ENOR

European Network on Oxysterols Research

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Oxysterols and Related Sterols in Chemistry, Biology & Medicine

2nd ENOR Symposium

Centre des Sciences du Goût et de l'Alimentation

20-21 September, 2012

Dijon, France

European Network on Oxysterols Research

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Dear participants, welcome to Dijon !

The ENOR meeting series was started with successful by our colleagues successively from Munich in 2010 and from Roma in 2011. As defined in 2010 the main goals of the ENOR meeting are :

- to provide an international forum for researchers to present their results of ongoing research (oral presentations, posters),
- to bring European oxysterols researchers together,
- to stimulate discussions and possible collaborations.

It is a great pleasure to receive you and your co-workers in Dijon at the University of Burgundy for this 2nd European Network on Oxysterols Research symposium.

Organization Committee

Luigi Iuliano,
Sapienza University of Rome, Italy

Gérard Lizard,
University of Burgundy / INSERM, Dijon - France

Athias Anne
CHU le Bocage – Dijon - France

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INRA Dijon -France

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University of Burgundy Dijon – France

Pais-de-Barros Jean-Paul
University of Burgundy Dijon - France
France

Anne Vejux,
University of Burgundy, Dijon – France

Index

Posters

Two poster awards will be attributed (1st price: 300 €, 2nd price: 100 €). All participants will design the 2 winners.

The communication will be published in a official issue of "Biochemical Pharmacology"

Program

September 20th, 2012

9:00-9:10 **Opening of the Symposium:** Luigi IULIANO / Gérard LIZARD

9:10-9:30 **Welcome**: Deans Faculty of Sciences, Medicine and Pharmacy, INRA director Dijon, INSERM director Dijon

9:30-10:30 **Opening lecture**
William J GRIFFITHS, Swansea University, UK
"Methods for oxysterol analysis: past, present and future"

10:30-11:00 **Coffee break**

Session 1 ***Analytical clues in oxysterol and phytosterol research (chairman: WJ GRIFFITHS)***

11:00-11:20 Silke MATYSIK, University Hospital of Regensburg, Germany
"Distribution of oxysterols, plant sterols and cholesterol precursors in lipoprotein fractions"

11:20-11:40 Steven Ray WILSON, University of Oslo, Norway
"Studying the role of oxysterols in hedgehog signaling using highly automated AFFL-SPE-LC-MS methodology"

11:40-12:00 Sabine BAUMGARTNER, Maastricht University, The Netherlands
"Oxyphytosterol formation in humans: how to identify high versus low oxidizers"

12:00-13:00 **Poster session** (Chairman: H. GUILLOU) – (5 minutes oral presentation per poster)

13:00-14:00 **Lunch (cafeteria Montmuzard)**

Session 2 ***Oxysterol receptors and enzymes (chairman: JM LOBACCARO)***

14:00-14:20 Charbel MASSAD, Université Paris-Descartes, France
"Opposite effect of LXR on myelination process in the central and peripheral nervous systems and interplay with Wnt pathway"

14:20-14:40 Marion WEBER-BOYAT, Minerva Foundation Institute for Medical Research, Helsinki, Finland
"Visualization of ORP-VAP complexes at putative membrane contact sites: novel clues to oxysterol-binding protein function"

14:40-15:00 Lionel BRETILLON, INRA - CNRS, UMR6265 Centre des Sciences du Goût et de l'Alimentation, Dijon, France
"When experimental data meet the clinics: evidence that CYP46A1 is specific to retinal neurons and related pathologies"

15:00-15:20 Ulf DICZFALUSY, Karolinska Institutet, Stockholm, Sweden
"4β-hydroxycholesterol as a marker for CYP3A4/5 induction"

15:20-15:40 Richard LATHE, State University of Pushchino, Russia
"Steroid symmetry"

15:40-16:00 Ingemar BJORKHEM, Karolinska Institutet, Stockholm, Sweden & Hadassah Hebrew University, Israel
"Is 27-hydroxycholesterol of importance for regulation of cholesterol homeostasis in brain and liver? Lessons from the CYP27A1 transgenic mouse"

16:00-16:20 ***Coffee break***

Session 3 ***Oxysterols and inflammation (chairwoman: NM O'BRIEN)***

16:20:16:40 Andreas W. SAILER, Novartis Institutes for BioMedical Research, Basel, Switzerland
"Oxysterols direct immune cell migration via EBI2"

16:40:17:00 Gabriella LEONARDUZZI, University of Turin, Italy
"Plaque oxysterols induce imbalanced up-regulation of MMP-9 in macrophagic cells contributing to plaque instability"

17:00-17:20 Peter GHAZAL, University of Edinburgh, UK
"Coupling of 25-hydroxycholesterol to the interferon antiviral response"

Session 4 ***Oxysterols and nervous system (chairman: I. BJORKHEM)***

17:20-17:40 Valerio LEONI, IRCCS Neurology Institute 'Carlo Besta', Milan, Italy
"Oxysterols and cholesterol metabolism in neurodegeneration. Evidence for anabolic impairment"

17:40-18:00 Dieter LUTJOHANN, University of Bonn, Germany
"Cerebral and extracerebral cholesterol metabolism and CSF markers of Alzheimer's Disease"

20:00 ***Social Event ; Gala dinner : Cellier de Clairvaux, Dijon, sponsored by the City of Dijon.***

September 21st, 2012

Session 5 ***Biological activities of oxysterols and phytosterols (Chairman: G POLI)***

9:00 - 9:20 Nora M. O'BRIEN, University College Cork, Ireland
"Oxidized derivatives of campesterol and dihydrobrassicasterol: cytotoxic and apoptotic potential in cell culture systems"

9:20 - 9:40 Laia ALEMANY, University of Valencia, Spain
"7-ketostigmasterol protects against 7-ketocholesterol cytotoxicity but induces inflammation to intestinal epithelial cells"

9:40-10:00 Folke SITBON, Swedish University of Agricultural Sciences, Sweden
"Oxysterol metabolism in plants"

10:00-10:40 ***Coffee break***

10:40-11:00 M. Manuel CRUZ SILVA, University of Coimbra, Portugal
"Oxysterol glycosylation: looking for a better cytotoxic profile"

11:00-11:20 Patrice RAT, Elodie OLIVIER, Université Paris Descartes, France
"Oxysterols induce cell death on human retinal pigment epithelial cells with P2X7 cell death receptor activation"

11:20-11:40 Gérard LIZARD, EA7270 / Université de Bourgogne, Dijon, France
"Absence of correlation between oxysterol accumulation in lipid rafts, Calcium rise and induction of GSK3-dependent apoptosis in 158N murine oligodendrocytes"

11:40-12:00 Ruth ANDREW, University of Edinburgh, UK
"Imaging neutral steroids in tissues by MALDI-FTICR"

12:00-12:45 *General Assembly - Round Table (ENOR present and future ENOR's activities)*

12:45-14:00 ***Lunch*** **(cafeteria Montmuzard)**

Session 6 ***Oxysterols and Cancer (Chairwoman: I VANDENBROUCKE)***

14:00-14:20 Jean-Marc LOBACCARO, Université Blaise Pascal, CNRS UMR 6293, GRed / Inserm UMR 1103, GReD, Centre en Nutrition Humaine d'Auvergne, Clermont-Ferrand/Aubière, France
"Cholesterol and prostate cancer: the dark side of the life"

14:20-14:40 Norbert BAKALARA, ENS de Chimie de Montpellier, France
"7β-hydroxycholesterol-induced energy stress leads to sequential opposing signaling responses and to death of C6 glioblastoma cells"

14:40-15:00 Marc POIROT, UMR 1037 Inserm / University Toulouse III, France
"Importance of the liver-X-receptor-β and cholesterol-5,6-epoxide metabolites in the induction by tamoxifen of triglyceride biosynthesis and cytotoxicity in breast cancer cells"

15:00-15:20 Sérgio P. BYDLOWSKI, University of Sao Paulo, Brazil
"In vitro toxicity of cholesterol, 7-ketocholesterol and Cholesterol-3β,5α,6β-triol in different hematological cancer cell lines"

15:20-15:30 ***Coffee break***

Session 7 ***Oxysterols in cardiovascular and metabolic diseases (Chairman: U. DICZFALUSY)***

15:30-15:50 Luigi IULIANO, Sapienza University of Rome, Italy
"Oxysterols and lipid metabolism in obesity and metabolic syndrome"

15:50-16:10 M. BAPTISSART, Université Blaise Pascal, CNRS UMR 6293, GRed, Clermont-Ferrand/Aubière, France
"Bile acid : a molecular link between liver and testis functions"

16:10-16:30 Gerd SCHMITZ, University Hospital of Regensburg, Germany
"Sterol precursors, oxysterols and steroid hormones in platelets and platelet extracellular vesicles in vascular and metabolic diseases"

16:30-16:50 David MASSON, INSERM 866 / Université de Bourgogne, Dijon, France
"Differential impact of oxysterol receptors LXR alpha and LXR beta on the regulation of cholesterol efflux in primary human macrophages"

16:50-17:10 Jacob VAYA, Migal – Galilee Technology Center and Tel Hai College, Israel
"The mutual effects between the human carotid plaque constituents and the blood components"

17:10-17:30 **Poster awards** and **Closing remarks**

Opening lecture

Methods for Oxysterol Analysis: Past, Present and Future

William J. Griffiths

Swansea University, UK

Oxysterols have traditionally been analysed by GC-MS. Classical methods were devised by Björkhem and Diczfalusy and by Sjövall and Axelson in Stockholm in 1980's and 90's. These methodologies rely on sterol extraction followed by separation of oxysterols from cholesterol and derivatisation with ultimate analysis by GC-MS [1,2]. Quantitation is performed via stable-isotope dilution. These methods represent the gold-standard for oxysterol analysis, and by minor modification are appropriate for bile acid precursors and bile acids themselves.

In more recent times, LC-MS and MS/MS methods, without derivatisation have become popular [3]. These offer the advantage of higher throughput, and the avoidance of derivatisation, but the MS/MS spectra of oxysterols are far less informative than those obtained by EI-MS. In the last decade, we and others have combined the merits of derivatisation with the through-put of LC-MS/MS, but with the penalty of increased labour. Many different derivatisation strategies have been exploited some relying on direct derivatisation of alcohol groups [4] and others on enzymatic conversion of alcohols to ketones followed by chemical derivatisation [5]. In this paper we will endeavour to highlight the salient points of the different analytical methods and offer thoughts on future analytical schemes.

1. Axelson M, Sjövall J. J Steroid Biochem. 1990 Aug 28;36(6):631-40
2. Dzeletovic S, Breuer O, Lund E, Diczfalusy U. Anal Biochem. 1995 Feb 10;225(1):73-80.
3. McDonald JG, Smith DD, Stiles AR, Russell DW. J Lipid Res. 2012 Jul;53(7):1399-409. Epub 2012 Apr 19.
4. Honda A, Yamashita K, Hara T, Ikegami T, Miyazaki T, Shirai M, Xu G, Numazawa M, Matsuzaki Y. J Lipid Res. 2009 Feb;50(2):350-7.
5. Griffiths WJ, Wang Y. Biochim Biophys Acta. 2011 Nov;1811(11):784-99.

Oral communications

Session 1

Analytical clues in oxysterol and phytosterol research

Distribution of oxysterols, plant sterols and cholesterol precursors in lipoprotein fractions

Silke Matysik, Gerd Schmitz

Institute of Clinical Chemistry, University Hospital Regensburg, F.-J.-Strauss-Allee 11, 93053 Regensburg, Germany

Cholesterol precursors and plant sterols have considerable potential as plasma biomarkers in several disorders of sterol metabolism and intestinal sterol absorption. Oxysterols are associated with atherogenesis, neurodegeneration, and inflammation.
A rapid method based on gas chromatography coupled to triple quad mass spectrometry is presented for the simultaneous analysis of these species in human plasma, including 24-, 25- and 27-hydroxycholesterol, 7-ketocholesterol lanosterol, lathosterol, 7-dehydrocholesterol, desmosterol, stigmasterol, sitosterol, and campesterol. The definition of highly sensitive precursor/product ion transitions, especially for coeluting substances, allowed fast GC run times of under 8.5 minutes. Using multiple reactions monitoring (MRM) mode, detection limits in the pg/ml range could be achieved for most compounds. The method was validated for accuracy, precision, and recovery and applied to patient stratification of disorders in cholesterol biosynthesis and/or cholesterol absorption in hypercholesterolemia. The method revealed associations of sterol species with thyroid dysfunction and type 2 diabetes.
Lipoproteins were isolated by salt gradient ultracentrifugation of human serum. Cholesterol precursors and plant sterols are mainly transported in association with HDL and LDL whereas side-chain oxidized cholesterol species have also been identified in lipoprotein free fractions. Application of LDL apheresis as therapy for phytosterolemia is shown in a case report.

Studying the role of oxysterols in hedgehog signaling using highly automated AFFL-SPE-LC-MS methodology

Steven Ray Wilson[1], Hanne Roberg Larsen[1], Anders Grimsmo[1], Elsa Lundanes[1], Stefan Krauss[2], Martin Strand[2]

[1]*Department of Chemistry, University of Oslo, Oslo, Norway*
[2]*Unit for Cell Signaling, SFI-CAST Biomedical Innovation Center, Oslo University Hospital*

The Hedgehog (Hh) pathway is a highly conserved signaling pathway that plays a vital role in embryonic development, and regulates the proliferation, migration and differentiation of cells. Hh is also involved in several human disorders and diseases, and has been implicated in a wide variety of cancers. Hence, Hh has increasingly become a target of anticancer treatments.

A number of oxysterols are activators of Hh, but it is unclear exactly how they influence the pathway. Therefore, precise methodology is required for studying oxysterols in tumors and cells as function of e.g. treatment and knock-out studies. In addition, tumors and cancer cell lines may include stem cell-like sub-populations with e.g. increased resistance to chemotherapy and invasive potential. Such sub-populations are available in limited amounts (e.g. less than 300,000 cells), requiring analysis methodology with high sensitivity and suitability for small samples.

For sensitive and precise analysis of oxysterols in cancer cells, we have designed a highy automated methodology, based on on-line solid phase extraction (SPE) of Girard T-derivatized oxysterols combined with liquid chromatography-mass spectrometry (LC-MS). To avoid extensive sample preparation steps, we have developed an automated self-washing on-line filtration feature (termed AFFL), which allows highly robust, direct injection of Girard T-treated cell lysates. Initial studies with our AFFL-SPE-LC-MS methodology has e.g. revealed correlations between oxysterol levels and cancer cell aggressiveness, as well as correlations between oxysterol levels and treatment with Hh antagonists.

Oxyphytosterol formation in humans; how to identify high vs. low oxidizers

Sabine Baumgartner[1], Ronald P. Mensink[1], Gertjan den Hartog[2], Dieter Lütjohann[3], Aalt Bast[2], Jogchum Plat[1]

[1]Maastricht University, Department of Human Biology, NUTRIM School for Nutrition, Toxicology and Metabolism
[2]Maastricht University, Department of Toxicology, NUTRIM School for Nutrition, Toxicology and Metabolism
[3]University of Bonn, Institute for Clinical Chemistry and Clinical Pharmacology

Consumption of both plant sterol- and stanol-enriched products reduces LDL-cholesterol concentrations. In contrast to plant stanols, plant sterols can result in the formation of oxyphytosterols, which have already been identified in human circulation. Preliminary findings from animal studies suggest that oxyphytosterols may, like oxidation products of cholesterol, be atherogenic. Therefore, the objective was to examine whether increased plant sterol and stanol consumption results in changed fasting serum oxyphytosterol concentrations.
A randomized, double-blind, cross-over study was performed in which 43 healthy subjects consumed for 4 weeks a plant sterol-enriched (3.0 g/d plant sterols), a plant stanol-enriched (3.0 g/d plant stanols), and a control margarine separated by wash-out periods of 4 weeks. Serum oxyphytosterol concentrations were determined in BHT-enriched EDTA-plasma via GC-MS.
Serum LDL-C concentrations were reduced by 0.29 mmol/L (-8.1%; $p<0.001$) after sterol consumption and by 0.26 mmol/L (-7.8%; $p<0.001$) after stanol consumption. Moreover, plant sterol consumption increased serum plant sterols and plant stanol consumption lowered serum plant sterol concentrations. However, plant sterol or plant stanol ester consumption did not change 7α-OH-sitosterol, 7α-OH-campesterol, 7β-OH-sitosterol, 7-keto-sitosterol and 7-keto-campesterol concentrations. On the other hand, plant stanol consumption reduced 7β-OH-campesterol by 0.07 ng/mL (~14%; $p<0.01$) compared with the control condition and by 0.07 ng/mL (~15%; $p<0.01$) compared with sterol consumption.
These results indicate that there was no relation between changes in serum plant sterol and oxyphytosterol concentrations. However, results indicated that subjects seem to have a consistent oxyphytosterol concentration independent from the intervention. Moreover, a clear difference was seen in oxyphytosterol concentration between what we defined as 'low and high oxidizers'. Interestingly, this was also reflected in oxycholesterol and oxLDL concentrations. By determining oxidative and anti-oxidative capacity markers, such as iron/copper status, vitamin E concentrations and TEAC values, we attempt to explain the difference between 'low and high oxidizers' in terms of oxyphytosterol concentrations.

Oral communications

Session 2

Oxysterol receptors and enzymes

Opposite effect of LXR on myelination process in the central and peripheral nervous systems and interplay with Wnt pathway.

Delphine Meffre, Julien Grenier, Joelle Makoukji, GG Shakleford, Michael Schumacher, Charbel Massaad

CNRS UMR 8194, Centre d'Etude de la Sensorimotricité, Université Paris Descartes, 45 Rue des Saints-Pères, 75006 Paris

Oxysterols are reactive molecules generated from the oxidation of cholesterol. Few data are available about their functions in myelination of nervous system. Our aim was to study the influence of oxysterols on myelin gene expression and myelin sheath formation by oligodendrocytes (central nervous system), and by Schwann cells (peripheral nervous system). We show by gas chromatography/mass spectrometry that oligodendrocytes and Schwann cells contain 24(S)-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol, and that they express their biosynthetic enzymes and receptors (Liver X Receptors: LXRα and LXRβ). We demonstrate that oxysterols activate the expression of central myelin genes (PLP and MBP) in oligodendrocytes, but inhibit peripheral myelin genes expression (MPZ, PMP22) in a Schwann cells.

In the CNS, we showed that the activating effects of oxysterols were restricted to the cerebellum and dependant of LXR presence as myelin gene expression was drastically reduced in this structure in LXR-KO mice. By, using organotypic cultures of cerebellum slices, we showed that oxysterols were able to stimulate myelin gene expression after lysolecithin-induced demyelination and enhance remyelination process.

In the PNS, the down-regulation of myelin genes is mediated either by LXRα or LXRβ, depending on the promoter context. Importantly, the knockout of LXR in mice results in thinner myelin sheaths surrounding the axons of the nerves (Makoukji et al, J Neuroci 2011). Oxysterols repress peripheral myelin genes via two mechanisms: by binding of LXRs to myelin gene promoters and by inhibiting the Wnt/beta-catenin pathway that is crucial for the expression of myelin genes (Tawk et al, J Neurosci 2011, Makoukji et al, PNAS 2012).

Altogether our results reveal new mechanisms of action of oxysterols and open new perspectives for the treatment of demyelinating diseases by targeting LXR.

Visualization of ORP-VAP complexes at putative membrane contact sites: Novel clues to oxysterol-binding protein function

Marion Weber-Boyvat, Vesa M. Olkkonen

Minerva Foundation Institute for Medical Research, Helsinki, Finland

Cytoplasmic oxysterol-binding protein/OSBP-related proteins (ORPs) are lipid binding/transfer proteins implicated in a variety of cellular functions: in lipid metabolism/transport, vesicle transport and signaling cascades. They possess a characteristic sterol-binding pocket, which shows different specificities for oxysterols/cholesterol. Oxysterols are formed via cholesterol oxidation or as side products of its biosynthesis. They have signaling functions in lipid metabolism, inflammation, and differentiation processes, and act as intermediates in bile acid and steroid hormone syntheses as well as means of removing excess sterol from cells.

Ten members of the OSBP/ORP family can be anchored to ER membranes either via interactions with type 2 integral ER membrane proteins called VAMP-associated proteins (VAPs) or via a transmembrane segment. In addition, most ORPs carry N-terminal plecktrin homology (PH) domains that bind PIPs and target other, non-ER membranes, suggesting that these proteins function in communication of the ER with other organelles at membrane contacts sites (MCS) with well documented roles in lipid syntheses/transport and calcium fluxes.

We investigated the spatial occurrence and regulation of interaction of OSBP/ORPs with the ER anchored VAMP-associated (VAP-A and B) proteins by employing the Bimolecular Fluorescence Complementation (BiFC) technique. We show that the interactions between the ORP and VAP proteins specifically localize to distinct organelle-ER membrane contact sites. The specificity of the obtained BiFC signal was confirmed by mutating the VAP protein binding site in the ORP proteins, which leads to clearly reduced ORP-VAP interactions in the BiFC technique and in VAP-A pull down experiments. Using previously described sterol-binding deficient ORP mutants we present data suggesting that sterol binding negatively regulates ORP-VAP interactions and thereby membrane contact site formation. Additionally the sterol bound status of ORP proteins can affect the localization of the ORP-VAP BiFC interaction to the organelle (ORP2, ORP9L) or of the organelle itself (OSBP, ORP1L).

When experimental data meet the clinics: evidence that CYP46A1 is specific to retinal neurons and related pathologies

Lionel Bretillon, Cynthia Fourgeux, Lucy Martine, Alain Bron, Catherine Creuzot-Garcher

Centre des Sciences du Goût et de l'Alimentation, UMR1324 INRA-6265 CNRS-Université de Bourgogne, Department of Ophthalmology, Dijon, France

Cholesterol-24S-hydroxylase, also known as CYP46A1, catalyzes the formation of 24S-hydroxycholesterol from cholesterol. In the brain, CYP46A1 is specific to neurons where it accounts for the elimination of the major part of cholesterol. The retina shares the common property with the brain to be composed of neurons. But it has also the specificity to present photosensitive cells, i.e. cones and rods, that are not *sensu stricto* neurons but establish synapses and functional connections with neurons in the one hand and pigmentary cells in the other hand.

Age-related Macular Degeneration (AMD) is the leading cause of visual loss in the Western populations after the age of 65yrs. Glaucoma is the second cause of blindness worldwide, affecting more than 60 million people nowadays. The etiology of AMD involves the impairment of cones, rods and pigmentary cells whereas the death of one type of neurons – retinal ganglion cells – is responsible for visual field loss in glaucoma. Glaucoma is therefore considered as a neurodegenerative disease on the contrary to AMD.

CYP46A1 expression has been localized in neurons and especially in retinal ganglion cells, while low or even no expression was found in photoreceptors and pigmentary cells. It was thus clearly understandable that CYP46A1 would be associated with glaucoma but not with AMD. The analysis of single nucleotide polymorphism in *CYP46A1* gene in populations suffering from retinal pathologies validated this assumption since an association, although of unknown origin, was found with glaucoma but not with AMD.

Experimental glaucoma was induced in the rat. The loss of retinal ganglion cells and reduced CYP46A1 and 24S-hydroxycholesterol amounts were expected in this model. On the contrary, CYP46A1 expression and 24S-hydroxycholesterol levels were maintained at steady states in the retina.

These findings highlight not only the importance of CYP46A1 in the retina but also its neuron-specificity.

4β-Hydroxycholesterol as a marker for CYP3A4/5 induction

Ulf Diczfalusy

Departmenty of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

Cytochrome P450 (CYP) 3A4 is an important drug metabolizing enzyme, involved in the metabolism of more than 50 % of all prescription drugs. There is a great inter-individual variability in enzyme activity, partly due to genetic factors. In addition, many drugs induce the enzyme activity. CYP3A4 converts cholesterol into the oxysterol 4β-hydroxycholesterol. We have suggested that 4β-hydroxycholesterol may be used as an endogenous marker for CYP3A4 activity. In experiments in healthy volunteers treated with the CYP3A4-inducer rifampicin we have compared 4β-hydroxycholesterol to other more established markers such as the urinary 6β-hydroxicortisol to cortisol ratio and midazolam clearance after oral administration. There was a good agreement between the different markers and also with the exogenous marker quinine metabolic ratio.

4β-Hydroxycholesterol was also used to monitor the induction of CYP3A activity in two different cohorts of HIV patients treated with efavirenz. Efavirenz is not as potent as rifampicin as an inducer of CYPA enzymes, but prolonged treatment resulted in highly elevated levels of 4β-hydroxycholesterol.

HIV patients coinfected with tuberculosis and treated with efavirenz and rifampicin were studied in order to the evaluate the combined effect of two different inducers and to monitor changes in CYP3A activity when the rifampicin treatment was ended. The combined treatment increased the activity compared to efavirenz treatment alone and upon completetion of the rifampicin teatment (but continued treatment with efavirenz) the induction dropped by a factor 1.5.

Together, these results show that 4β-hydroxycholesterol is a promising marker for CYP3A activity useful for the estimation of enzyme induction. Potential fields of use include personalized medicine and screening of new drug candidates for induction properties.

Steroid symmetry

Richard Lathe

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Steroids and sterols are largely planar molecules, but substitutions at different positions protrude above or below the backbone. The issue was raised of whether rotationally symmetrical positions are recognized by enzymes that do not discriminate between the different symmetrical forms (Lathe, Steroids 67, 967, 2002). This has now been confirmed; some enzymes do not discriminate between the 11-beta and 7-alpha positions – the latter being the predominant site of spontaneous sterol oxidation and thus an ancestral regulatory modification. We also discuss the finding that the Alzheimer disease-related enzyme, HSD17B10, not only catalyzes oxidoreduction of long-chain molecules that can fold into a steroid-like structure, but also efficiently metabolize both 17-beta and 3-alpha substituted molecules. These findings raise questions not only concerning the extent of the recognition site that is bound by steroid and sterol-metabolizing enzymes, but also as to the identity of the molecules that originally drove the evolution of the binding site.

Is 27-hydroxycholesterol of importance for regulation of cholesterol homeostasis in brain and liver? Lessons from the CYP27A1 transgenic mouse

Ingemar Björkhem[1], Zeina Ali[1], Maria Olin[1], Maura Heverin[1], Eran Leitersdorf[2]

[1]Division of Clinical Chemistry, Department of Laboratory Medicine, Karolinska Institute, Sweden;
[2]Department of Medicine B and Department of Human Genetics, Hadassah Hebrew University Hospital, Israel

The oxysterol 27-hydroxycholesterol (27OH) is an inhibitor of cholesterol synthesis and an activator of LXR when added to cultured cells. The enzyme system responsible for formation of 27OH, CYP27A1, is present in most tissues and 27OH is one of the dominating oxysterols in circulation of mouse and man. The role of 27OH as a physiological regulator under *in vivo* conditions is controversial, however.

In the present work we have utilized a previously described mouse model with overexpressed human CYP27A1. The levels of 27OH are about 6-fold higher than normal in the circulation, 4-fold higher in the liver and about 12 times higher in the brain. With two exceptions (cyp7b1 in females and abca1 in males) all LXR target genes investigated in the liver were unaffected or changed in a direction opposite to that expected for an LXR activation. Cholesterol precursors were increased, suggesting increased cholesterol synthesis. In the brain, the HMG CoA reductase and HMG CoA synthase mRNA levels were slightly increased as a consequence of the overexpression. In accordance with this the levels of some of the cholesterol precursors were also increased. In the brain the LXR target genes were unaffected by the overexpression or slightly changed in a direction opposite to that expected for LXR activation. The level of 24S-hydroxycholesterol, the dominating oxysterol in the brain, was reduced by about 25%.

The effects of the overexpression of CYP27A1 on cholesterol synthesis in brain and liver are likely to be due to consumption of cholesterol with a compensatory increase in synthesis. The results do not favour the hypothesis that 27OH is important as a physiological inhibitor of cholesterol synthesis or activator of LXR under in vivo conditions.

Oral communications

Session 3

Oxysterols and inflammation

Oxysterols direct immune cell migration via EBI2

Andreas W. Sailer[1], Juan Zhang[1], Tangsheng Yi[2], Birgit U. Baumgarten[1], Silvio Roggo[1], Francois Gessier[1], Thomas Suply[1], Jason G. Cyster[2], Klaus Seuwen[1]

[1]Novartis Institutes for BioMedical Research, Basel, Switzerland
[2] Howard Hughes Medical Institute, University of California San Francisco, CA, USA

We and others have recently identified 7α, 25-dihydroxycholesterol (7α, 25-OHC) as a potent and selective agonist of EBI2[1,2] (Epstein-Barr virus induced gene 2). The EBI2 receptor is a G protein-coupled receptor that is required for humoral immune responses and has been linked to autoimmune disease. Functional activation of EBI2 by 7α, 25-OHC and closely related oxysterols was verified by monitoring second messenger readouts and saturable, high affinity radioligand binding. Furthermore we find that 7α, 25-OHC and closely related oxysterols act as chemoattractants for immune cells expressing EBI2 by directing cell migration *in vitro* and *in vivo*. A key enzyme required for the generation of 7α, 25-OHC is cholesterol 25-hydroxylase (Ch25h)[1]. Similar to EBI2 receptor knockout mice, mice deficient in Ch25h fail to position activated B cells within the spleen to the outer follicle and mount a reduced plasma cell response after an immune challenge.
In order to delineate the physiological function of the oxysterol / EBI2 pathway we try to determine the sites of oxysterol production. In addition, we have isolated tool compounds which can be used to address the role of the oxysterol/EBI2 pathway in health and disease.

[1] Hannedouche et al. Nature 475(2011)524
[2] Liu et al. Nature 475(2011)519

Plaque oxysterols induce imbalanced up-regulation of MMP-9 in macrophagic cells contributing to plaque vulnerability

Simona Gargiulo, Paola Gamba, Barbara Sottero, Gabriella Testa, Giuseppe Poli, Gabriella Leonarduzzi

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An imbalance in the matrix metalloproteinases/tissue inhibitors of metalloproteinases (MMPs/TIMPs) contributes to destabilization and eventual rupture of atherosclerotic plaques. Among MMPs expressed in atherosclerostic lesions, MMP-9 has been consistently implicated in the pathophysiology of plaque vulnerability. Oxidative modifications of low density lipoproteins (LDLs), major carriers of cholesterol in human circulation, provide them with several pro-atherogenic properties. Among the different molecules present in oxidized LDLs, cholesterol oxidation products, known as oxysterols, are potentially responsible for their pro-atherogenic effects.

To clarify whether oxysterols might play a role in plaque destabilization, we investigated, in human promonocytic U937 cells, the effect of an oxysterol mixture, of composition similar to that found in advanced human carotid plaques, on expression and activity of MMP-9 and the potential mechanisms involved. A marked increment of MMP-9 gene expression and protein levels, but not of its endogenous inhibitors TIMP-1 and TIMP-2, was observed. Using antioxidants or specific inhibitors or siRNAs, we demonstrated that the oxysterol mixture induces MMP-9 expression through: i) over-production of reactive oxygen species (ROS), likely by NADPH oxidase and mitochondria, via protein kinase C; ii) up-regulation of mitogen-activated protein kinases signaling pathways; iii) up-regulation of activator protein-1 and nuclear factor-κB-DNA binding. Moreover, oxysterols induce the expression of various inflammatory molecules, such as IL-1β, IL-8 and TNF-α, presumably through TLR4 involvement. These inflammatory molecules appear to contribute to plaque destabilization by enhancing MMP-9 expression.

These results suggest that oxysterols, accumulating in advanced atherosclerotic lesions, significantly contribute to plaque vulnerability by promoting MMP-9/TIMP-1 and TIMP-2 imbalance and inflammatory molecules release.

Coupling of 25 hydroxycholesterol to the inteferon antiviral response

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Recent studies suggest that the sterol metabolic network participates in the interferon (IFN) antiviral response. However, the molecular mechanisms linking IFN with the sterol network and the identity of sterol mediators remains unknown. Here we report a role for macrophage synthesis and secretion of 25-hydroxycholesterol (cholest-5-en-3β,25-diol, 25-HC) as an antiviral lipid; directly linked to the IFN-stimulated-gene response through recruitment of Stat1 to the *Ch25h* promoter. Utilizing quantitative profiling of all known naturally occurring oxysterols upon infection or IFN-stimulation we reveal 25-HC as the only physiologically produced oxysterol. We find that 25-HC is a specific, broadly potent inhibitor of viral infection and spread, while other oxysterols, including ligands for LXR and Ebi2 are ineffectual. We further demonstrate using transcriptional regulatory-network analysis, genetic interventions and chromatin immunoprecipitation experiments that Stat1 directly couples *ch25h* regulation to IFN. Our studies underscore a new physiological role for 25-HC as a sterol-lipid effector of an innate immune antiviral pathway.

Oral communications

Session 4

Oxysterols and nervous system

Oxysterols and cholesterol metabolism in neurodegeneration. Evidence for anabolic impairment

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Brain cholesterol is involved in cell membrane structure, signal transduction, neurotransmitter release, synaptogenesis and membrane trafficking. Impairment of brain cholesterol metabolism were described in neurodegenerative diseases, such as Alzheimer (AD), Huntington (HD), Diseases, Multiple Sclerosis (MS), Parkinson Disease (PD) and other rare diseases. Since the blood-brain barrier (BBB) efficiently prevents cholesterol uptake from the circulation into the brain, de novo synthesis is responsible for almost all cholesterol present there. In case of AD altered distribution of membrane cholesterol is associated with higher formation and deposition of amyloid. Excess of cholesterol is converted into 24S-hydrocycholesterol (24OHC) by cholesterol 24-hydroxylase (CYP46A1) expressed in neural cells of the brain 24OHC decrease the formation of amyloid, contrasted by 27-hydroxycholesterol, which brain levels depend by BBB function. In the mature brain neuron down regulate their cholesterol synthesis and rely on delivery of cholesterol from astrocytes by ApoE lipoproteins.
In HD, the mutated huntingtin (mtHtt) interfere with LXR-SREBP pathway resulting in reduced brain cholesterol synthesis which can contribute to the toxicity of mtHtt. Also disturbances of fatty acid metabolism were described. Disturbance of mitochondria and energetic metabolism were found associated to reduced synthesis of lipids.
In Panthotenate kinase 2 (PANK2) deficiency, reduced levels of CoA synthesis were found related to a general impairment in cholesterol and fatty acids synthesis with lower plasma levels of oxysterols (24OHC, 27OHC, 7αOHC).
In several animal models of neurodegenerative diseases reduced cholesterol synthesis was found together with impairment of anabolic metabolism and intermediate metabolism.
A systematic metabolic approach can offer new information in studies of pathogenesis, biomarkers discovering, and therapeutic strategy definition. The combined study of oxysterols and sterols in plasma collected from patients revealed in all neurodegenerative diseases alteration of whole body cholesterol homeostasis, oxidative stress, disturbance of brain cholesterol turnover. Hypercholesterolemia was finally described as a major risk factor for AD and ongoing studies are investigating the possible pathogenetic mechanism

Cerebral and extracerebral cholesterol metabolism and CSF markers of Alzheimer's Disease

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Running title: Cholesterol Metabolism and sAPP

The disturbances of the cholesterol synthesis and metabolism described in Alzheimer's disease (AD) may be both a consequence of the neurodegenerative process and a contributor to the pathogenesis. These putative relationships and their underlying mechanisms are not well understood.
The aim of this study was to evaluate the relationship between the cerebral and extracerebral cholesterol synthesis and metabolism and the AD pathology as reflected by CSF markers in humans.
We evaluated the relationships between the plasma and the cerebrospinal fluid (CSF) concentrations of cholesterol, the cholesterol precursors lanosterol, lathosterol and desmosterol, and the cholesterol elimination products 24S-hydroxycholesterol and 27-hydroxycholesterol, and the CSF markers for AD pathology Aß1-42 and p-tau181 in 86 subjects with normal cognition and in 107 AD patients. CSF desmosterol, cholesterol and 24S-hydroxycholesterol in the AD group, and CSF 24S-hydroxycholesterol in the control group correlated with the p-tau181 levels. Neither CSF nor plasma concentrations of the included compounds correlated with the CSF Aß1-42 levels. In multivariate regression tests including age, gender, albumin ratio, number of the APOEε4 alleles, and diagnosis, p-tau181 levels independently predicted the CSF desmosterol, cholesterol and 24S-hydroxycholesterol concentrations. The associations remained significant for CSF cholesterol and 24S-hydroxycholesterol when analyses were separately performed in the AD group.
The results suggest that alterations of CNS cholesterol de novo genesis and metabolism are related to neurodegeneration and in particular to the cerebral accumulation of phosphorylated tau.

Keywords: Amyloid beta, tau, cerebrospinal fluid, cholesterol, 24S-hydroxycholesterol, 27-hydroxycholesterol

Oral communications

Session 5

Biological activities of oxysterols and phytosterols

Oxidised derivatives of campesterol and dihydrobrassicasterol: Cytotoxic and apoptotic potential in cell culture systems.

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Compared to other phytosterols, campesterol is more readily absorbed by rat jejunal villus cells and across brush border membranes. At similar levels of intake, it is present at higher concentrations than other phytosterols in human serum, both at baseline and also following dietary supplementation with sterol enriched spreads. The methyl group at the C24 position of campesterol may exist as either of two epimers, 24α-methylcholesterol (campesterol) or 24β-methylcholesterol (dihydrobrassicasterol). The epimers are difficult to isolate and studies investigating the biological activity of campesterol have not generally distinguished between the two forms. We have demonstrated that the oxidised products of β-sitosterol and stigmasterol are toxic to U937 cells in a similar manner but to a lesser extent than their cholesterol oxide counterparts. Here we report on the cytotoxic effects of the oxidised derivatives of mixtures of campesterol and dihydrobrassicasterol in both the U937 and HepG2 cell lines. The parent compounds consisted of a campesterol: dihydrobrassicasterol mix at a ratio of 2:1 (2CMP:1DHB) and a dihydrobrassicasterol: campesterol mix at a ratio of 3:1 (3DHB:1CMP). A range of oxide derivatives were synthesised from these parent compounds. The 2CMP:1DBH oxides were more cytotoxic in the U937 cells than the 3DBH:1CMP oxides but the difference in cytotoxicity was less marked in the HepG2 cells. The order of toxicity of the individual oxidation products was found to be similar to that previously observed for cholesterol, β-sitosterol and stigmasterol oxidation products in the U937 cell line. There was an increase in apoptotic nuclei in U937 cells incubated with the 7-keto and 7β-OH derivatives of both 2CMP:1DHB and 3DHB:1CMP and also in the presence of 3DHB:1CMP-triol and 2CMP:1DHB-5β,6β-epoxide. An additional oxidation product synthesised from 2CMP:1DHB, 5,6,22,23-diepoxycampestane, was cytotoxic but did not induce apoptosis. These results indicate the importance of campesterol oxides in the overall significance of phytosterol oxide cytotoxicity.

7-ketostigmasterol protects against 7-ketocholesterol cytotoxicity but induces inflammation to intestinal epithelial cells

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Plant sterols (PS) are added to functional foods by their potential to reduce cholesterol-induced cardiovascular diseases. These compounds are structurally similar to cholesterol and prone to oxidation leading the formation of phytosterol oxidation products. However, previous of our research evidenced that 7-kstigmasterol together with 7-kcholesterol reduced the cytotoxic effects caused by 7-kcholesterol (1).
The objective of the present study was to achieve further characterization and comparison of biological effects caused by 7-kstigmasterol to those caused by 7-kcholesterol concerning their inflammatory potential and effects on cholesterol metabolism.
In this study, Caco-2 cell cultures (5 days post-seeding) were challenged (60 µM/3h) to 7-kstigmasterol or 7-kcholesterol and alterations in mitochondrial membrane potential ($\Delta\Psi m$) and pro-inflammatory cytokines (TNF-α, IL-1β, IL-8) production were measured by ELISA. In addition changes in expression (mRNA) levels of ACAT and HMG-CoA, were evaluated by reverse transcription qPCR techniques. These effects related to cholesterol metabolism, were linked to ATP/NADH production and NF-κB/DNA-binding by using specific inhibitors such as rotenone and pyrrolidine dithiocarbamate (PDTC), respectively.
At the concentration level used (60 µM), Caco-2 cultures did not exhibit negative alterations in $\Delta\Psi m$. Only pre-incubation with rotenone caused a more marked increase in proportion of depolarized mitochondria in those cultures incubated with 7-kcholesterol than 7-kstigmasterol. Otherwise, 7-kstigmasterol exposure induced higher production of all pro-inflammatory markers quantified than 7-kcholesterol. In cell cultures pre-treated with rotenone both 7k-derivatives down-regulated ACAT and HMG-CoA expression levels. However, different expression patterns appeared in cell cultures pre-treated with PDTC where only 7-kstigmasterol down-regulated both enzymes.
From these data it can be concluded that 7-kderivatives-mediated effects on $\Delta\Psi m$ are aggravated by alterations of cell redox status and the higher pro-inflammatory potential of 7-kstigmasterol than 7-kcholesterol. 7-kstigmasterol inhibited ACAT and HMG-CoA either in cell cultures deprived of ATP/NADH or those with an inhibited translocation of NF-κB, whereas this effect was not observed for 7-kcholesterol.

(1): Alemany et al., 2012. *Food Chemical and Toxicology* 2012 (*In press*, FCT-D-12-00361R2).

Oxysterol metabolism in plants

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Compared to what is known about sterol homeostasis in mammals and yeast, knowledge about this process in plants is limited. Homeostatic regulation of sterols in plants is of interest not only from the perspective of membrane function, but also from the role of sterols in certain plant species as metabolic precursors of defense metabolites, such as saponins and glycoalkaloids. Oxysterols may constitute intermediates in these biosynthetic pathways, although little is known about their presence in plants.

To explore mechanisms in plant sterol homeostasis, we have increased the turnover of sterols in Arabidopsis and potato plants by overexpressing three mouse cDNA encoding cholesterol hydroxylases (CHs), hydroxylating cholesterol at the C-7, C-24 or C-25 positions.
Compared to the wild type, the three types of Arabidopsis transformant showed varying degrees of phenotypic alteration, the strongest one being in CH25 lines, which were darkgreen dwarfs resembling brassinosteroid mutants. Gas chromatography-mass spectrometry (GC-MS) analysis of extracts from wild-type Arabidopsis plants revealed trace levels of α and β forms of 7-hydroxycholesterol, 7-hydroxycampesterol, and 7α-hydroxysitosterol. The expected hydroxycholesterol metabolites in CH7-, CH24-, and CH25 transformants were identified and quantified using GC-MS. Additional hydroxysterol forms were also observed, particularly in CH25 plants. In CH24 and CH25 lines, but not in CH7 ones, the presence of hydroxysterols was correlated with a considerable alteration of the sterol profile and an increased sterol methyltransferase activity in microsomes. Moreover, CH25 lines contained clearly reduced levels of brassinosteroids, and displayed an enhanced drought tolerance. Equivalent transformations of potato plants with the CH25 construct increased hydroxysterol levels, but without the concomitant alteration of growth and sterol profiles observed in Arabidopsis.
The results demonstrate oxysterols as natural sterol metabolites in plants, and suggest that an increased hydroxylation of cholesterol and/or other sterols in Arabidopsis triggers compensatory processes, acting to maintain sterols at adequate levels.

Oxysterol Glycosylation: Looking for a Better Cytotoxic Profile

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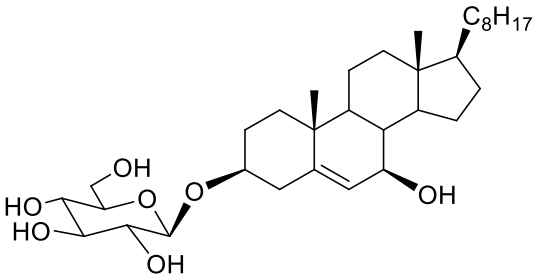
Oxysterols have been gaining increasing attention in Medicinal Chemistry due to their wide range of biological effects, including cytotoxic activity in tumor cells.
Steryl glycosides are widely distributed, although they are commonly known to be mainly present in higher plants. They belong to a group of molecules called saponins, which exert a wide range of biological activities, such as membrane-permeabilisation, immunostimulant, hypocholesterolaemic and anticarcinogenic, among others.



The cytotoxicities exerted by steryl glycosides as well as by novel synthetic counterparts reinforce the potential of oxysterol glycosyl derivatives as anticancer agents and prompted us to the synthesis of several glucosyl oxysterol derivatives.
A library of glucosylated oxysterol and their acyl glucosyl derivatives was prepared and evaluated *in vitro* for cytotoxicity with the aim to gain insight about the influence of the glycosyl moiety and respective acylation on the known cytotoxicity of oxysterols [1-3], on both cancer and normal cells. The compounds exhibit antiproliferative activity in a dose-dependent manner and in the low micromolar range as the corresponding oxysterols previously studied by us.

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Oxysterols induce cell death on human Retinal Pigment Epithelial cells with P2X7 cell death receptor activation

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Age related Macular Degeneration (AMD) is the first cause of blindness in the elderly of northern countries. Clinically, this pathology is characterized by a progressive death of retinal pigmented epithelial cells along with the formation of proteolipidic deposits called drusen. Molecular mechanisms leading to this disease are poorly understood but constituents of drusen are believed to play a crucial role in the pathogenesis of AMD. Among them, oxysterols are compounds known for their toxic properties on various cells leading to numerous degenerative pathologies. The aim of our study was to characterize the effects of two oxysterols on Retinal Pigment epithelial cells in order to determine their implication in the DMLA process. Basal amount of oxysterols was assessed by Mass spectrometry. After 48 hours of incubation with 7-ketocholestérol or 25-hydroxycholestérol on human retinal pigmented epithelial cells (ARPE-19 cells), we observed cell death mechanisms with mitochondrial injury (Transmembrane potential , succinate deshydrogenase) assessed with flow and cold light cytometry.
Retinal cell death is associated with caspases activation (caspases 3/8/9). Moreover, a significant increase of P2X7 receptor is observed with 7-ketocholesterol and 25-hydroxycholesterol (respectively +1050% and +590% vs control). P2X7 purinoreceptor is a cell death receptor located in lipid raft domain in cellular membrane. P2X7 cell death receptor is involved in chronic inflammatory (inflammasome induction) and degenerative diseases (Alzheimer's, AMD, rheumatoid arthritis).
Recent study show that the ß-amyloid peptide induces oxysterol formation by oxidative stress in retina, and our study indicates that oxysterols can stimulate P2X7 cell death receptor in retinal degenerative mechanism. This work establishes first insights of the central role that oxysterols could have in the development of AMD. Moreover for the first time, we present the role of P2X7 receptor in the mechanisms of cell death induced by oxysterols on human retinal cells.

Absence of correlation between oxysterol accumulation in lipid rafts, Calcium rise and induction of GSK3-dependent apoptosis in 158N murine oligodendrocytes

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There are now evidences that oxidized derivatives of cholesterol, 7-ketocholesterol (7KC) and 7β-hydroxycholesterol (7β-OH), are increased in the plasma of patients with neurodegenerative diseases leading to demyelination of the central nervous system (CNS). It was therefore of interest to precise the effects of these oxysterols on oligodendrocytes, the myelin forming cells in the CNS.
To this end, 158N murine oligodendrocytes were treated for 24 - 48 h in the absence and presence of 7KC or 7β-OH (10 - 20 µg/mL). In those conditions, we observed an induction of apoptosis characterized by a condensation / fragmentation of the nuclei, a mitochondrial depolarization and a caspase-3 activation. In contrast, under treatment with 27-hydroxycholesterol (27-OH), no cell death induction was observed. When the cells were stained with Fura-2, no Calcium rise was observed with the different oxysterols used whereas a strong signal was detected in the presence of ionomycine used as positive control. Noteworthy, at concentrations inducing apoptosis on 158N cells, 7KC but not 7β-OH accumulated in lipid rafts. Moreover, 27-OH, which was not cytotoxic, was mainly detected in lipid rafts. Interestingly, 7KC and 7β-OH-induced apoptosis is dependent of the Glycogen Synthase Kinase-3 (GSK3), a serine/threonine kinase which is a key regulator of apoptosis in neuronal cells.
Altogether, our data demonstrate that the ability of oxysterols to trigger GSK3 dependent apoptosis, involving a wide range of signal transduction pathways, is independent from the increase of calcium level and from their accumulation into lipid rafts.

Key words: apoptosis, calcium, lipid rafts, oligodendrocytes, 7-ketocholesterol, 7β-hydroxycholesterol, 27-hydroxycholesterol.

Imaging neutral steroids in tissues by MALDI-FTICR

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Quantitation of neutral steroids in tissues has historically involved extraction of analytes from homogenised biopsies. Efficiencies of recovery can be poor and valuable information about distribution of the hormones within component cell-types is lost. Matrix assisted Laser Desorption Ionisation (MALDI) imaging has been developed to visualize compounds within intact tissue sections, being particularly successfully applied to small molecular weight drugs. Neutral steroids and sterols however are poorly detected as they are not readily ionised and susceptible to ion-suppression. Formation of permanently charged derivatives enhances signal intensity by electrospray ionisation and here, we applied on-tissue derivatisation to image steroids within tissues using Fourier transform Ion cyclotron Mass Spectrometry Imaging (MALDI-FTICR-MS).

Steroids were imaged in sections (10µm) of rat adrenal glands, cut in an OCT-free cryostat embedded in gelatin (10%w/v). Haematoxylin and Eosin staining was performed on adjacent sections. MS images (150-1000amu, positive ion) were acquired using a 12T SolariX (FTICR-MS), with spatial resolution of 150µm and resolving power of 105. Limits of quantitation (LOQs) were assessed by standard spotting using the dry-droplet technique. Neutral steroids, corticosterone (the active rodent glucocorticoid) and its inert metabolite (11-dehydrocorticosterone) were poorly detected within tissues; with LOQs of 1-5 µg. Signals were boosted by 106 by formation of permanently charged derivatives of the A-ring keto moiety leading to LOQs of 0.5-5pg. α-Cyano-4-hydroxycinnamic acid, applied by spray coating, was the matrix producing the best signal to noise. Steroidal derivatives were identified in rat adrenal glands and localisation correlated well with histological zones; glucocorticoids were mainly detected in the zonae reticularis and fasciculata, their principal sites of synthesis. Mass accuracy of all derivatives demonstrated differences of <10 ppm from their theoretical monoisotopic masses.

On-tissue derivatisation MALDI-FTICR-MS is a promising *exvivo* high sensitivity, high resolution and label-free methodology allowing detection of multiple alpha-beta unsaturated keto-steroids in multicellular metabolic tissues.

Oral communications

Session 6

Oxysterols and Cancer

Cholesterol and prostate cancer: the dark side of the life

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In mammals, cholesterol is indispensable for steroid synthesis. It could also be found in lipid raft together with sphingomyeline, ganglioside and proteins such as caveolins and receptor tyrosine kinase family members. This signaling platform regulates cellular homeostasis. Cholesterol has a preponderant role in lipid raft coalescence, which increases the size of these platforms. Regulation of cholesterol homeostasis is thus of primary importance.
In many signaling alterations, cholesterol-enriched rafts promote cell transformation, tumor progression, angiogenesis and metastasis. In LNCaP prostate cancer cells, cholesterol content in membrane decreases apoptosis in particular via the modulation of EGFR/ERK and EGFR/AKT pathways. Mice xenografted with LNCaP cells display an increase of AKT phosphorylation, promoting then survival when fed a cholesterol-enriched diet. Simvastatin, a HMG-CoA reductase inhibitor, decreases raft-associated AKT phosphorylation. Even though accumulation of cholesterol in solid tumors was described more than a century ago, the link between cholesterol and promotion and/or progression of prostate cancer (PCa) started to be studied at the end of the 20th century.
Liver X Receptors (LXRs) are transcription factors activated by oxysterols derived from cholesterol (Viennois et al. 2012). We (Pommier et al. 2010) and others demonstrated an anti-proliferative and pro-apoptotic effect of LXRs activation in PCa cell lines suggesting a protective role of these receptors in cancer. We showed that it was possible to downregulate AKT phosphorylation in lipid rafts of human PCa cells LNCaP by activating LXRs. Replenishment of cell membranes with exogenous cholesterol antagonized these effects, showing that cholesterol was a key modulator in this process. Altogether, pharmacological modulation of LXRs activity could thus reduce prostate tumor growth (Viennois et al. 2011) by enhancing apoptosis in a lipid raft-dependent manner.

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7β-Hydroxycholesterol-induced energy stress leads to sequential opposing signaling responses and to death of C6 glioblastoma cells

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We have shown previously that 7β-hydroxycholesterol (7βHC) is toxic to transformed but not to quiescent astrocytes. It is not so much 7βHC as its esters, produced in the endoplasmic reticulum via Acyl-CoA : cholesterol acyltransferase, that induce cell death. The oleate ester of 7βHC, 7β-hydroxycholesteryl-3-oleate, inhibits experimental glioblastoma growth in rat brain. We further demonstrated that following exposure to 7βHC, AMP-Kinase is activated transiently in rat glioblastoma cells (C6) followed by Akt activation and cell death. During the period of AMPK activation (5-8 hr after the beginning of 7βHC treatment) cell cycle is slowed down since BrdU incorporation and the expression of cyclins D and E, known to be up-regulated in cancers, diminish. At the same time mitochondrial inner membrane potential increases and mitochondrial fusion is observed, suggesting increased oxidative phosphorylation in response to increased ATP demand. Intracellular ATP concentrations remain fairly stable until AMPK de-activation and Akt activation (8-15 h) at which moment both ATP levels and glucose uptake collapse. AMPK activation by AICAR also reduces cell proliferation, but is not toxic to C6 cells. We suggest that 7βHC-induced activation of AMPK switches the metabolism in C6 cells from a Warburg regime into the regular oxidative phosphorylation regime found in normal brain cells. The subsequent as yet unidentified steps implicating Akt will be studied next.

Importance of the liver-X-receptor-β and cholesterol-5,6-epoxide metabolites in the induction by tamoxifen of triglyceride biosynthesis and cytotoxicity in breast cancer cells

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The microsomal antiestrogen binding site (AEBS) is a multifunctional complex incorporating both cholesterol biosynthesis enzymes and cholesterol-5,6-epoxide hydrolase (ChEH). The AEBS was shown in breast cancer cells to be involved in Tamoxifen (Tam) induction of differentiation characteristics such as triacylglycerol (TG) biosynthesis and cytotoxicity. The objective of the present study was to decipher the molecular mechanisms involved in these effects. We found that LXRβ controlled the induction of TG biosynthesis and part of the cytotoxicity induced by Tam and by the selective AEBS ligand PBPE (N-pyrrolidino-(phenylmethyphenoxy)-ethanamine, HCl) in MCF-7 cells. AEBS ligands induced the biosynthesis of 5,6α-epoxy-cholesterol (5,6α-EC), 5,6β-epoxy-cholesterol (5,6β-EC), and 5,6α-epoxy-cholesterol-3β-sulfate (5,6-ECS) due to the dual activation of cholesterol autoxidation and ChEH inhibition. 5,6α-EC and 5,6α-ECS stimulated LXRβ-dependent TG biosynthesis and cytotoxicity. 5,6β-EC induced LXRβ-independent cytotoxicity. Knock-down of SULT2B1b in MCF-7 cells reduced their sensitivity to Tam, PBPE and 5,6α-EC. Treatment of SULT2B1b-negative MDA-MB-231 breast cancer cells with Tam and PBPE induced the accumulation of 5,6α-EC and 5,6β-EC responsible for LXR-dependent TG biosynthesis and LXR-independent cytotoxicity respectively. Cells were sensitized to Tam and PBPE by re-expression of SULT2B1b or addition of 5,6-ECS . Altogether, these data showed that the LXRβ, cholesterol autoxidation into 5,6α-EC and 5,6β-EC, and production of 5,6-ECS by SULT2B1b are implicated in the pharmacology of Tam and AEBS ligands in MCF-7 cells. This study delineates a novel signaling pathway that provides a new rationale to improve the clinical use of Tam and related compounds

In vitro toxicity of cholesterol (Chol), 7-ketocholesterol (7KC) and Cholesten-3β,5α,6β-triol (Chol-triol) in different hematological cancer cell lines

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Oxysterols are characterized by the presence of additional oxygenated groups in the cholesterol structure and can be endogenously produced by enzymatic and nonenzymatic oxidative processes or absorbed from diet sources. Nonenzymatic oxidations of cholesterol occur mainly at ring B producing, among others, 7KC and chol-triol. They exhibit a number of biologic activities including inhibition of cellular proliferation and cytotoxicity. We evaluated the cytotoxic effects of cholesterol, 7KC and chol-triol on several hematological cancer cell lines. In vitro effect of cholesterol and oxysterols (10 nM to 100 µM final concentrations) was studied on Jurkat (acute T cell leukemia), Granta-519 (mantle cell lymphoma), Molt-4 (acute lymphoblastic leukemia) and Raji (Burkitt's lymphoma) cells. After 24 h cytotoxicity was evaluated by MTT reduction assay. Sensitivity to 7KC and chol-triol varied among cell lines, Jurkat being the most sensitive to 7KC ($IC50_{7KC}$ 26.64 ± 8.95 µM) and Molt-4 to chol-triol ($IC50_{chol\text{-}triol}$ 10.67 ± 2.71 µM). Granta-519 cell line showed the lower sensitivity for both compounds ($IC50_{7KC}$ 74.7 ± 30.2; $IC50_{chol\text{-}triol}$ 24.45 ± 4.31 µM). Morphological observation during the incubation period indicated that cells treated with 7KC and chol-triol did not proliferate and showed increased death, whereas cells treated with cholesterol showed a normal morphology and proliferation rate. Cholesterol did not reduce cell viability in any cell line tested, even though Raji cell line showed a higher sensitivity to cholesterol concentration variations. Chol-triol has shown to be more cytotoxic than 7KC. In conclusion, different hematological cancer cell lines exhibit different cytotoxic sensitivity to cholesterol, 7KC and chol-triol. The mechanisms behind cell death mediated by these oxysterols in these cell lines remain to be investigated.

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Oral communications

Session 7

Oxysterols in cardiovascular and metabolic diseases

Oxysterols and Lipid Metabolism in Obesity and Metabolic Syndrome

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It is now emerging that, as the major storage site for lipids, adipose tissue has also a role in metabolic regulation through lipid signaling. There are two prevailing views about the role of adipose tissue lipid metabolism in metabolic syndrome. First, storage of lipids in adipose tissue has been suggested to protect other organs from exposure to excessive lipids and thereby reducing the risk of lipotoxicity. Second, fatty acids derived from adipose tissue, particularly under obese conditions, could disrupt the function of peripheral tissues, resulting in muscle insulin resistance or hepatic steatosis. Very recently, lipidomics technology applied to animal models revealed that the long chain saturated fatty acid palmitoleate (C16:1n7) acts as a lipid hormone that strongly stimulate muscle insulin action and suppresses hepatosteatosis. Another lipid class that is an emerging candidate for controlling lipid metabolism is the oxysterol class. In particular oxysterols generated via free radicals have been hypothesized as the oxidative stress link between obesity and atherogenesis. Increasing evidence suggests that oxysterols act as ligands of liver X receptors (LXR), transcription factors with key roles in lipid metabolism. Consistent with Cao's study who demonstrated that the monounsaturated fatty acid palmitoleate (C16:1n7) acts as a lipid hormone that strongly stimulate muscle insulin action and suppresses hepatosteatosis, we have recently reported a significant higher concentration of plasma palmitoleate in Cystic Fibrosis patients compared to controls. This information provided by Cystic Fibrosis, as a clinical model opposite to obesity, strongly supports the relevance of Cao's data at clinical level. To date, neither extensive studies of oxidative stress nor the interplay between them and fatty acids has been reported in obesity and metabolic syndrome. Therefore, the present study was designed to use a lipidomic approach to examine whether fatty acid metabolism, cholesterol metabolsim and oxidative stress are altered in obesity in vivo and whether such abnormalities are associated with each other and with characteristics of the disease state.

Bile acid : a molecular link between liver and testis functions

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Alterations in male reproductive function appear as a social problem since 25% of couples are sterile after one year without contraception trial. Male infertility factors are present in 50% of these couples. Thus, it is of major importance to clearly define the molecules that could have a negative impact on fertility.
A series of epidemiological data shows that men with impaired liver have a higher risk of developing infertility. In addition, a bile duct ligation to mimic liver induced a decrease in plasma testosterone and testis weights, associated with a loss of germ cells dysfunction in several species (rabbit, rat, mouse). Besides, increased plasma concentrations of bile acids are a common feature of most liver diseases. We thus decided to test the hypothesis that bile acids could be part of the deleterious signal on testis in the context of cholestasis.
We demonstrate that increased plasma concentrations of bile acids impair male fertility in mice. Thus, we aim: 1) to determine the molecular mechanisms involved in the changes induced by the bile acids in the testes, 2) to analyze the role of bile acid receptors in the pathophysiology and 3) to measure the impact of bile acids on germ cells.

Sterol precursors, oxysterols and steroid hormones in platelets and platelet extracellular vesicles in vascular and metabolic diseases

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We established *in vitro* models for megakaryocytic differentiation into pro-platelets, shedding of platelets, release of platelet extracellular vesicles (PL-EV) EVs, and for analysis of *in vitro* platelet senescence in human haemapheretic platelet preparations, analyzing platelets, PL-EVs and surrounding autologous plasma over 5 days storage and agitation by transcriptomics, lipidomics, proteomics and miRNA patterning. The newly developed assays were then applied to patients with vascular- and metabolic disease (Diabesity) and Neurodegenerative disorders (AD, Parkinson).
The proteome analysis of freshly isolated platelets and platelet extracellular vesicles before and after senescence revealed modulation of enzymes connected to cholesterol and sterol metabolism acetyl-Coenzyme A acetyltransferase 2 (ACAT2), phosphomevalonate kinase (PMVK), farnesyl diphosphate synthase (PMVK), lanosterol synthase (LSS) and three hydroxysteroid (17-beta) dehydrogenase (HSD17B) species. HSD17B3 catalyzing the final step in testosterone synthesis (10% of testis activity) was found down regulated during platelet maturation while the type 12 enzyme (HSD17B12) is reciprocally induced. Among other steroid hormones and sterols (such as lanosterol, desmosterol, 7-ketocholesterol) which have been measured in platelets and PL-EVs especially estrogens are dependent on gender and age. Substantial amounts of E2 were found in platelets that can be released in vitro and in vivo to transfer the E2 signal to other estrogen receptor positive cells such as endothelial cells and leukocytes at the site of thrombosis or in the circulation.
PL-EVs were fractionated into 5 subclasses, differentially expressing free cholesterol, sphingomyelin, dihydrosphingomyelin, glycerophospholipid-, plasmalogen- and lysophosphatidyl choline-lipid species, as well as caveolin-1 and apolipoproteins A-I, J and E, predominant in subfractions 3-5. Mitochondrial marker proteins and cardiolipin were enriched in subfractions 4-5, indicative for autophagic vesicles. Neurodegeneration-related proteins amyloid beta precursor protein (APP) and alpha-synuclein enriched in subfractions 3-4 and 1-2, respectively. The distribution of oxysterols, especially of 24-hydroxycholesterol which is associated to neurodegenerative diseases, and cholesterol precursors are currently investigated.

Differential impact of oxysterol receptor LXR alpha and LXR beta on the regulation of cholesterol efflux in primary human macrophages.

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Objective/Rationale: Pharmacological activation of Liver X Receptors (LXRs) α and β is promising in the treatment of atherosclerosis since it can promote cholesterol efflux from macrophages and prevent foam cell formation. However, the development of LXR agonists has been limited by undesirable side-effects such as hepatic steatosis mediated by LXRα activation. Our aim was therefore to determine the relative contribution of LXRα and LXRβ to the control of cholesterol efflux in human primary macrophages.
Methods and Results: LXRα and/or LXRβ expression was suppressed by small interfering RNA in human primary macrophages treated or not with synthetic LXRα/β dual agonists. We observed that LXRβ silencing had no impact on the expression of LXR-target genes and cholesterol efflux. In contrast, LXRα silencing significantly reduced the response of LXR-target genes to LXR agonist and inhibited cholesterol efflux to ApoA-I, HDL_2 or to endogenous ApoE. Most importantly, no differences were observed between LXRα and LXRα/β knockdown conditions, suggesting that LXRβ activation was unable to rescue for LXRα deficiency in human primary macrophages.
Conclusion: We demonstrate here that in contrast to earlier mouse studies, LXRα is the major isoform involved in the control of macrophage cholesterol efflux in humans, with no competitive or compensatory effect of LXRβ.

Key words: LXR; Cholesterol efflux; Primary human macrophages.

The mutual effects between the Human carotid plaque constituents and the blood components

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Human carotid plaque components interact directly with circulating blood elements and thus they might affect each other. As part of ongoing study, the nature and the consequences of these mutual interactions is investigated in order the identify markers in the blood (levels of oxidized and non-oxidized lipids, HDL, PON1, HbA1c) which will provide information on the plaque stability. PON1 is a HDL-associated enzyme with anti-atherogenic properties and many of the antiatherogenic properties of the HDL are attributed to PON1. The aims of the present study were to identify the PON1 inhibitor in lipids lesion extract (LLE) and explore the mechanism of the inhibition. Human carotid plaques were obtained from patients undergoing routine endarterectomy, and the lesions were ground and extracted. Addition of antioxidants or electrophiles to LLE did not prevent PON1 inhibition. LLE was unable to inhibit a PON1 mutant lacking Cys284, whereas it did inhibit all other PON1 mutants tested. The inhibitor in the LLE was identified as linoleic-acid hydroperoxide (LA-OOH) and inhibition was specific to this hydroperoxide. During its inhibition, PON1 acted like a peroxidase enzyme, reducing LA-OOH to LA-hydroxide via its Cys284. A similar reaction occurred with external thiols, such as DDT or cysteine, which also prevented PON1 inhibition and restored enzyme activity following inhibition. Thus, the anti-atherogenic properties of HDL could be, at least in part, related to the sulfhydryl-reducing characteristics of its associated PON1, which are further protected and recycled by the sulfhydryl amino acid cysteine.

Posters

Analysis of bioactive oxysterols in newborn mouse brain by liquid chromatography - mass spectrometry.

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Unesterified cholesterol is a major component of plasma membranes. In the brain of the adult it is mostly found in myelin sheaths where it plays a major architectural role. In the newborn mouse little myelination of neurons has occurred and much of this sterol comprises a metabolically active pool. In the current study we have accessed this metabolically active pool and using liquid chromatography - mass spectrometry have identified cholesterol precursors and metabolites. While desmosterol and 24S-hydroxycholesterol represent the major precursor and metabolite respectively, other steroids including the oxysterols 22-oxocholesterol, 22R-hydroxycholesterol and 20R,22R-dihydroxycholesterol and the C21-neurosteroid progesterone were identified. 24S,25-Epoxycholesterol formed in parallel to cholesterol was also found to be a major sterol in newborn brain. Like 24S- and 22R-hydroxycholesterols, and also desmosterol, 24S,25-epoxycholesterol is a ligand to the liver X receptors which are expressed in brain. The desmosterol metabolites (24Z),26-, (24E),26- and 7alpha-hydroxydesmosterol were identified in brain for the first time.

Amyloid-β peptide neurotoxicity is potentiated by 4-hydroxynonenal and 24-hydroxycholesterol

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Alzheimer's disease (AD) is a neurodegenerative disease histologically characterized by extracellular deposits of amyloid-β (Aβ) in the form of senile plaques, and intracellular inclusions of hyperphosphorylated tau in the form of neurofibrillary tangles. AD begins with the abnormal processing of amyloid precursor by the sequential enzymatic action of the β- and γ-secretase, which leads to an excessive production of Aβ peptides among which is the aggregation-prone and damaging Aβ42 specie.
A growing body of evidence suggests a link between lipid peroxidation and AD. The brain is particularly vulnerable to oxidative stress, which is responsible for the formation of cholesterol oxidation products (oxysterols), and highly reactive aldehydes, among which the most relevant to brain pathophysiology appears to be 4-hydroxynonenal (HNE). HNE production in the brain is stimulated by Aβ and, conversely, Aβ production is up-regulated by this aldehyde. Because elevated levels of HNE have been found in the brain of AD patients, it has been proposed as a biomarker of AD. Moreover, 24-hydroxycholesterol (24-OH), which is the main cholesterol oxidation product in the brain, has been shown to enhance Aβ neurotoxicity in human differentiated neuroblastoma cell lines, as well as augmenting ROS generation.
In our work we observed the ability of HNE and 24-OH to potentiate Aβ cytotoxicity as determined *in vitro* using neuron-like cells derived from human dental-pulp progenitor cells. Cell pre-incubation with the aldehyde or the oxysterol strongly enhanced Aβ uptake and intraneuronal accumulation, by up-regulating a cluster of membrane receptors, composed by CD36, β1-integrin and CD47. Consequently, the two lipid peroxidation products markedly potentiate Aβ neurotoxicity, in terms of necrosis; this event was confirmed by the employment of specific antibodies against CD36 or β1-integrin. These data support a primary involvement of altered brain lipid metabolism in the pathogenesis of AD.

LXRs are implicated in cerebellar re/myelination

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Liver X receptors (LXRα and β) are nuclear receptors implicated in lipid metabolism. Oxysterols (oxidized forms of cholesterol) are natural ligand of LXRs. Within the central nervous system (CNS), their accumulation has been correlated with neurodegenerative episodes in multiple sclerosis or Alzheimer disease. Their role in myelination process is poorly understood.
Oligodendrocytes are the myelinating cells of the CNS. They form myelin sheaths that enwrap axons, supporting axonal integrity and fast conductance of the nervous influx. Particularly rich in phospholipids and cholesterol, this layered structure is stabilized by specific myelin proteins such as Proteolipid Protein (PLP) or Basic Myelin Protein (MBP).

Here we demonstrate that LXRs are crucial for the myelination of the cerebellum. LXRα/β double knock-out mice (LXR dKO) exhibited thinner myelin sheaths and reduced expression of myelin genes in the cerebellum. The administration of an LXR agonist (TO901317) stimulated the expression levels of the two major central myelin genes (PLP and MBP), in the cerebellum but not in the spinal cord or the corpus callosum. We also showed that TO901317 stimulated promoter activity, mRNA and protein accumulation of PLP and MBP in primary cultures of oligodendrocytes as well as in the oligodendroglial cell line 158N. Interestingly, this positive effect of LXR on myelination elicited by oligodendrocytes is in contrast with our previous observations in Schwann cells (Makoukji et al, 2011). LXR activation was accompanied with profound modifications of oligodendrocyte cell shape, suggesting a maturation of the oligodendrocytes. Finally, using organotypic cultures of cerebellum, we showed that LXR activation by TO901317 stimulated myelin gene expression after lysolecithin-induced demyelination, enhanced oligodendrocyte differentiation and accelerated the remyelination process.
Our results indicate that LXRs are positive regulators of myelination, and might be pharmacological targets for remyelination therapies of the CNS.

Interplay between LXR and Wnt/β-catenin signaling in the negative regulation of peripheral myelin genes by oxysterols

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Oxysterols are reactive molecules generated from the oxidation of cholesterol. Their implication in cholesterol homeostasis and in the progression of neurodegenerative disorders is well known, but few data are available for their functions in the peripheral nervous system. Our aim was to study the influence of oxysterols on myelin gene expression and myelin sheath formation in peripheral nerves. We show by gas chromatography/mass spectrometry that Schwann cells and sciatic nerves contain 24(S)-hydroxycholesterol, 25-hydroxycholestrol and 27-hydroxycholesterol and that they express their biosynthetic enzymes and receptors (liver X receptors LXRα and LXRβ). We demonstrate that oxysterols inhibit peripheral myelin gene expression [myelin protein zero (MPZ) and peripheral myelin protein-22 (PMP22)] in a Schwann cell line. This downregulation is mediated by either receptors LXRα and LXRβ), depending on the promoter context, as suggested by siRNA strategy and chromatin immunoprécipitation assays in Schwann cells and in the sciatic nerve of LXR knock-out mice. Importantly, the knock-out of LXR in mice results in thinner myelin sheaths surrounding the axons. Oxysterols repress myelin genes via two mechanisms: by binding of LXRs to myelin gene promoters and by inhibiting the Wnt/β-catenin pathway that is crucial for the expression of myelin genes. The Wnt signaling components (Disheveled, TCF/LEF, β-catenin) are strongly repressed by oxysterols. Furthermore, the recruitment of β-catenin at the levels of the MPZ and PMP33 promoters is decreased. Our data reveal new endogenous mechanisms for the negative regulation of myelin gene expression, highlight the importance of oxysterols and LXR in peripheral nerve myelination, and open new perspectives of treating demyelinating diseases with LXR agonists.

High sensitivity measurement of oxysterols with robust automatic filtration /filter backflush solid phase extraction liquid chromatography

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On-line techniques e.g. column switching in liquid chromatography have many benefits, such as increased possibility of automation, and an increase in sensitivity as large volumes may be injected on micro bore columns without a loss in chromatographic performance. However, depending on the matrix, the pre-injection treatment and amount injected, column switching can increase back pressure and cause clogging. A manual procedure, such as filtration or SPE is often included in the method to avoid clogging.
To overcome the back pressure build up problem, we have developed an Automated Filtration and Filter-Flush (AFFL) system (Fig. A). An injected sample passes through a union containing a stainless steel filter prior to SPE trapping. The filter stops any particular matters from reaching the SPE (Fig. B). When the trapped analytes are transferred to the analytical column by column switching, a third pump back-flushes the filter (Fig C). In this way, all particles are removed from the filter after every injection. This reduces back-pressure build up.
With this technique, Girard T derivatives of oxysterols can be analyzed on LC MS systems without time consuming manual sample clean up. Large volumes can be injected giving low limits of detection. All sample preparation is performed in one vial, and less analyte is lost during sample preparation steps. The technique is currently in use for studying oxysterol levels in e.g. cancer cell side populations, tumor etc.

Effect on serum sterol oxides after the intake of a phytosterol-enriched beverage

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A method for the determination of serum sterol oxidation products (SOPs) by CG-MS/MS was validated. Briefly, lipid fraction from 700 µL of serum were extracted which was saponified at room temperature after the addition of BHT and the internal standard (19-hydroxycholesterol). The unsaponifiable fraction was extracted with diethylether, and then purified by SPE. Cholesterol and phytosterol oxidation products (COPs and POPs) were derivatised into trimethylsilylether derivatives for the GC-MS/MS analysis.
POPs identification was performed from the fragmentation patterns of POPs, obtained by thermooxidation from phytosterol standards, by comparison with commercialized COP standards and by their analysis by TLC. For the quantification of SOPs, calibration curves were performed with COP standards (7α-and 7β-hydroxycholesterol, cholestanetriol), obtaining good linearity ($r>0.985$) for all the oxides.
The analytical parameters of the method were: accuracy (by recovery assays with COPs) ranged between 84-123.5%, intraday (n=5) and interday (n=10) precisions were 1.9-9.7 and 2.2-12.5% (expressed as RSD), respectively, and limit of detection ranged between 0.04-36 ng/mL.
The method was applied to serum samples obtained from healthy post-menopausal women (n= 10, 50-65 years of age) who ingested a 0.8% phytosterol-enriched fruit and milk-based beverage (79% β-sitosterol, 5.6% campesterol, 1.5% stigmasterol) for 4 weeks. Blood samples were drawn at week zero (W0) and at the end of intervention period (W4).
The median and minimum-maximum serum oxides levels (µg/mL), respectively, were: 7α-hydroxycholesterol (W0: 0.52, 0.09-1.118, and W4: 0.441, 0.085-1.338), 7β-hydroxycholesterol (W0: 0.771, 0.054-1.221, and W4: 0.581, 0.064-1.435), cholestanetriol (W0: 0.047, 0.03-0.639, and W4: 0.067, n.d.-0.904), 7α-hydroxysitosterol (W0: 0.042, n.d.-0.064, and W4: 0.047, 0.039-0.104), 7β-hydroxysitosterol (W0: 0.036, n.d.-0.071, and W4: 0.034, n.d.-0.044), and sitostanetriol (W0: 0.021, n.d.-0.084, and W4: 0.041, n.d.-0.064).

The ANOVA test performed showed that the intake of the beverage did not produced a statistically significant increase ($p>0.05$) on serum contents of SOPs, which implies the safe consumption of this kind of functional beverages.

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Regulation of oxysterols formation in macrophages by the endosomal phospholipid bis(monoacylglycero)phosphate

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Atherosclerosis is a major vascular complication in cholesterol-related diseases such as type 2 diabetes and metabolic syndrome. It is defined as the progressive occlusion of the lumen due to the formation of atherosclerotic plaque in the subendothelial space, according to a complex and evolving process called atherogenesis. This phenomenon results primarily from increased oxidative stress that leads to the accumulation of oxidized LDL and thus cholesterol in the subendothelial macrophages. The oxidation products of cholesterol, called oxysterols, are involved in the regulation of cholesterol cellular homeostasis. Some oxysterols, especially associated with oxidized LDL, exert pro-apoptotic effects on macrophages thereby promoting atherogenesis.
Bis(monoacylglycero)phosphate (BMP) is a phospholipid localized specifically in late endosomes, a central cellular compartment of LDL-cholesterol metabolism. Our previous studies have shown that BMP plays an important role in intracellular cholesterol transport in macrophages, more specifically in the regulation of cholesterol efflux.
The objective of this study was to characterize the effects of BMP on cell viability and cholesterol metabolism in relation with oxidative stress induced by high concentration of oxidized LDL. BMP content in murine RAW 264.7 macrophages was specifically increased by supplementation with its precursor dioleoyl-phosphatidylglycerol (DOPG). The consequences of BMP accumulation were measured in terms of oxidized LDL-induced toxicity and oxidative cholesterol metabolism. Different tests (MTT assay and TUNEL method) have shown a protective effect of BMP against the toxic effect induced by oxidized LDL. Moreover, the analysis of oxysterols profiles using GC-MS/MS and radioactive labeling, highlighted a specific reduction of pro-apoptotic oxysterols, 7β-hydroxycholesterol and 7-oxocholesterol, in DOPG-supplemented cells compared with control cells. All together, these data suggest that BMP exerts a protective action against the pro-apoptotic effect of oxidized LDL via a reduced production of intracellular pro-apoptotic oxysterols.

Olesoxime, a cholesterol/oxysterol-like compound, promotes oligodendrocyte progenitor cell differentiation and remyelination in MOG-induced experimental autoimmune encephalomyelitis

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Olesoxime is a novel neuroprotective compound that shares structure homology with cholesterol and oxysterols. It prevents neurodegeneration and promotes repair in multiple models of neurodegenerative diseases (Bordet et al. 2007, 2008). Olesoxime is currently being evaluated for its ability to delay disease progression in patients suffering from spinal muscular atrophy. Very recently, we showed that olesoxime accelerates oligodendrocyte progenitor cell (OPC) maturation and accelerates myelination in naïve mice (Magalon et al. 2012). In vitro studies demonstrated that olesoxime acts directly on OPCs to promote differentiation even in the absence of neurons. Interestingly olesoxime also promotes remyelination and functional recovery in preclinical models of CNS demyelination making it a promising drug candidate to address neuroprotection and remyelination in demyelinating disorders such as multiple sclerosis (MS) or leukodystrophies. Here olesoxime was further tested for its ability to favour functional recovery in MOG-induced experimental autoimmune encephalomyelitis (EAE) mice.

EAE induced a significant increase in serum cytokines, especially IFN-γ and TNF-α, which was not impacted by olesoxime treatment. Likewise, olesoxime treatment did not delay disease onset or clinical score at disease peak. However, it significantly improved clinical scores during the relapsing phase. Similar improvement in overall body weight change over the 50 days was observed with olesoxime treatment. Demyelination in the spinal cord was too low to assess a treatment effect. In contrast, a significant increase in the number of mature oligodendrocytes could be observed in optic nerves of olesoxime-treated EAE mice, as well as a small but significant increase in the myelin content. Altogether these results strengthen the therapeutic interest of olesoxime for MS. Recently LXRs activation by oxysterols was shown to regulate cholesterol homeostasis in oligodendrocytes potentially impacting on myelination (Nelisen et al. 2012). The role of LXRs in olesoxime mechanism of action will be further investigated along with other potential targets.

Analysis of Oxysterols in Plasma Using Stable Isotope Labelled Derivatives

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The analysis of oxysterols in plasma is complicated by their low abundance (pg/mL to ng/mL), especially when compared to cholesterol (mg/mL), lack of chromaphore, and poor ionisation profile in mass spectrometry. Some of these problems can be overcome by chemical derivatisation prior to analysis by either GC/MS or, more recently, LC/MS.
Our group has developed an analytical strategy based on oxidation with cholesterol oxidase followed by derivatisation with the Girard P reagent. We have termed this EADSA (enzyme-assisted derivatisation for sterol analysis). The Girard P derivative incorporates a permanently charged quaternary ammonium ion leading to greatly enhanced sensitivity in ESI-MS. Additionally, predictable fragmentation yields structurally informative MS^n spectra that allow identification of up to 40 metabolites.
Here we report an extension of our original EADSA procedure using a series of isotope labelled derivatives that allow direct comparison of several samples in a single LC/MS run. This improves accuracy by negating matrix effects and any variability in the performance of the instrument. At the same time this method allows higher throughput by decreasing instrument time.
To illustrate our method we present rapid diagnostic tests for cerebrotendinous xanthomatosis (CTX, deficiency in CYP27A1), hereditary spastic paraplegia (HSP/SPG5, deficiency in CYP7B1), and Smith-Lemli-Opitz syndrome (SLOS, deficiency in 7-DHC).

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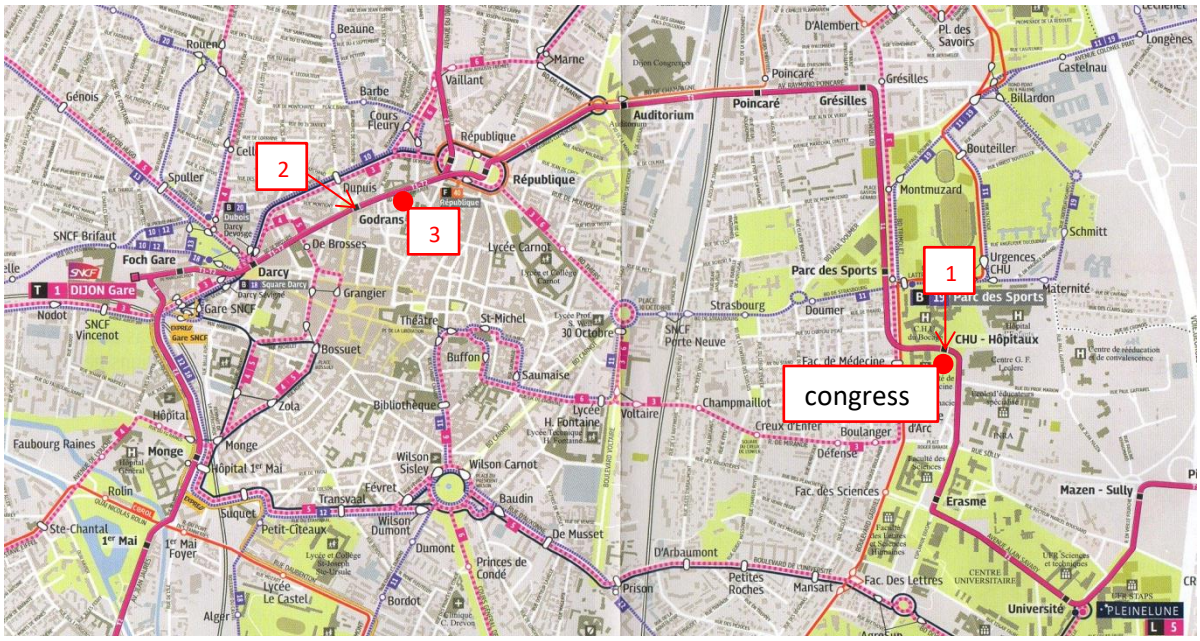
Gala dinner (map)



1. Tramway station departure (CHU – Hôpitaux)
2. Tramway station stop (Godrans)
3. and walk to the “Cellier de Clairvaux“ (27, boulevard de la Trémouille)

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Gérard Lizard, Jean-Paul Pais de Barros, Anne Vejux

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