperatures with MFs in the µT range elicit only marginal responses, and it is unlikely that radical pairs formed in aqueous biological milieu at physiological temperatures are sufficiently long-lived to evoke magnetoreception.

The cryptochrome-based RPM is believed to involve blue light activation of a flavin adenine dinucleotide (FAD) cofactor followed by electron transfer (ET) from a conserved triad of tryptophan residues. Cryptochrome has an unusually high tryptophan content, with many more than the RPM triad highly conserved; there are also many highly conserved tyrosine residues. Thus, intramolecular ET involving additional aromatic residues in cryptochrome likely extends beyond the canonical triad to the solvent edge of the protein.

The ascorbyl radical has been proposed as a potential radical pair partner in the RPM. It is known that ascorbate "repairs" radicals on aromatic residues in proteins. Ascorbate, which is present at millimolar concentrations in many cell types, is likely to transfer an electron to the ultimate amino acid radical formed during one-electron reduction of FAD in cryptochrome. The flavin semiquinone radical formed upon one-electron reduction of FAD may be reoxidized to the resting state via ET to O_2 to form $O_2^{\bullet-}$. Increased levels of the ascorbyl radical and $O_2^{\bullet-}$ would contribute to oxidative stress responses in the cell and thereby play a role in carcinogenesis.

Acknowledgement: This project has received funding from the European Union's Seventh Programme for research, technological development, and demonstration under grant agreement No. 282891.

A9: Regulation of endothelial cell death by hypothiocyanous acid

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Cardiovascular disease is caused by oxidative stress in the artery wall, mediated by the release of unique oxidants from neutrophils. These oxidants target the endothelial cells lining blood vessels, driving plaque formation and eventual rupture, and causing heart attacks and strokes. This continual oxidative stress in the vessel wall can result in cell death, however the mechanism of death is critical in determining disease outcome. We have found that hypothiocyanous acid (HOSCN), one of the major oxidants produced by neutrophils, may cause cell death through necroptosis.

Necroptosis is best described as a programmed, regulated form of necrosis now considered to be the most likely outcome of extreme cell injury. Most work has been done using the TNFα receptor-mediated cell death pathway in conjunction with apoptosis inhibitors. Although it is a controlled process, necroptosis nevertheless results in membrane rupture and the release of cell contents into the tissues, thus eliciting an inflammatory response. Necroptosis occurs in the absence of caspase activity, through the activation of specific kinases (receptor interacting proteins, RIP), and the mixed lineage kinase domain-like (MLKL) pseudokinase. Determination of which cell death pathway will result hinges on the function of caspase 8 which has several activities and is central to cell survival and apoptosis signalling.

We know that high levels of HOSCN are produced by neutrophils, especially in smokers, however little is known of how it affects the endothelial cell itself and how it causes disease. We have found that HOSCN triggers a “survive or die” response which is determined by the level of HOSCN exposure. We have shown that HOSCN inhibits caspases (and therefore apoptotic death) at very low concentrations, and that it affects cell signalling machinery and elements of the necroptotic pathway. We propose that HOSCN may therefore be a physiological trigger of necroptosis.

A10: An increase in endocannabinoid content by inhibition of fatty acid amide hydrolase alleviates nitrergic-related neuroinflammation in the hippocampus following acute restraint stress

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Increasing evidence suggests that immune dyshomeostasis following stress plays a major role in the pathophysiology of stress-related illnesses. Long term exposure to stress has been demonstrated to cause neuroinflammation through a sustained overproduction of free radicals, including nitric oxide, via increased inducible nitric oxide synthase (iNOS) activity. We have previously demonstrated that iNOS activity and mRNA expression are also intensely upregulated in the hippocampus following just 4 hours of acute restraint stress. Interestingly, interactions have been shown between the nitrergic and endocannabinoid system following stressful or aversive situations. Similar to nitric oxide, the endocannabinoids are synthesised on demand with preclinical findings suggesting that cannabinoid receptor agonists and endocannabinoid enhancers inhibit nitrergic activity and display antidepressant-like properties. Specifically, pharmacological enhancement of endocannabinoid content by fatty acid amide hydrolase (FAAH) inhibitor, PF-3845, has been demonstrated to alleviate neuroinflammation by decreasing the number of iNOS-expressing microglia following traumatic brain injury. Although cannabinoid modulation during pathological conditions has been shown, the acute physiology has not been well characterised. The present study examined the effects of systemically injected PF-3845 in the modulation of hippocampal nitrergic and inflammatory-related genes after acute stress. Following vehicle or PF-3845 injections (5 mg/kg in normal saline; i.p.), outbred male Wistar rats aged 5-6 weeks postnatal were exposed to 0 (control), 60, 240, or 360 minute restraint stress (n=5-6 per group). The hippocampus was cryodissected and assayed for relative expression of nitrergic and inflammatory-related genes. The results demonstrate that pretreatment with the FAAH inhibitor rapidly reduces restraint stress-induced plasma corticosterone release. This was accompanied by a reduction in the stress-induced proinflammatory response including iNOS, interleukin-1β, interleukin-6, and cyclooxygenase-2 mRNA in the hippocampus. Furthermore, transcriptional potential of NF-κB was inhibited by PF-3845 suggesting that enhanced endocannabinoid levels in the hippocampus have an overall antioxidative and immunosuppressive effect following acute stress exposure.

A11: Significance of the myeloperoxidase isoforms to human health

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Myeloperoxidase (MPO) is a bactericidal enzyme of white blood cells that functions by generating reactive oxygen species, including hypohalous acids and free radicals that are used to kill pathogens. Recent evidence, however, suggests that excessive generation of the oxidants produced by MPO is linked to tissue damage in many diseases, especially those associated with the inflammatory response.

Given these affects, considerable interest has emerged in the role of MPO and its downstream products in a variety of inflammatory diseases. In cardiovascular disease, MPO sticks to the endothelium where it promotes oxidation of lipoproteins, resulting in formation and destabilization of coronary plaques.

Distinct forms of MPO isolated from human white blood cells are resolved chromatographically. However, the evidence accounting for the suggested structural and functional differences between the isoenzymes is unconvincing and the differences remain unclear. What is evident is that the isoforms of MPO are differently charged and will therefore stick to the endothelium with varying affinity. Consequently, the most charged form of MPO is more likely to adhere to the endothelium and cause oxidative stress.

In this research, we are investigating the structural and functional differences between the various forms of MPO. The isoforms of MPO have been successfully isolated and examined by mass spectrometry. Our results suggest that post-translational modifications could be predominantly responsible for the variation between the isoforms of MPO. Furthermore, we are exploring the 'MPO isoenzyme profile' of individuals to quantify the ratio of forms of MPO and relate to outcome measures including the presence of cardiovascular disease and death.

A.12 Mechanistic role of the second and third coordination sphere of cysteine dioxygenase

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Cysteine dioxygenase (CDO) is a non-heme mononuclear iron enzyme which catalyses the first step of oxidative cysteine metabolism by oxidising cysteine to cysteine sulfinic acid. Oxidation takes place at the iron centre which is in close proximity to the side-chains of residues cysteine 93 and tyrosine 157. C93 and Y157 can form a thioether crosslink which is observed in both recombinant CDO and CDO derived from rat liver lysate. Not all protein contains the crosslink and it has been shown previously that the rate of multiple turnover reaction depends upon the fraction of crosslink.[1,2] However, bacterial CDOs are unable to form this crosslink because glycine is highly conserved at an equivalent position to cysteine 93. This shows that the crosslink is not a prerequisite for catalysis. Recent studies using variants of rat CDO where C93 had been substituted by alanine have suggested a relationship between k_{cat} and the crosslink.[3] To further assess this relationship , we produced a C93G variant of rat CDO which converts rat CDO into a bacterial-type CDO.[4] Crystallographically, we could show that there were no large structural rearrangements. Spectroscopic and kinetic data using Mössbauer spectroscopy, HPLC and a chromogenic assay[5] showed that this enzyme efficiently forms a catalytically competent *ES* complex with cysteine and turns over. Michaelis-Menten kinetics have shown that the C93G variant is just as active as wild-type but with a shift in the pH optimum.[4] This work has now been expanded to include other variants that allow us to probe the other component of the crosslink, tyrosine (Y157F) or a highly conserved histidine involved in a hydrogen bonding network to the tyrosine (H155Q/N). Results suggest the presence of two ionisable groups in the active site that are responsible for activity of cysteine dioxygenase.

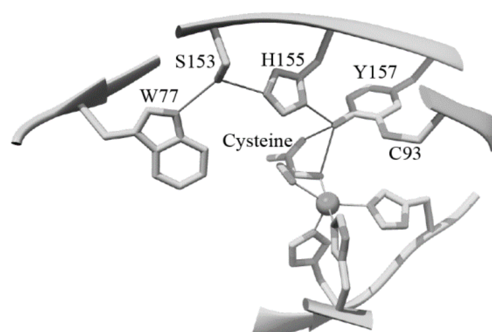

Figure - Active site of cysteine dioxygenase showing the hydrogen bonding network and interactions with bound substrate.

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A13: Kinetic analysis of the reaction of hypohalous acids with disulfide bonds

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Myeloperoxidase generates hypohalous acids (HOX, X=Cl,Br,SCN) from halide and pseudohalide ions using H_2O_2. HOCl and HOBr are potent oxidants involved in the killing of invading pathogens. These oxidants react rapidly with many biological targets and generate a wide spectrum of damage. In contrast, HOSCN is a less reactive and more selective oxidant. The controlled production of these species is crucial in maintaining good health, but uncontrolled or inappropriate generation at sites of inflammation can cause tissue damage with this being associated with multiple inflammatory diseases.

Previous studies have reported that thiols (RSH) and thioethers (RSR') (e.g. the amino acids Cys and Met) are major targets for these oxidants and that these reactions are rapid. Reaction has also been reported with disulfide bonds (e.g. cystine), with these reactions occurring with lower rate constants, though the effect of electronic and structural effects on these rate constants have not been determined.

In this study we have determined rate constants for the reaction of oxidants with a range of compounds containing disulfide (S-S) bonds in different environments, ranging from model compounds to proteins, using stopped-flow spectrophotometry or competition kinetic approaches with fluorescently-tagged methionine (Fmoc-Met) or TNB as the competitive substrate.

It is shown that the rate constants for these reactions at pH 7.4 vary dramatically with both the oxidant and environment of the disulfide bond. The reactivity of disulfides was lower than with most free thiols, and decreases in the order HOBr > HOCl > HOSCN > H_2O_2. However some disulfide bonds are markedly more reactive than others (with *k* values varying by several orders of magnitude), indicating that some disulfide bonds, including those in proteins, are considerably more reactive and susceptible to oxidation than others. These processes result in the formation of thiosulfinates [RS(O)SR'] that can undergo further reactions.

A14: Oxidative damage to the extracellular matrix protein fibronectin by myeloperoxidase-derived oxidants

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The extracellular matrix (ECM) is comprised of a complex mixture of glycosaminoglycans and proteins, such as fibronectin, that are important for the structure and function of the artery wall. The ECM regulates cell activity via cell-ECM interactions and binding of cytokines and enzymes. Fibronectin possesses multiple functional domains, including cell-binding (CBF) and heparin-binding fragments (HBF). In chronic inflammatory diseases such as atherosclerosis, activated leukocytes generate oxidants including hypochlorous (HOCl) and hypothiocyanous (HOSCN) acids that can modify ECM materials. The hypothesis for this study was that HOCl and HOSCN would modify fibronectin resulting in endothelial cell dysfunction (an early and defining marker of atherosclerosis) and contribute to a weakening of lesion structure, thereby enhancing atherogenesis and plaque rupture.

The results obtained in this study indicate that there is a loss of antibody reactivity against the CBF and HBF domains of fibronectin, as detected by Western blotting and enzyme-linked immunosorbent assay (ELISA), when the protein is exposed to increasing molar ratios of HOCl or HOSCN. HOCl treatment resulted in a decrease in tryptophan and methionine concentrations and a corresponding increase in methionine sulfoxide, changes that are characteristic of protein modification by HOCl, as well as protein cross-linking/aggregation. These modifications are accompanied by a loss of endothelial cell adhesion to HOCl-modified fibronectin, consistent with modification to the cell-binding domain. Studies on advanced human atherosclerotic lesions has provided evidence for co-localisation of the fibronectin cell-binding fragment (CBF) epitope, with epitopes recognised by an antibody raised against HOCl-damaged proteins, consistent with *in vivo* damage to fibronectin.

These data support the hypothesis that fibronectin, and particularly its functional CBF and HBF domains are susceptible to modifications by HOCl (and to a lesser extent HOSCN), with this resulting in changes its structure, function and biological properties. These changes may contribute to endothelial dysfunction and plaque rupture.

A15: Edaravone, a potent free radical scavenger, reacts with peroxynitrite to produce predominantly 4-NO-edaravone

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3-Methyl-1-phenyl-2-pyrazolin-5-one (edaravone) is used in clinical treatment of acute brain infarction to rescue the penumbra, based on its ability to prevent lipid peroxidation by scavenging lipid peroxyl radicals. Here, we show that edaravone also reacts with peroxynitrite to yield 4-NO-edaravone as the major product and 4-NO_2-edaravone as a minor product. We observed little formation of 3-methyl-1-phenyl-2-pyrazolin-4,5-dione (4-oxoedaravone) and its hydrate, 2-oxo-3-(phenylhydrazono)-butanoic acid (OPB), which are the major free radical-induced oxidation products of edaravone, suggesting that free radicals are not involved in the reaction with peroxynitrite. The reaction of peroxynitrite with edaravone is approximately 30-fold greater than with uric acid, a physiological peroxynitrite scavenger (reaction rate $k = 1.5 \times 10^4\ M^{-1}s^{-1}$ vs. $480\ M^{-1}s^{-1}$). These results suggest that edaravone functions therapeutically as a scavenger of peroxynitrite as well as lipid peroxyl radicals, which is consistent with a report that edaravone treatment reduced levels of 3-nitrotyrosine in the cerebrospinal fluid of patients with amyotrophic lateral sclerosis.

A16: Characterising the reactions of diselenides with reactive oxidants generated during chronic inflammation

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Introduction: Chronic inflammation can result in the production of a diverse array of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), peroxynitrous acid (ONOOH), peroxides and free radicals. Inappropriate or excess formation of these species, or failure of the body's protective mechanisms, can lead to localised oxidative stress and host tissue damage. Recent kinetic data have shown that selenium-containing compounds react rapidly with a range of biological oxidants, and selenoethers (e.g. selenomethionine) can undergo rapid recycling. Thus, selenium-containing species have the potential to act as beneficial antioxidants that may attenuate inflammation.

Aims: To characterise the mechanisms, products and kinetics of the reactions of biological oxidants with a range of stable diselenide (RSeSeR) species.

Methods: The reactions of the model diselenides selenocystamine (SeCyst) and diselenodipropionic acid (DSePA) with HOCl, H_2O_2 and other hydroperoxides have been assessed via UV/vis spectroscopy. A fluorescently-labelled diselenide (Fmoc-selenocystine) has been used to characterise the products of diselenide oxidation by UPLC and mass spectrometry.

Results: Oxidation of SeCyst and DSePA by HOCl and H_2O_2 results in cleavage of the diselenide bond as evidenced by the loss of absorption at ca. 300 nm, and the detection of low molecular mass species by mass spectrometry. These changes occur more readily with HOCl than with H_2O_2, with second order rate constants determined for reaction with H_2O_2 ($k \sim 10^{-4}\ M^{-1}\ s^{-1}$ and $10^{-2}\ M^{-1}\ s^{-1}$ for SeCyst and DSePA, respectively). UPLC and LC/MS/MS studies of Fmoc-selenocystine reveal that the seleninic acid ($RSeO_2H$) is a major oxidation product generated by HOCl and H_2O_2, with evidence for dose- and time-dependent production of a range of other oxidation products.

Conclusions: Diselenides are rapidly oxidised by biologically-relevant oxidants. Thus diselenides could act as efficient antioxidants *in vivo*, thereby preventing oxidative damage associated with inflammatory diseases, including atherosclerosis.

A17: Cysteine and glutathione hydroperoxides

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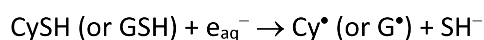
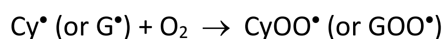
Biological functions of both cysteine and glutathione depend primarily on the presence of thiyl groups, with the former crucial to the function of numerous proteins and the latter able to repair much of cellular damage induced by radicals. Oxidation of the –SH groups results in formation of sulfur and carbon radicals, with the latter converted in the presence of dioxygen eventually to hydroperoxides. This was never tested for either molecule.

Hydroperoxides were measured in GSH and Cys exposed to radiation-generated species e_{aq}^{-}, $HO^{\bullet}$, $H^{\bullet}$ and H_2O_2 in the presence of air. In both, hydroperoxides were generated in high yields not by the $HO^{\bullet}$ but by the electrons, in the following reactions:



CySH (or GSH) + $e_{aq}^{-} \rightarrow Cy^{\bullet}$ (or $G^{\bullet}$) + SH^{-}



$Cy^{\bullet}$ (or $G^{\bullet}$) + $O_2 \rightarrow CyOO^{\bullet}$ (or $GOO^{\bullet}$)

$CyOO^{\bullet}$ (or $GOO^{\bullet}$) + CySH (or GSH) $\rightarrow$ CyOOH (or GOOH)

The results may be significant for biological processes involving leakage of electrons from the normal electron transfer chains.

A18: Inactivation of thiol-dependent enzymes by urate hydroperoxide

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There are links between high serum urate (hyperuricemia) and many inflammatory diseases, yet the mechanism is obscure. Urate, the product of purine and ATP break down, builds up in plasma because humans lack the enzyme uricase to convert it to allantoin, which is freely excreted. Urate may benefit health by acting as an antioxidant that scavenges reactive oxygen species. However, hyperuricemia is associated with gout, metabolic syndrome and cardiovascular disease. Oxidative stress is also associated with all these inflammatory diseases.

During oxidative stress urate is converted to several reactive electrophiles, including urate hydroperoxide. This novel oxidant could contribute to the adverse effects of urate. Urate hydroperoxide is formed when urate is oxidized to a radical that subsequently combines with superoxide. Neutrophils, and xanthine oxidase along with peroxidases can produce urate hydroperoxide. Previous studies characterized the formation of urate hydroperoxide and its oxidation of small biomolecules (1-3). In this investigation, we explored oxidation of thiols and the thiol-dependent enzyme glyceraldehydes-3-phosphate dehydrogenase (GAPDH) by urate hydroperoxide. The effectiveness of urate hydroperoxide as an thiol oxidant was compared with taurine chloramine (4).

Ellman's assay for reduced thiols was used to measure depletion of cysteine residues on GAPDH by urate hydroperoxide and taurine chloramine. GAPDH was exposed to oxidants in a dose-dependent manner, then assayed by measuring its ability to catalyse the production of NADH. Mass spectrometry was used to identify specific modifications of GAPDH.

Urate hydroperoxide oxidized exposed thiols on GAPDH and fully inactivated the enzyme at a ratio about 5:1. Half of its activity was recovered by reduction with DTT. Hence, urate hydroperoxide inactivates GAPDH by reversible and irreversible routes. GAPDH increased in molecular mass by 132 Da with exposure to urate hydroperoxide, indicating the formation of a GAPDH-urate adduct. In comparison, taurine chloramine inactivated GAPDH at approx. 10:1 and DTT reduction recovered all activity. Formation of urate hydroperoxide during inflammation and its subsequent oxidative reactions may explain some of the adverse effects of hyperuricemia.

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A19: CamKIIδ regulation of human coronary artery smooth muscle cell proliferation: implication for a role in the pathogenesis of atherosclerosis

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Atherosclerosis remains the major cause of cardiovascular disease. Reactive oxygen species (ROS) are highly damaging compounds that promote inflammation and it is now well recognized that sub-acute, chronic inflammation drives smooth muscle cell activation that is central to atheroma formation. What is unclear is how excessive ROS in an atherosclerotic milieu signals the activation of the smooth muscle cells. A potential candidate is calmodulin kinase II (CaMKII), a nodal signaling protein. In the presence of inflammation and oxidative stress mediators, CaMKII can become irreversibly modified by phosphorylation leading to its chronic activation that results in apoptosis, inflammation and oxidative stress responses in cardiac cells. Whether it has such a pathological role in vascular smooth muscle cells and therefore, could be a significant contributor to the pathogenesis of atherosclerosis, is completely unknown. The aim of this study was to determine whether CaMKII was expressed in human coronary artery smooth muscle cells (HCASMC), and moreover, demonstrate the presence of the pathological form of CaMKII, phosphorylated-CaMKII (P-CaMKII). Upon showing P-CaMKII was elevated in HCASMC, we determined that CaMKII overexpression led to a 15-fold ($p<0.001$) increase in the expression of the inflammatory response mediator, interleukin-6 (IL-6). In the presence of inflammatory cytokine, TNF-α, the effect of CaMKII on IL-6 expression was dramatically augmented (18-fold, $p<0.05$). In conclusion, this study provides first evidence of the expression of CaMKII in HCASMC, and importantly that the pathological phosphorylated from of CaMKII exists in these cells. Over-expression of CaMKII drives IL-6 expression. Together, these findings implicate CaMKII as a novel target for controlling the progression of early atherosclerotic plaque formation.

A20: Heart transplantation in the 21st century –marginal donors and sicker recipients: Initial clinical experience with pharmacological conditioning of brain dead donor hearts with glyceryl trinitrate and erythropoietin

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Introduction: Older and sicker individuals are now being accepted as recipients for heart transplant resulting in a shrinking pool of 'standard criteria' donor hearts. This has necessitated increased consideration and acceptance of 'marginal' donor hearts for transplantation. The aim of the present study was to examine the effect of glyceryl trinitrate (GTN) and erythropoietin (EPO) present at cardiac arrest and during storage, (pharmacological conditioning), on graft recovery, primary graft dysfunction (PGD) and survival in heart transplants performed at St Vincent's Hospital.

Methods: Donor hearts retrieved between August 2010 and November, 2013 were arrested, perfused with and stored in Celsior supplemented with 0.1mg/ml GTN and 5 U/ml EPO (n=61). Historical comparisons were made between the supplemented group and hearts stored in Celsior alone (April 2005-July 2010; n=104) and modified St Thomas' solution (STS) (January 2000-March 2005; n=100). Donor, recipient and procedural risk factors for PGD were determined for each group, and post-transplant use of mechanical circulatory support (MCS), length of hospital stay and survival out to 12 months were compared between groups.

Results: Hearts stored in Celsior (±[GTN+EPO]) were retrieved from a higher proportion of donors aged >50yr (25%; $p<0.05$). Increased use of MCS pre-transplant (36%, $p<0.05$) was observed in the Celsior supplemented group only. Implantation of these hearts required increased cross clamp times (111min, $p<0.05$). Use of MCS within 24h post-transplant was 32.0%, 31.7% and 24.6% in STS, Celsior and supplemented Celsior groups respectively. There were no differences in the length of hospital stay between groups. Survival at 1-month was 92%, 95% and 98%; at 3-months 89%, 91% and 93%; and at 12-months 86%, 89% and 90% respectively.

Conclusions: Pharmacological conditioning with EPO and GTN maintained survival in higher risk recipients receiving hearts from a pool of older, 'marginal' donors and allowed comparable or reduced use of MCS post-transplant.

A21: Formation and metabolism of N-hexanoyl and propanoyl-phosphatidylethanolamine adducts

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Some lipid peroxidation products show a high reactivity against primary amino groups of biomolecules, such as phosphatidylethanolamine (PE), leading to the formation of lipid-PE adducts. In this study, we focused on two alkylamide-type adducts, *N*-hexanoyl phosphatidylethanolamine (HEPE) and *N*-propanoyl phosphatidylethanolamine (PRPE), which are formed by reaction of lipid hydroperoxides with PE. HEPE can be formed by the reaction of peroxidized n-6 polyunsaturated fatty acid (PUFAs) with PE. PRPE is considered to be generated from peroxidation of n-3 PUFAs with PE. However, these PE adducts are difficult to be directly detected. Then we measured *N*-hexanoylethanolamine (HEEA)/*N*-propanoylethanolamine (PREA) by treatment of HEPE/PRPE with commercial phospholipase D (PLD) by liquid chromatography-mass spectrometry. As a model system, human low-density lipoprotein (LDL) was oxidized by copper ion and the formation of HEPE or PRPE in the LDL was then investigated by measuring HEEA or PREA. As a result, HEEA, but not PREA, was detected, presumably because the amount of n-3 PUFAs was too small in the LDL used. Next, liposomes equally containing HEPE and PRPE were incorporated into RAW264.7 macrophages and the secretion of HEEA or PREA into culture medium was analyzed. In contrast to the results with LDL, PREA was only detected in the cell culture medium. The formation of PREA from PRPE may be fast, as compared with that of HEEA from HEPE, suggesting that PRPE is a more favorable substrate for the PLD enzymes, such as *N*-acyl-phosphatidylethanolamine PLD (NAPE-PLD), which promotes the release of *N*-acylethanolamine from NAPE. In addition, the commercial PLD hydrolyzed PRPE more effectively than HEPE. The biological functions of lipid-PE and EA adducts are currently under investigation.

A22: Intrinsic oxidative stress induces accumulation of ubiquitinated and carbonylated proteins in mouse erythrocytes

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Reactive oxygen species (ROS) are produced under various conditions such as inflammation and hypoxia-reperfusion injury, and they are involved in a variety of diseases including anemia and renal failure. Hemoglobin is a major protein in red blood cells (RBCs), and suffers autoxidation, which results in the production of superoxide. Without Superoxide dismutase 1 (SOD1), the radical chain reaction initiated by superoxide oxidatively damages RBCs, and ultimately accelerates their destruction. We have previously demonstrated that SOD1-deficient RBCs show a shortened life span that is approximately 60%-70% that of the RBCs of wild-type mice, suggesting that the oxidative stress-induced massive destruction of RBCs is an underlying mechanism for autoimmune hemolytic anemia. In the current study, we examined the issue of how elevated ROS are involved in the destruction of RBCs and the onset of anemia from the view point of the proteolytic removal of oxidatively-damaged proteins. We found that poly-ubiquitinated and carbonylated proteins had accumulated and undergone aggregation in RBCs from SOD1-deficient mice. Although the protein levels of the three catalytic components of the proteasome, β1, β2, and β5, were not significantly altered, their proteolytic activities were decreased in the SOD1-deficient RBCs. These data suggest that SOD1 deficiency triggers the dysfunction of the proteasomal system, which results in the accumulation of the aggregation of poly-ubiquitinated proteins. We conclude that an oxidative stress-induced malfunction in the scavenging activity of proteasomes accelerates the accumulation of damaged proteins, oxidative modification, and shorten their lifespan, ultimately resulting in the development of anemia.

A23: Oxidative post-translational modifications of calprotectin during inflammation

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White blood cells known as neutrophils are responsible for promoting tissue damage in numerous inflammatory diseases. Neutrophils are a major source of reactive oxidants and are likely contributors to the oxidative damage associated with a variety of diseases in which inflammatory cells participate. Calprotectin is a heterodimer of S100A8 (A8) and S100A9 (A9) proteins present in the cytosol of neutrophils. It is a calcium-binding protein that inhibits metalloproteinases, has antibacterial and antifungal activities, and induces apoptosis in malignant and non-malignant cell cultures. It has also been shown to inhibit bacterial growth through the chelation of nutrient manganese, zinc and iron. Calprotectin levels increase in inflamed tissues, and recent work has shown that calprotectin is oxidatively modified during neutrophil activation. This project focuses on identifying specific oxidative modifications of purified calprotectin, and whether these modified products can be detected in clinical samples and subsequently used as biomarkers for various inflammatory diseases.

Calprotectin was purified from the neutrophils of patients with haemochromatosis. Purified calprotectin was then treated with increasing concentrations of HOCl and resolved by SDS-PAGE. The presence of reducible higher molecular mass structures was observed with increasing concentrations of HOCl. Molecular masses of 26 kDa and 24 kDa corresponded to the presence of A9-A9 homodimers and A8-A9 heterodimers respectively. Mass spectrometry was subsequently used to confirm the presence of these dimers. The A8-A9 heterodimer observed was then identified as a disulfide crosslink between the cysteine residues of TCK (A9) and LLETECPQYIR (A8). Immunoblotting with antibodies to A8 and A9 was also used to further support these findings. The A8-A9 crosslink was also detected by immunoblot in bronchoalveolar lavage (BAL) fluid from patients with cystic fibrosis (CF).

These results are extremely promising, and indicate that the formation of reducible disulfide crosslinks could be used as a potential biomarker for the extent of infection and inflammation in patients with CF. Further research is required to develop a quantitative method for measuring this crosslink in patient samples, and to determine how the formation of these crosslinks inhibit binding of essential metals and lead to loss of nutritional immunity.

A24: Quantum chemistry studies for the formation and reactions of nitrated fatty acids

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Fatty acids (FAs) are common building components of lipids. In the past two decades, nitrated fatty acid (NO_2-FA) has been recognized for their cell-signaling functions. Recently, endogenous presence of NO_2-oleic acid (NO_2-OA) and NO_2-linoleic acid (NO_2-LA) in olive oil and olive was confirmed by mass spectroscopy. The electrophilic NO_2-FA exhibits their signaling function via reversible Michael addition reactions with presumably thiol and amino groups (i.e., cysteine and histidine) in proteins. Under acidic gastric digestive conditions, nitration of lipids resulted in nitro conjugated linoleic acid (NO_2-CLA). In contrast with the bis-allylic form of linoleic acid, CLA has been recognized to undergo nitration at a much faster rate than NO_2-LA. In addition to these reactions, it has been reported that nitrogen oxide is released from NO_2-FA under aqueous conditions, while it is hydrophobically stabilized. We have endeavoured to investigate mechanisms involved in these biologically important reactions. Quantum chemistry approaches, including wave function based ab initio theories and density functional theory, have been applied to investigate these reactions.

A25: Up regulation of Nrf2 expression in 5-fluorouracil-resistant colon cancer cells by interaction of DNA demethylases and histone methyltransferases

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Recently, we reported that nuclear factor erythroid 2-related factor 2 (Nrf2) is upregulated through the activity of the DNA demethylase ten-eleven translocation 1 (TET1) in the 5-fluorouracil-resistant colorectal cancer cell line SNUC5/5-FUR. In the present study, the mechanisms of Nrf2 induction involving histone modifications were examined in SNUC5/FUR cells. The histone acetyltransferase (HAT) and histone deacetylase (HDAC) were increased and decreased, respectively, in SNUC5/FUR cells. The histone methyltransferase MLL was induced and trimethylated at histone H3 lysine 4, whereas G9a was downregulated, and the resultant dimethylation of histone 3 lysine 9 was decreased. Small interfering RNA (siRNA)-mediated MLL knockdown downregulated Nrf2 and HO-1 to a greater extent than silencing of HAT1. Host cell factor 1 (HCF1) was upregulated in SNUC5/5-FUR cells, and a significant interaction was observed between HCF1 and MLL, which are subunits of the histone methyltransferase COMPASS-like complex. Furthermore, upregulation of O-GlcNAc transferase (OGT) in SNUC5/5-FUR cells was associated with the interaction between HCF1 and MLL. The interaction between OGT and TET1 was associated with the activity of DNA demethylases and histone methyltransferases, leading to the upregulation of Nrf2 and 5-FU resistance.

Poster Abstracts
Session B

B1: Urate-4,5-dioxetane is the precursor of parabanic acid, a singlet oxygen specific oxidation product of uric acid

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Singlet oxygen is generated by not only photosensitization but also two electron oxidation of hydrogen peroxide induced by hypochlorite or peroxynitrite and can damage lipids, proteins, and nucleic acids in vivo. Uric acid is believed to be one of the most important scavengers of singlet oxygen. We have demonstrated that parabanic acid is a singlet oxygen specific oxidation product of uric acid and it is hydrolysed to oxaluric acid. In this study, we found that oxidation of uric acid by singlet oxygen produces urate-4,5-dioxetane and its chemical structure was confirmed by LC/MS/MS analysis. Isolated urate-4,5-dioxetane was converted to parabanic acid by a β-cleavage reaction of dioxetane. Since the detection of urate-4,5-dioxetane, parabanic acid, and oxaluric acid at pmol levels was achieved, we are currently applying this method to biological samples.

B2: Effects of ADDX^R a novel frying oil additive on heated vegetable oil-induced hypertension in a rat model

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ADDX® is a biophenol-rich plant extract that was proven to be able to prevent oil oxidation and absorption. Prolonged consumption of heated vegetable oil was demonstrated to cause hypertension, enhanced vasoconstriction, impaired vasorelaxation and reduced nitric oxide bioavailability. The present study, investigated the effects of ADDX® supplementation on heated vegetable oil-induced hypertension in Sprague Dawley rats. It was found that incorporation of ADDX® in a ratio of 1:10 was able reduced systolic blood pressure (BP) in the five-times-heated palm oil (5HPO) significantly ($p < 0.05$) but not in ten times heated palm oil (10HPO). The blood pressure lowering effect of ADDX in 5HPO was associated with attenuation of vasoconstriction response to phenylephrine (PE) and prevented the loss ($p<0.05$) in plasma nitrite. These changes were not seen in 10HPO. However, ADDX® had no significant effect on endothelium-dependent and endothelium-independent vasorelaxation response to acetylcholine (ACh) and sodium nitroprusside (SNP). These finding suggest that ADDX prevent the blood pressure raising effect of 5HPO which was associated with the increment in nitric oxide (NO) bioavailability that may dampen the vasoconstriction effect of PE. The inability of ADDX® to give significant effects on blood pressure, vascular reactivity and nitric oxide in 10HPO may be due higher degree of oxidative stress which may requires higher dose of ADDX.

B3: 3, 7, 3′, 4′-tetrahydroxyflavone induces cell death and endoplasmic reticulum stress in human non-small lung cancer cells through inhibition of the MAPK signaling pathway

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3, 7, 3′, 4′-tetrahydroxyflavone (THF), a dietary flavonoid compound is currently being investigated for its anti-cancer effect in various cancer models including lung cancer. Recent studies have shown that THF induces cell growth inhibition and apoptosis in human non-small cell lung cancer cell line, NCI-H460. The present study, we investigated the ER-stress induced apoptotic effects of THF against the NCI-H460 cells. THF induced mitochondrial ROS and characteristic signs of ER stress: ER staining, mitochondrial Ca2+ overloading, expression of ER stress-related proteins, phosphorylation of RNA-dependent protein kinase-like ER kinase and inositol requiring enzyme 1, Glucose-related protein (GRP) 78, spliced X-box-binding protein 1 (XBP1), phospho-eukaryotic initiation factor 2α (peIF2A), cleavage of activating transcription factor 6, and induction of the pro-apoptotic factors, CCAAT/enhancer-binding protein-homologous protein (CHOP) and caspase-12. Moreover, down-regulation of CHOP and ATF6 by siCHOP RNA an siATF6 attenuated THF-induced cytotoxicity. In addition, THF reduced the phosphorylation of ERK, JNK, p38 MAPK in the NCI-H460 cells. Furthermore, silencing of MAPK signal pathway failed to induce cell death indicating that THF induced cytotoxicity is mediated ER-stress through MAPK signaling pathway. In summary, our results indicated that THF induced cell death and ER stress in NCI-H460 cells, which was mediated by the induction of the MAPK signaling pathway.

B4: A comparative study of fine particulates, PAH exposure and oxidative DNA damage in cooks exposed to three types of cooking oil fumes

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Objectives: To evaluate how repeated frying oils (RFOs) and restaurant waste oils (RWOs) affect PAH emission in particulates and their association with oxidative DNA damage.

Methods: Production and elimination rates of UFP and PM2.5 from three types of cooking oil fumes (COF) were determined. Air samples were analyzed for PAH content. Urine samples collected from different groups of cooks and unexposed controls (n = 236), all male and non-smokers of similar age, were analyzed for 1-hydroxypyrene (1-OHP) and 8-hydroxy-2′-deoxyguanosine (8-OHdG). Participant health status, BMI, occupational history, alcohol, and fruit consumption were assessed 24 hours before sampling.

Results: The production and emission rates of UFP and PM2.5 were higher for exposed groups than for the reference group, with the RFO-frying group having the highest values. The concentrations of summed PAHs were in the order of RFO-frying group > RWO-frying group > deep-frying group > unexposed control group. The levels of 1-OHP and 8-OHdG were highest in the RFO-frying group. A significant correlation between the natural logarithms of the urinary 1-OHP and 8-OHdG concentrations (ln 1-OHP and ln 8-OHdG) was observed for all of the exposed groups but not for the control group. A clear association between ln 1-OHP and PAH exposure levels was also observed, and ln 1-OHP and ln 8-OHdG were also significantly correlated in multiple regression analysis. Interactions between UFP, PM2.5 and PAH exposure were non-significant.

Conclusions: Exposure to particulate PAHs or other possible compounds in COF of RFO and RWO may cause differentially increased oxidative DNA damage.

B5: Towards the identification of the mammalian ascorbate-stimulated plasma membrane ferricyanide reductase

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Eukaryotic cells possess transplasma membrane electron transfer (tPMET) systems capable of reducing extracellular electron acceptors at the cost of cytosolic electron donors. tPMET has been suggested to be involved in non−transferrin−bound iron (NTBI) reduction and uptake. We have, however, shown, that at least in the case of the ascorbate-stimulated tPMET activity, this is not the case and that transplasma membrane ascorbate/dehydroascorbate (DHA) cycling can instead promote NTBI reduction and uptake by human erythroleukemia (K562) cells and rodent astrocytes. Ascorbate-stimulated ferricyanide reduction, in contrast to ferric citrate reduction, is dependent on intracellular ascorbate. We have previously reported on an ascorbate-stimulated plasma membrane ferricyanide reductase in human erythroleukemia (K562) cells and rodent primary astrocytes. We hypothesised the enzyme responsible to be a member of the cytochrome b_{561} family. We therefore aimed to establish whether members of the cytochrome b_{561} family of proteins are expressed at the plasma membrane of K562 cells. We employed Western blot analyses in whole cell extracts from K562 and PC12 cells to establish the expression of the cytochrome b_{561} proteins: duodenal cytochrome b_{561} (Dcytb), lysosomal cytochrome b_{561} (Lcytb), chromaffin granule cytochrome b_{561} (CGcytb), gene product 101F6, ferric chelate reductase 1 (FRRS1, SDR-2). We then employed indirect immunofluorescence labelling and confocal microscopy to analyse the subcellular localisation of the cytochrome b561 isozymes expressed in these cells. PC12 cells expressed all isoforms and K562 expressed all isoforms except Lcytb. Confocal microscopy of K562 cells showed all isoforms in intracellular compartments, but only Dcytb was expressed at the plasma membrane. Our data suggest Dcytb to be the identity of the long-sought ascorbate-stimulated plasma membrane ferricyanide reductase. Future siRNA knock down of this enzyme should establish the molecular identity of the reductase.

B6: Red blood cell transfusion: Does the storage solution sufficiently replace the native plasma?

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Red blood cell transfusion is life-saving for severely ill and injured patients. However, it has been shown recently that transfusion can increase morbidity and mortality due to the prolonged storage time of blood. Previous studies have also indicated that red blood cells undergo structural and molecular changes during storage that affect their lifespan and proper function.

We aimed to characterise markers of red blood cell aging during storage and determine whether the SAGM (saline, adenine, glucose, mannitol) storage solution is a sufficient replacement for the native plasma that is removed during blood processing and subsequently used by other patients. To test this, leukocyte-reduced blood from multiple donors was collected and stored under standard blood banking conditions in plasma or SAGM storage solution, and analysed weekly for biochemical and functional analysis.

The antioxidant capacity of stored red blood cells decreased over time after an initial spike observed in the first week. Samples stored in SAGM had a significantly lower antioxidant capacity than plasma samples at all time points measured. We observed increased oxidation of peroxiredoxin 2 during the storage period, with SAGM samples exhibiting significantly higher oxidation than plasma samples. Red blood cell lysis similarly increased over time under both conditions but was significantly higher in SAGM samples at later time points. Deformability of red blood cells decreased during storage, although no significant difference was detected between the two conditions.

Our data suggest that red blood cells are more oxidatively stressed and prone to lysis when stored in SAGM than their native environment (plasma). As plasma is also a valuable product, it is impractical to store all blood samples with it. We suggest that red blood cells collected from rare donors be stored with plasma, so that the function and quality of these cells can be preserved for longer.

B7: Peroxiredoxin 2 oxidation in obese patients before and after bariatric surgery

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The incidence of morbid obesity, with its associated metabolic and health consequences, has been steadily increasing in the developed world. Bariatric surgery is the most effective way of treating obese patients and is associated with a rapid reversal of metabolic dysfunction following surgery. As such, it provides a useful model for investigating the underlying biochemical processes associated with obesity. Oxidative stress and inflammation are both linked to obesity. The antioxidant protein peroxiredoxin 2 (Prx2) is exquisitely sensitive to oxidation by hydrogen peroxide, and can act as an endogenous marker of oxidative stress. We have previously shown that oxidised Prx2 accumulates in erythrocytes exposed to hydrogen peroxide or activated neutrophils [1, 2]. The objective of this pilot study was to assess the redox status of Prx2 in erythrocytes collected from 12 morbidly obese subjects both before and after bariatric surgery, with a view to correlating Prx2 oxidation with other disease parameters. Erythrocyte Prx2 oxidation was elevated in patients immediately prior to surgery. These values decreased over the following 1, 3, 6 and 12 months, with many of the subjects within the normal range by 6 months. The decrease in oxidised Prx2 was closely associated with a decline in body mass index (BMI) of the subjects. To investigate the mechanism of increased oxidation, markers of inflammation (C-reactive protein) and Type II diabetes (HbA1c) were measured, but there was no significant correlation with the redox status of Prx2. A larger cohort is required to confirm the Prx2 results, and to explore the mechanisms associated with increased oxidative stress.

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B8: Oxidative stress and abnormal cholesterol metabolism in patients with sepsis

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Sepsis is one of the major cause of death in the hospital. Therefore, more appropriate treatment is desired. Increased oxidative stress in patients with sepsis has been demonstrated since its onset is triggered by infection and inflammation. Alternations in lipid metabolism are also reported. In this study we focused on time-course changes in oxidative stress, cellular oxidative damage, and cholesterol metabolism. For these evaluations, we analysed redox status of plasma coenzyme Q10 (CoQ10), plasma levels of free fatty acids (FFA) and their composition, and plasma ratio of free cholesterol (FC) to cholesterol esters (CE). Eighteen patients (age = 74.8) were hospitalized and diagnosed as sepsis. Blood samples were collected at h0, h6, h24, h48, and h168. Compared to age-matched healthy control (n = 67, age = 75.5), patients with sepsis at baseline showed a significant increased percentage of oxidized CoQ10 (%CoQ10) in total CoQ10 (9.6 vs 41.4) and a significant decreased percentage of polyunsaturated fatty acid (%PUFA) in total FFA (23.2 vs 16.0), indicating an increased oxidative stress and cellular oxidative damage. Interestingly, percentage of palmitoleic and oleic acid compositions significantly increased (4.2 vs 5.3 and 33.6 vs 40.9). Although plasma FC level was remained unchanged (1.26 vs 1.05 mM), a significant decrease in CE level was observed (3.47 vs 2.04 mM). Therefore, the ratio of FC/CE increased significantly (0.36 vs 0.61), suggesting an impairment of cholesterol esterification catalysed by lecithin-cholesterol acyltransferase and liver function. Although time dependent decreases in FFA level, %16:1, and %18:1 were observed in some patients, %CoQ10 and FC/CE ratio remained approximately unchanged. Therefore, amelioration of these two markers should be an important target of treatment.

B9: Study on supplementation of free or esterified lutein related to the same effect on serum and macular pigment density in healthy Japanese individuals

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Purpose: To examine whether either free or esterified lutein supplementation after 3 months affects MPOD and the relative serum lutein response in healthy Japanese individuals.

Methods: Eighteen healthy subjects were randomized, double-blind study to either 10 mg of orally administered free lutein or lutein ester daily for up to 3 months. After that supplementation was stopped and check was carried out in the 6th month. Macular pigment optical density (MPOD) levels were measured by resonance Raman spectroscopy at baseline and 3 and 6 months after the start of supplementation. Serum lutein in fasting was obtained at baseline and after 3 and 6 months after the start of supplementation. Wilcoxon signed-rank test was used in the study.

Results: In free lutein group, the MPOD levels increased 41% at 3 months after supplementation compared with baseline. In ester lutein group, the MPOD levels increased 12% at same period. However, there was no statistical differences.

In serum, there is no statistically significant difference among both groups at baseline. A significant difference in serum lutein is seen in an ester group 3 months later.

The MPOD levels increased at 3 and 6 months after supplementation compared with baseline. But there was no statistical differences between at 3 and 6 months. Although the volunteers did not take lutein from 3 month to 6 month, the MPOD levels were going up statistically.

Conclusion: In normal healthy Japanese individuals, free and esterified lutein supplementation increased MPOD levels and serum lutein 3 months later.

B10: Effects of morinda citrifolia leave extracts on cardiovascular profile in ovariectomized rats fed thermoxidized diet

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Cardiovascular diseases is one of the major causes of morbidity and mortality in developed countries over the last several decades. The major risk factors of cardiovascular disease are hyperlipidemia, hypertension, and diabetes. The underlying pathology of this atheromatous vascular disease is known to be mediated by inflammatory condition resulting in denudation of the lining of the blood vessels leading to atherosclerosis. Atherosclerosis is known to be abrogated by simvastatin (ST). The present study aimed to investigate the influence of Morinda citrifolia (MC) leaf extract, on blood pressure(BP), lipid profile(LP) and C-reactive protein(CRP) levels in the serum of rats fed with heated palm oil, the thermoxidized diet. Thirty two female *Sprague-Dawley* rats (200-280 g) were equally assigned into four groups as follows: Sham Control-group with normal rat chow; Ovariectomized(OVX)-group fed with normal rat chow mixed with thermoxidized diet; Ovariectomized(OVX+MC)-group and Ovariectomized(OVX+ST)-group fed with similar diet as OVX group but supplemented with MC and, simvastatin(ST) respectively. The treatment duration continued for four months. Thereafter, the rats were sacrificed and serum was sent for biochemical analyses. The body weights of the OVX, OVX-MC and OVX-ST groups were not significantly different from one another but were significantly raised compared to the sham control group. The systolic BP of the OVX group was significantly raised, while the diastolic BP did not differ significantly from the rest of the groups. The only significant biochemical parameter seen was the significant decrease in the high-density lipoprotein (HDL) and a significant increase in the TC/HDL ratio of the OVX group compared to the rest of the groups. There were no significant change in the total cholesterol (TC), low-density lipoprotein (LDL) and CRP values in all the groups of rats. In conclusion, MC supplementation at this dose and this duration improved the systolic BP and lipid profile comparable to ST, but not the CRP.

B11: Investigating the peroxidase reaction mechanism of human cytochrome c

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Cytochrome *c* is essential for ATP production and has a role in apoptosis. Cytochrome *c* has peroxidase activity which is believed to help trigger apoptosis. However, cytochrome *c* has a different active site compared to other haem peroxidases and the reaction mechanism is unclear. In 2008, we reported the first naturally occurring mutation in cytochrome *c* (G41S) in a NZ family with mild autosomal dominant thrombocytopenia. Both the G41S mutation and recombinant G41A and G41T mutations increase the peroxidase activity of cytochrome *c*. We hypothesise that by studying these proteins we will define the chemistry of the peroxidase reaction and the mechanism by which mutation of residue 41 increases activity.

Cytochrome *c* catalyses H_2O_2-dependent oxidation of various substrates. To gain insights into the peroxidase mechanism, we determined the effect of H_2O_2 on cytochrome *c*. H_2O_2 causes degradation of cytochrome *c* as monitored by decrease in Soret band absorbance (410 nm) and iron release. The rate of loss of the soret band and iron was highest for the G41T variant (G41T > G41S > G41A > WT). To investigate the mechanism of haem loss we undertook LC- MS/MS analysis. This showed oxidation of the axial coordination ligand, Met80. H_2O_2-induced loss of axial coordination was confirmed by monitoring the 695 nm absorbance band, which depicts coordination between Met80 and Fe(III). Interestingly the presence of a small molecule substrate protected the protein from H_2O_2-induced damage. Taken together these results suggest that a cascade of events takes place in the vicinity of the haem in response to H_2O_2, and that mutation of cytochrome *c* at residue 41 increases accessibility of the haem to H_2O_2. Next, we will use Mössbauer spectroscopy to investigate the iron oxidation state, and determine whether the same changes occur in the presence of the physiological substrate cardiolipin.

B12: Neutrophil extracellular trap formation is elicited in response to cold physical plasma

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Upon wounding, neutrophils are the first line of defense against invading microorganisms. Their toxic arsenal of molecular weapons is, however, also linked to chronic inflammation such as occurs in non-healing wounds. These wounds often fail multiple treatments and are frequently colonized with multi-resistant pathogens. Recent studies suggest that exposure of chronic wounds to cold physical plasma – a novel treatment option in skin disease – may accelerate healing. Cold physical plasma is an ionized gas with a multitude of characteristics including the presence of hydrogen peroxide and other reactive oxygen and nitrogen species. Plasma exhibits strong antimicrobial effects but it is hypothesized that it also stimulates wound-resident skin and immune cells. Currently, it is unknown how plasma modulates neutrophil behavior. We therefore studied how plasma treatment affects the viability and functions of peripheral blood neutrophils. Plasma-treatment resulted in the oxidation of intracellular fluorescent redox probes but the viability, metabolic activity, and oxidative burst efficacy of the neutrophils were only marginally affected. Killing of phagocytosed *Pseudomonas aeruginosa* and *Staphylococcus aureus* was unaffected. Intriguingly, we found that plasma induced profound extracellular trap formation. This was inhibited by the presence of catalase during plasma treatment but was not replicated by adding an equivalent concentration of hydrogen peroxide. Concentrations of IL-8 were also significantly increased in supernatants of plasma-treated neutrophils. Both, NETs and IL-8 release may aid antimicrobial activity and spur inflammation at the wound site.

B13: Glutathionylation of the active site cysteines of peroxiredoxin 2 and recycling by glutaredoxin

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2-Cys peroxiredoxins (Prxs) are thiol proteins that react extremely rapidly with peroxides and act as antioxidants and regulators of redox signaling. The disulfides of Prxs are recycled by thioredoxin/ thioredoxin reductase. Although reactions of Prx2 with other thiols are known, their physiologically relevance is not established.

We investigated whether Prx2 forms mixed disulfides with GSH, and whether GSH plus glutaredoxin 1 (Grx1) provide an alternative mechanism for Prx2 recycling. Prx2 became glutathionylated on its active site Cys residues when the disulfide form was incubated with GSH, and when the reduced protein was treated with H_2O_2 in the presence of GSH. The latter reaction occurred via the sulfenic acid, which reacted with GSH with a rate constant of 500 M^{-1} s^{-1}. This rate constant was determined by using resolving cysteine mutant Prx2 C172S. This is fast enough for physiological concentrations of GSH to inhibit Prx disulfide formation and to protect against further oxidation (hyperoxidation) to the sulfinic acid. Mono- and di- glutathionylation was observed and the peroxidatic and resolving Cys residues were both modified. Glutathionylation was reversed by Grx1, and GSH/Grx1 supported peroxidase activity. Glutathionylated Prx2 was detected in erythrocytes from Grx1 knockout mice when they were challenged with H_2O_2.

Our results suggest that glutathionylated Prxs could be involved in redox signaling and support the concept that Prxs transmit signals via a mixed disulfide relay. GSH/Grx1 provide an alternative mechanism to thioredoxin and thioredoxin reductase for Prx2 recycling.

B14: Elevated seminal plasma myeloperoxidase is associated with a decreased sperm concentration in healthy young men

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Inflammatory leukocytes, such as neutrophils, are a major source of oxidative stress in semen. Upon activation these phagocytic cells generate superoxide and hydrogen peroxide as part of their oxidative burst. In addition, neutrophils contain large amounts of the heme enzyme myeloperoxidase (MPO) with which they convert hydrogen peroxide to hypochlorous acid and other highly reactive oxidants. Neutrophil and macrophage oxidants have the potential to damage sperm and adversely affect sperm functions vital for achieving fertility, such as motility. They may also damage sperm DNA. We carried out a pilot study in which we measured sperm fertility parameters in 13 healthy young men, on three occasions over a three month period and correlated these with myeloperoxidase levels in semen. Because vitamin C is a potent scavenger of reactive oxygen species and has other anti-inflammatory activities, participants were supplemented with 250 mg/d vitamin C after baseline measurements were taken. Semen parameters (i.e. volume, concentration, total count, viability and morphology) were assessed using WHO guidelines, sperm motility was assessed by computer-aided sperm analysis (CASA) and sperm DNA damage was assessed using the sperm chromatin structure assay (SCSA). Seminal vitamin C was assessed by high-performance liquid chromatography (HPLC) and myeloperoxidase protein levels and activity were determined using enzyme-linked immunosorbent assay (ELISA).

Eight of the 12 participants had high levels of myeloperoxidase in their seminal plasma (15-250 ng/ml). Strong inverse correlations were observed between seminal plasma myeloperoxidase levels and sperm counts ($r = -0.69$, $P < 0.0001$) and also percentage of rapidly motile sperm ($r = -0.44$, $P = 0.021$) over the duration of the study. No effect of vitamin C supplementation was observed on any of the parameters measured. A larger study is required to confirm the observed correlations between seminal myeloperoxidase levels and decreased sperm quality.

B15: Myeloperoxidase-derived oxidants promote necrotic cardiac myocyte death via sustained calcium accumulation and mitochondrial dysfunction

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It is well established that calcium (Ca^{2+}) overload and disruption to mitochondrial respiration are the two major contributing factors resulting in cardiac muscle damage following ischaemia reperfusion (I/R) injury. Furthermore, it is known that neutrophils and macrophages infiltrate heart tissue following I/R injury, resulting in the longer-term detrimental remodelling of heart tissue. During this inflammatory response these activated phagocytes release the haem enzyme myeloperoxidase (MPO), resulting in the production of hypohalous acids, primarily the oxidants hypochlorous acid (HOCl) and hypothiocyanous acid (HOSCN). Little is known, however, of the specific role these MPO-derived oxidants play in cardiac myocyte damage, dysfunction and death following I/R injury. In this study, exposure of cardiac myocyte-like H9c2 cells to (patho)-physiological levels of the oxidants promoted an immediate dose-dependent increase in cytosolic Ca^{2+} accumulation that was coupled with a sustained loss of mitochondrial inner trans-membrane potential ($\Delta\Psi_m$) and depletion of the cellular thiol pool, culminating in an increase in necrotic cell death at the higher oxidant doses tested. Collectively, these results indicate a potential role for MPO-derived oxidants in mediating sustained cardiac dysfunction following myocardial I/R injury.

B16: Tumour redox stress triggers a rapid mechanism of intracellular multidrug resistance *via* lysosomal P-glycoprotein

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Introduction: The most characterised mechanism of cancer cell resistance is drug efflux through plasma membrane transporters (Eckford et al., 2009). One such pump, P-glycoprotein (Pgp), also serves an intracellular resistance role (Yamagishi et al., 2013). In this case, Pgp bound to lysosomal membranes transports drugs into the acidic lumen, which acts as a "safe house" to prevent chemotherapeutics, such as Doxorubicin (DOX; Fig. 4.1A), reaching their intracellular target.

We examined how stress present within the tumour microenvironment, namely variation in the availability of glucose, regulated the formation of lysosomes *via* induction of the endocytosis pathway, and consequently how this increased lysosomal DOX trapping, resulting in enhanced cancer cell resistance.

Interestingly, in contrast to the "safe house" trapping of DOX, a class of novel thiosemicarbazones have been shown to "hijack" the lysosomal Pgp pump to increase drug accumulation in this organelle, resulting in enhanced cytotoxicity (Jansson et al., 2015). These agents potently react with copper to generate reactive oxygen species (ROS) that, in turn, cause lysosomal membrane permeabilisation (LMP) and apoptosis.

Results: These studies showed that glucose variation-induced stress stimulate endocytosis, thereby redistributing plasma membrane Pgp to the lysosomal membrane. This lysosomal Pgp was confirmed to be actively transporting DOX into the lysosome where it becomes trapped. Consequently, Pgp-expressing cells became more resistant to DOX treatment. In contrast, the thiosemicarbazones became more toxic to multidrug resistant (MDR) cells in the presence of more lysosomes as they were able to cause more LMP, which is necessary for their induction of apoptotic cell death.

Conclusion: These studies highlight the rapid intracellular MDR response of tumour cells to glucose variation-induced stress. This intracellular Pgp-resistance mechanism was utilised by agents that can "hijack" Pgp in the lysosomal compartment to increase lysosomal damage and overcome MDR.

B17: Assessing inhibition of macrophage migration inhibitory factor by isothiocyanates

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Isothiocyanates are a class of phytochemicals that occur in cruciferous vegetables, and have been shown to exhibit anti-inflammatory and anti-cancer activity in both cell and animal models. Macrophage migration inhibitory factor (MIF) was discovered as a major target of isothiocyanates. MIF is a highly conserved pro-inflammatory cytokine that has been associated with several human diseases including sepsis, cardiovascular disease and cancer. MIF has an unusually reactive N-terminal proline that bestows the protein with tautomerase activity. Isothiocyanates covalently modify this proline and inhibit MIF activity. In this study we have used a library of novel isothiocyanates to investigate their ability to inhibit the tautomerase activity of MIF, both with recombinant human MIF and in cell culture. IC_{50} values as low as 0.25 µM were observed in cells. In cellular models of liver fibrosis, we have shown that isothiocyanates also inhibit the biological activity of MIF. There was no correlation between MIF inhibition and isothiocyanate cytotoxicity, suggesting independent pathways. Finally, we have shown that the reactive N-terminal proline makes MIF susceptible to other biological electrophiles, including epicatechins. This provides a novel mechanism to explain the anti-inflammatory activities of these dietary phytochemicals.

B18: Assessment of myeloperoxidase activity in arterial tissue of mouse models of vascular inflammation and atherosclerosis

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There is compelling evidence linking myeloperoxidase (MPO)-mediated oxidation to the augmentation of arterial inflammation and vascular dysfunction in atherosclerosis. Currently, 3-chlorotyrosine (3-Cl-Tyr) is considered the gold standard biomarker to assess MPO activity *in vivo*. However, the utility of this biomarker is limited by the low reaction rate of tyrosine residues with HOCl (~40 $M^{-1}s^{-1}$) and the fact that 3-Cl-Tyr is prone to further metabolism. Previously, we reported that MPO activity can be assessed *in vitro* and *in vivo* with high specificity and greater sensitivity compared with 3-Cl-Tyr by measuring the MPO/H_2O_2/Cl^-- and HOCl-mediated conversion of hydroethidine (HE) to 2-chloroethidium (2-Cl-E^+) by LC/MS/MS (*J Biol Chem* 2014;289:5580). Here we describe the utility of HE to 2-Cl-E^+ conversion to measure MPO activity in mouse arteries *ex vivo*. Using models of vascular inflammation and atherosclerosis plaque rupture in apolipoprotein E-deficient mice, 2-Cl-E^+ was measured in arterial homogenates following *ex vivo* treatment with HE and glucose/glucose oxidase for 30 minutes at 37°C. In the vascular inflammation models, 2-Cl-E^+ was detected readily in inflamed arteries whereas it was absent in sham-treated animals. In the plaque rupture model, 2-Cl-E^+ was detected in arterial segment with unstable plaques and in atherosclerotic lesions at the aortic sinus, whereas 2-Cl-E^+ was not detected in arteries free of lesions. Using these models we investigated the effect of the MPO-inhibitor, AZM198 (a 2-thioxanthine), on MPO-mediated conversion of HE to 2-Cl-E^+ in arteries. We observed *ex vivo* formation of 2-Cl-E^+ in inflamed arteries to be significantly lower in drug-treated than control mice. Together with our earlier studies, these findings demonstrate that *ex vivo* conversion of HE to 2-Cl-E^+ offers a specific and sensitive assay to measure MPO activity in arteries of mouse models of atherosclerosis inflammation-induced endothelial dysfunction.

B19: Prosaposin regulates mitochondrial concentration of coenzyme Q10 and the rates of electron transfer

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Coenzyme Q10 (CoQ10) is a key component of the mitochondrial electron transfer chain and an important antioxidant. It is interesting to know how CoQ10 is delivered from one membrane to the other, especially to the mitochondrial inner membrane where mitochondrial electron transport system exists. We have found that glycoprotein saposin B (SapB) and its precursor prosaposin (Psap) bind CoQ10 in human cells. To elucidate the physiological role of this complex, we made stable transfectants (Tf) of HepG2 that express wild-type human Psap. A western blotting analysis confirmed the overexpression of prosaposin in the HepG2 cells. Concomitantly CoQ10 contents increased in Tf cells. CoQ10 content in mitochondria also increased in Tf. Since electron transport system exists in mitochondria, rate of oxygen consumption in Tf was analyzed. Rate of oxygen consumption in Tf was greater than that in parent. Rate of electron transfer from complex I to complex III increased in Tf. Rate of electron transfer from complex II to complex III also increased in Tf. Interestingly, mitochondrial percentage of oxidized CoQ10 in total CoQ10 decreased in Psap Tf cells, suggesting that CoQ10 redox balance in mitochondria is modulated by Psap overexpression. These results imply that CoQ10 binding protein prosaposin regulates mitochondrial concentration of coenzyme Q10 and the rates of electron transfer.

B20: Detection of uric acid adducts on peptides and proteins

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Uric acid is the major end-product of purine metabolism in humans and can be pro-oxidant or antioxidant under different circumstances. Upon oxidation, uric acid forms allantoin, amongst other species, and this is the most detectable uric acid oxidation product *in vivo*. We have shown, using liquid chromatography-mass spectrometry, elevated allantoin levels in biological fluids in patients with inflammatory conditions, including cardiovascular disease, gout, and rheumatoid arthritis. However, allantoin is rapidly excreted by the body so it is limited to being a biomarker of acute inflammation.

We aimed to identify a product of uric acid oxidation that has a longer half-life than allantoin and is a better biomarker of oxidative stress. Uric acid is oxidized to several electrophiles that could react with nucleophiles on proteins. Therefore, we investigated uric acid adduct formation on peptides and proteins upon oxidation by the neutrophil enzyme myeloperoxidase and hydrogen peroxide. In peptides, we detected M+140 adducts and some M+166 adducts. Lysine residues and N-terminal amines were the adduct sites. In proteins, we found multiple urate adducts formed on ubiquitin and other small proteins. Upon tryptic digestion, we detected these adducts on the lysine residues of the proteins. Currently, we are determining the structure of the M+140 adducts and whether they are present on plasma proteins in patients with inflammatory diseases. The addition of urate oxidation products onto proteins could potentially be an ideal barometer of oxidative stress and may also affect protein functionality.

B21: Inhibition of the pro-atherogenic effects of Serum Amyloid A (SAA)-induced endothelial cell dysfunction by pharmacological blockade of NF-kB

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The acute phase protein serum amyloid A (SAA) is a biomarker of inflammation. Elevated circulating SAA in chronic inflammatory disorders such as diabetes mellitus is associated with endothelial dysfunction and the earliest stages of atherosclerosis. Binding of SAA to cell receptors leads to increased production of mRNA encoding tumor necrosis factor (TNF), tissue factor (TF) and cytokines such as interleukin-6 (IL-6); the proteins produced by these genes display pro-inflammatory and pro-thrombotic activities that may be involved in promoting endothelial dysfunction. Pharmaceutical blockade of receptors for SAA, either alone or in combination, only partially inhibits SAA-mediated endothelial dysfunction. Activation of the transcription factor NFκB appears to be a central canonical pathway for SAA-mediated endothelial cell dysfunction. Targeting NFκB, using the specific inhibitor BAY11-7082, may be more effective at inhibiting SAA-mediated endothelial dysfunction than a pharmaceutical cocktail that targets its receptors. Human carotid artery endothelial (HCtAE) cells were cultured to confluence under normoglycaemic (5mM glucose) or hyperglycaemic conditions (25mM glucose) conditions and pre-incubated (1.5 h; 37°C) with 10μM BAY11-7082 or vehicle (control) followed by 10μg/mL SAA (4.5 h; 37°C). Notably, TF and TNF gene expression increased markedly in HCtAE cells after SAA stimulation whereas HCtAE cells pre-treated with BAY11-7082 (with SAA) showed TF and TNF mRNA levels similar to that determined in control cells (minus SAA). In the absence of BAY11-7082, both intracellular TNF and IL-6 protein increased following incubation with SAA as determined by immunofluorescence microscopy. This SAA activity was inhibited by BAY11-7082. ELISA analysis of HCtAE cell pellets and overlying supernatant confirmed the immunofluorescence data with SAA activity inhibited by BAY11-7082; IL-6 showed a greater sensitivity to the drug than TNF. Together these data suggest that inhibition of NFκB activation may protect the endothelium from the action of SAA by inhibiting pro-inflammatory and pro-thrombotic pathways.

B22: Detection of oxidation products of edaravone at *f*mol levels by LC/MS/MS

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We have demonstrated that a free radical scavenger drug, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) produces reactive oxygen species-specific oxidation products: 4-oxoedaravone and its hydrolysed product, 2-oxo-3-(phenylhydrazono)-butanioc acid (OPB) by peroxyl radicals; 4-NO-edaravone and 4-NO_2-edaravone by peroxynitrite; 4-Cl-edaravone by hypochlorite. Therefore, these products can be a good marker of each ROS formation in vivo. In this study, HPLC equipped with triple-quadrupole mass spectrometry (LC/MS/MS) was performed to analyse these products. Previous LC/MS/MS analysis, OPB, 4-NO-adduct, 4-NO_2-adduct or 4-Cl- adduct was synthesized by edaravone oxidation induced by AMVN, peroxynitrite, and sodium hypochlorite respectively. Each compound was isolated and purified by reverse phase HPLC. When OPB, 4-NO-adduct, 4-NO_2-adduct, or 4-Cl-adduct was analysed by LC/MS/MS, precursor anion corresponding to each compound was detected at Q1 MS. Its m/z value was -205, -202,-218, and -207 respectively. Furthermore, each specific fragment anion was also detected at Q3 MS, and its m/z value was -92, -42, -46, and -35 respectively. Using these fragment anions, high-sensitive analysis was performed. As the results, every product was detectable at least *f*mol level. We are currently applying this method to biological samples.

B23: Biochemistry of redox stress responses: identification of disulfide-linked signalling intermediates

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Hydrogen peroxide is considered the major signalling molecule in redox signalling. Previous work from our lab has shown that peroxiredoxins can transmit signals from hydrogen peroxide to downstream signalling partners via the formation of transient inter-molecular disulfide bonds. One of these important downstream signalling molecules is Mitogen Activated Protein Kinase Kinase Kinase 5 (MAP3K5). MAP3K5 has been widely reported to form a complex with its inhibitor, Thioredoxin 1 (Trx1), and a larger multi-protein complex when under redox stress. Using recombinant Peroxiredoxin 1 (Prx1), MAP3K5 and Trx1 as a model system, we seek to clarify the precise mechanism of this signalling axis, and investigate how widespread this mechanism is. To date, we have successfully expressed and purified a variety of recombinant human MAP3K5 constructs in *Escherichia coli*. We have also established that the N-terminal regulatory region of MAP3K5 can form disulfide-linked homodimers as well as disulfide-linked homo-oligomers. These species are all reducible by recombinant human Trx1. Additionally, we have successfully utilised a kinetic trap-variant of Trx1 to isolate a disulfide-linked MAP3K5-Trx1 species. Using a variety of biophysical and mass spectrometric techniques, we aim to identify which cysteine residues in MAP3K5 are needed for this interaction and thus infer how hydrogen peroxide and peroxiredoxins can act on such substrates to transmit a redox signal.

B24: Analysis of nitrated proteins induced in isolated beef heart mitochondria by peroxynitrite by means of immunochemistry and mass spectrometry

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Mitochondria are both the source and target of free radicals. They generate nitric oxide and superoxide which vigorously interact to form the nitrating agent peroxynitrite that leads to the formation of nitrotyrosine in affected proteins. The content of nitrated mitochondrial proteins is expected to increase at several pathologies accompanied by free radical generation. To model this situation we incubated isolated beef heart mitochondria with peroxynitrite in vitro. The quantitative assay of nitrated proteins was performed using ELISA with monoclonal antibodies against protein nitrotyrosine. The concentration of nitrotyrosine in the soluble proteins was about four fold higher than in the membrane ones. The same antibodies were used to find nitrated proteins employing chemiluminescence detection on blots after 2D electrophoresis. The positively labeled proteins were traced on the gel and the protein spots were excised from the Coomassie-stained gels, and then processed to obtain tryptic digests that were concentrated and analyzed by nano-HPLC apparatus coupled to a quadrupole – time of flight mass spectrometer by nanoelectrosprayer. Mass spectra corresponding to each signal from the total ion current chromatogram were averaged, enabling an accurate molecular mass determination. Data were processed using ProteinScape software. Proteins were identified bycorrelating tandem mass spectra to the IPI and SwissProt databases, using the MASCOTsearching engine. We have chosen 12 spots that corresponded to positively labeled proteins and 9 spots that did not label with antibodies. Among the positive group both soluble and membrane-bound proteins contained nitrotyrosine. Nitrotyrosine was confirmed with the highest score in ATP synthase subunit beta and in serum albumin. All the 9 non labeled proteins contained tyrosine localized in similar 3D structural motifs as in the positively labeled proteins. Though it appears that protein nitration by peroxynitrite is not random, the rules determining which protein will be nitrated are not clear.

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B25: Serum amyloid a receptor blockade and incorporation into high density lipoprotein modulates its pro-inflammatory and pro-thrombotic activities in vascular endothelial cells

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Introduction: Atherosclerosis is characterized by the accumulation of lipids and fibrous materials in the vascular wall and is the pathological basis of coronary artery disease. The acute phase protein Serum Amyloid A (SAA) induces expression of pro-inflammatory and pro-thrombotic mediators such as ICAM-1, VCAM-1, IL-6, IL-8 MCP-1 and tissue factor (TF), which act in concert to induce endothelial dysfunction-a precursor to atherosclerosis.

Aims: To inhibit SAA stimulation of human carotid artery endothelial cells (HCtAEC) using pharmacological blockade of known SAA receptors and compare these pharmacological approaches with added high-density lipoprotein (HDL).

Methods: HCtAEC were treated with antagonist to SAA receptors formyl peptide receptor-like-1 (FPRL1), receptor for advanced glycation-endproducts (RAGE) and toll-like receptor-2/4 (TLR2/4) before SAA stimulation and the activity of these inhibitors was compared to added HDL (250 μg/mL), which is known to mitigate SAA-stimulated pro-inflammatory and pro-thrombotic responses on HCtAEC.

Results: Exposure of HCtAEC to SAA increased the gene expression of TF, NFκβ and TNF ($p<0.001$) and protein levels of TF and VEGF ($p<0.001$) in HCTAEC, which was abrogated to different extents by pharmacological inhibitors WRW4 (antagonist to FPRL1), endogenous secretory RAGE (RAGE) and OxPapC (TLR2/4) either alone or in combination. By contrast, HDL consistently showed the highest efficacy ($p<0.001$) often abrogating SAA-induced responses to baseline levels, when compared to the pharmacological inhibitors.

Discussion: Sequestering of SAA by HDL confers complete protection against SAA-mediated atherogenic changes to the endothelium by preventing FPRL1, RAGE and TLR2/4 receptor activation. Increasing circulating HDL levels may prevent SAA-mediated endothelial dysfunction and ameliorate atherogenesis.

B26: Increased HO-1 activity with no evidence of oxidative stress in the early pathogenesis of alzheimer's disease

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Introduction: Alzheimer's disease (AD) is characterised by the presence of plaques, tangles and neuronal loss. At autopsy, the superior temporal gyrus (STG) of AD cases contains plaques, but few tangles and moderate neuronal loss. Therefore, the STG may represent an early-stage in AD pathogenesis or possibly be an area in the AD brain resistant to disease progression. We previously reported evidence suggestive of accumulating H_2O_2 in our AD cohort; heme oxygenase -1 (HO-1) is induced by H_2O_2 and may play a role in neuronal survival.

Aims: To evaluate gene and protein expression/activity of heme oxygenase 1 and associated proteins, and damage in STG tissues from age, gender and APOE ε4-matched AD cases (n=20) *vs* controls (n=20).

Method: Gene regulation was assessed with Quantitative-PCR and related to protein levels (ELISAs and Western Blotting) and corresponding activity (where appropriate) in STG homogenates. Oxidative damage was judged by 4-hydroxynonenal (4-HNE) immunoreactivity, whereas activity of caspase 3/7 and the mammalian target of rapamycin **(**mTOR**)** were assessed using commercial kits.

Results: Nrf-2 and HO-1 mRNA increased ($p<0.05$) in AD *vs* controls; by contrast no difference in constitutive HO-2 expression was observed. Total HO activity increased 2.8-fold in the AD STG ($p<0.05$; as judged using an ex vivo biochemical assay) and correlated with tangle-density ($p<0.04$). Protein expression of p-ERK1/2 and Nrf-2 was significantly increased in the AD cohort ($p<0.05$). A significant decrease in the anti-apoptotic signaling molecule *p*-Akt ($p<0.05$) was coincident with an increase in pro-apoptotic caspase-3/7-activity increased ($p<0.05$) in the AD STG. No evidence of oxidative damage was detected, as revealed by unchanged 4-HNE levels using immunohistochemistry. Similarly, IDO1/2 and mTOR activity was unchanged.

Discussion: Decreased cell viability was observed in the AD STG. The lack of change in lipid peroxidation judged by 4-HNE levels and inflammatory levels by IDO activity suggests possible resistance of the STG to further AD pathology; possibly due to increased HO-1 activity in response to accumulating H_2O_2 via the p-ERK signaling pathway. These data suggest that HO inducers may be a potential therapy for early stage AD.

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