Leibniz
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Final Report
Leibniz Competition

Systems level analysis of inositol messengers

Application number: K216/2016

Period under review:

2017-2020

Leibniz Institute in charge:

Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP)

Project leaders:
Dorothea Fiedler
Hartmut Oschkinat
Volker Haucke

Reporting guidance

The final report must be submitted to Leibniz Headquarters no later than 6 months after the end of the authorised project and must not exceed 5 pages in length.

The report should provide information about the project's progress, in terms of the milestones and objectives of both the project itself and of the overarching Leibniz objectives of the Leibniz Competition grants, for the Senate's Competition Committee (SAW). The SAW takes note of the reports and may use them to inform its view of the project's progress in dealings with the project leader.

In addition to the progress report, please provide an executive summary (max. 4 500 characters) and information on outcomes and achievements (publication list, knowledge transfer items, etc), measures of equal opportunity and internationalization as well as networking via our electronic application system.

The final report may be written in German or English.

Please observe the following requirements in terms of formatting and page layout:

- Page margins: The right, left and top margins should be set to 2.5 cm, the bottom margin should be 2 cm.
- Font and font size: Please select Arial as the font, and 11 pt for copy text, 14 pt for the main heading, and 11 pt for subheadings.
- Line spacing: Single line spacing. The line spacing after headings and paragraphs is 6 pt.
- Tables: The same rules apply to any tables, charts, etc.

Contents

1. Achievement of objectives and milestones

The proposed research sought to apply a novel transdisciplinary approach that combined (bio)chemical synthesis of ^{13}C-labeled inositol and inositol phosphates (InsPs) with metabolic labeling, followed by nuclear magnetic resonance spectroscopy (NMR), to enable the quantitative detection of inositol phosphates in cells and *in vivo*.
To achieve this ambitious goal, the proposal first sought to synthesize and characterize ^{13}C-labeled *myo*-inositol and phosphorylated versions thereof (Aim 1). Due to its benign nature, ^{13}C-labeled inositol was subsequently to be used for metabolic labeling of mammalian cells, and cell lysates – or membrane fragments – were to be analysed for labeled InsPs and PtdInsPs (phosphatidyl inositol phosphates) by NMR spectroscopy (Aim 2). We next wanted to showcase the power of this method by investigating the interplay of InsPs and PtdInsPs in the control of endolysosomal membrane homeostasis and nutrient signaling (Aim 3) and in the regulation of insulin signaling in cells and in vivo (Aim 4).
Aim 1 was completed successfully, and also Aim 2 was a success with regards to the soluble inositol phosphates (InsPs). Detection of labeled PtdInsPs by NMR was initially tested on yeast cells with and without the application of dynamic nuclear polarisation (DNP) MAS NMR at room temperature and 100 K, respectively, with the aim to step quickly to the analysis of mammalian cells. Labeled *myo*-inositol was employed, however, leading to complex signal pattern in MAS NMR spectra, even for the isolated inositol preparations in the solid state whereas solution NMR showed only individual peaks. In the course of this work considerable improvements of DNP technology were achieved to provide the required sensitivity but the signal multiplication problem occurring even at room temperature could not be solved.
Since Aims 3 and 4 hinged on the detection of both InsPs and PtdInsPs by NMR, we altered our strategy accordingly. Instead, the Haucke lab has focussed on dissecting the role of $InsP_3$ and of the $InsP_3$-specific 5-phosphatase in the regulation of cell physiology. The work uncovered a crucial function for INPP5A-mediated $InsP_3$ hydrolysis in the control of lipid exchange at membrane contact sites

2. Activities and obstacles

The Fiedler lab has a keen interest to provide a comprehensive and mechanistic picture of the signaling functions of inositol poly- and pyrophosphate messengers (InsPs and PP-InsPs). The analysis of these molecules still constitutes a significant challenge because they are devoid of an appropriate spectroscopic handle. As a first step of the proposed research, the Fiedler lab developed a high-yielding and scalable synthesis of ^{13}C-labeled *myo*-inositol from ^{13}C-labeled glucose. The labelled inositol was then further converted to labelled inositol polyphosphates by chemical means. By incorporating ^{13}C into the inositol scaffold, the inositol polyphosphates were readily detected by NMR at physiological concentrations, and within complex mixtures. The benign nature of ^{13}C-labeled inositol allowed for metabolic labeling of mammalian cells, followed by detection of the generated inositol phosphates, without the need for separation, enrichment, or the use of radioactively labeled compounds (*Chem Sci* **2019**). Instead of uniformly ^{13}C-labeled glucose, other labelling patterns of glucose can of course also be used as starting materials, providing the corresponding *myo*-inositol and InsPs (*Methods Enzymol* **2020**).
Due to the ease of detection by NMR, the labeled inositol phosphates were then used to optimize a chemoenzymatic approach to obtain PP-InsPs. Scalable syntheses of these high-energy metabolites did not exist to that point, but careful optimization of the reaction parameters for the biochemical transformations, coupled to a convenient precipitation protocol for purification, afforded the desired compounds in 100-250mg bathes (*Biochemistry* **2019**).
In collaboration with the Hothorn and the Schaaf groups, the ^{13}C-labeled inositol phosphates proved instrumental in biochemical assays characterizing plant inositol phosphate kinases, and their regulation by phosphate, ADP, and ATP (*eLife* **2019**, *Nat Commun* **2021**, *Mol Plant* **2021**). Another useful application of the ^{13}C-labeled InsPs and PP-InsPs is as stable isotope labeled internal standards for the quantification of endogenous InsP and PP-InsP levels by capillary electrophoresis electrospray ionization mass spectrometry and liquid chromatography mass spectrometry (*Nat Commun* **2020**, *ACS Pharmacol Transl Sci.* **2021**). Furthermore, ^{13}C-labeled *myo*-inositol could be utilized to follow *myo*-inositol transport in the small intestine of laying hens, and to characterize a novel pathway for inositol conversion by intestinal microbes (Br. Poult. Sci. **2021**, *Nat Commun*. **2021**). In all, the labeled reagents greatly facilitate the analysis of this otherwise spectroscopically silent group of molecules, and hold great promise to comprehensively analyze soluble, inositol-based signaling molecules under normal and pathological conditions.

When lipid extracts of mammalian cells – labelled with ^{13}C-*myo*-inositol – were analysed by NMR in solution, labelled PtdIns and PtdInsP were detectable as well. However, the investigation of the *in-vivo* situation by

analysing membrane patches or even whole cells, starting with yeast as test system, lead to a surprise: even when using an inositol precursor only ^{13}C-labeled in two positions, several sets of signals were detected which covered the full spectral range expected for the desired inositol derivatives, so that a distinction became difficult. Moreover, even four sets of signals were obtained for simple, solid and pure *myo*-inositol preparations when investigated by MAS NMR at room temperature. This urged us to investigate this finding more closely, however, ways to improve the situation were so far not found. After installation of a DNP device (microwave source) for our 800 MHz NMR spectrometer we are now on the way to revisit the situation.

The Haucke lab subsequently focussed on dissecting the role of $InsP_3$ and of the $InsP_3$-specific 5-phosphatase in the regulation of cell physiology. Work within the project has essentially focussed on two main aspects of INPP5A/ $InsP_3$ function that relate to the ability of $InsP_3$ to activate $InsP_3$ receptors in the endoplasmic reticulum (ER), leading to increased cytoplasmic calcium levels. Using a combination of genetic, cell biological and biochemical approaches including lipid mass spectrometry we could show that INPP5A-depleted cells indeed accumulate $InsP_3$ well above the levels measured in control cells resulting in calcium efflux from ER stores and a blockade of clathrin-independent endocytosis of Shiga toxin. We could further demonstrate that the $InsP_3$-induced inhibition of clathrin-independent endocytosis was an indirect consequence of the depletion of cholesterol and the cholesterol-associated glycolipid Gb3 from the cell surface due to the calcium-induced dissociation of oxysterol binding protein from the Golgi complex and from membrane contact sites between the ER and the Golgi complex. A similar phenotype was observed in cells upon sustained receptor signaling, i.e. of G protein-coupled receptors. Our findings have thus uncovered a crucial function for INPP5A-mediated $InsP_3$ hydrolysis in the control of lipid exchange at membrane contact sites (Malek et al., *Nat Commun* **2021**).
In further, so far unpublished work (Malek et al, submitted) we have addressed how elevated calcium levels induced by the accumulation of $InsP_3$ alter the transcriptional landscape and, thereby, the autophagy-lysosome system. By combined light and electron microscopy as well as immunoblotting we have shown that calcium efflux from ER stores upon depletion of INPP5A leads to the induction of lysosome biogenesis. We further demonstrate that this mechanism involves the calcium-regulated protein phosphatase calcineurin and the nuclear translocation and elevated transcriptional activity of TFEB/ TFE3, key transcription factors that regulate the expression of lysosomal and autophagy genes. These findings thus reveal a crucial function for INPP5A-mediated $InsP_3$ hydrolysis in the control of lysosome biogenesis via TFEB/ TFE3, thereby contributing to our understanding how signaling-active cells are able to maintain their lysosome content under conditions of active receptor and nutrient signaling (Malek et al., submitted).
In a third set of studies, we have teamed up with the Oschkinat and Fiedler laboratories to detect alterations in membrane-integral PP-InsPs. As explained above these works so far have not led to publishable results caused by occurrence of multiple sets of signals for the employed inositol derivatives even at room temperature.

3. Results and successes

PUBLICATIONS
(group members funded by grant in bold face, corresponding author underlined)

Harnessing ^{13}C-labeled myo-inositol to interrogate inositol phosphate messengers by NMR
Harmel RK, **Puschmann R**, **Trung MN**, Saiardi A, Schmieder P, **Fiedler D**.
Chem Sci. **2019**, 10, 5267.

Scalable chemoenzymatic synthesis of inositol pyrophosphates
Puschmann R, **Harmel RK**, **Fiedler D**.
Biochemistry **2019**, 58, 3927.

Two bifunctional inositol pyrophosphate kinases/phosphatases control plant phosphate homeostasis
Zhu J, Lau K, **Harmel RK**, **Puschmann R**, Broger L, Dutta AK, Jessen HJ, Hothorn LA, **Fiedler D**, Hothorn M.
Elife **2019**, 8, e43582.

Analysis of metabolically labeled inositol phosphate messengers by NMR
Puschmann R, **Harmel RK**, **Fiedler D**.
Methods Enzymol. **2020**, 641, 35.

Delivery of myo-Inositol Hexakisphosphate to the Cell Nucleus with a Proline-Based Cell-Penetrating Peptide
Li M, **Puschmann R**, Herdlitschka A, **Fiedler D**, Wennemers H.
Angew. Chem. Int. Ed. **2020**, 59, 15586.

Analysis of Inositol Phosphates Metabolism by Capillary Electrophoresis Electrospray Ionization Mass Spectrometry (CE-ESI-MS)
Qiu D, Wilson MS, Eisenbeis VB, **Harmel RK**, Riemer E, Haas TM, Wittwer C, Jork N, Shears SB, Schaaf G, Kammerer B, **Fiedler D**, Saiardi A, Jessen HJ.
Nat Commun. **2020**, 11, 6035.

Inositol pyrophosphates promote the interaction of SPX domains with the coiled-coil motif of PHR transcription factors to regulate plant phosphate homeostasis
Ried MK, Wild R, Zhu J, Pipercevic J, Sturm K, Broger L, **Harmel RK**, Abriata LA, Hothorn LA, **Fiedler D**, Hiller S, Hothorn M.
Nat Commun. **2021**, 12, 384.

Identification of Small-Molecule Inhibitors of Human Inositol Hexakisphosphate Kinases by High-Throughput Screening
Liao G, Ye W, Heitmann T, Ernst G, DePasquale M, Xu L, Wormald M, Hu X, Ferrer M, **Harmel RK**, **Fiedler D**, Barrow J, Wei H.
ACS Pharmacol Transl Sci. **2021**, 4, 780.

ITPK1-dependent generation of inositol pyrophosphates is required for systemic regulation of phosphorus homeostasis
Riemer E, Laha D, **Harmel RK**, Gaugler P, Pries V, Frei M, Hajirezaei M, Parvin N, Krusenbaum L, Schneider R, Jessen HJ, Saiardi A, **Fiedler D**, Schaaf G, Giehl RFH.
Mol. Plant **2021**, accepted.

Investigation of a potential electrogenic transport-system for myo-inositol in the small intestine of laying hens
Röhm K, Gonzalez-Uarquin F, **Harmel RK**, Trung MN, Diener M, **Fiedler D**, Huber K, Seifert J.
Br. Poult. Sci. **2021**, accepted.

A novel pathway for inositol conversion into propionate and acetate by Anaerostipes supports its potential health impact
Bui NTP, Mannerås-Holm L, **Puschmann R**, Wu H, Troise AD, Boeren S, Bäckhed F, **Fiedler D**, de Vos WM.
Nat Commun. **2021**, accepted.

Efficiency of Water-Soluble Nitroxide Biradicals for Dynamic Nuclear Polarization in Rotating Solids at 9.4 T: bcTol-M and cyolyl-TOTAPOL as New Polarizing Agents
Geiger MA, Jagtap AP, Kaushik M, Sun H, **Stöppler D**, Sigurdsson ST, Corzilius B, **Oschkinat H.**
Chemistry **2018,** 24, 13485.

Inositol triphosphate-triggered calcium release blocks lipid exchange at endoplasmic reticulum-Golgi contact sites.
Malek M, Wawrzyniak AM, Koch P, Lüchtenborg C, Hessenberger M, Sachsenheimer T, Jang W, Brügger B, **Haucke, V.**
Nat Commun. **2021**, 12, 2673.

Inositol triphosphate-triggered calcium release from the endoplasmic reticulum triggers lysosome biogenesis via TFEB/ TFE3
Malek M, Wawrzyniak AM, Puchkov D, **Haucke V.**
Submitted

COMPLETED THESES AND DISSERTATIONS
Minh Nguyen Trung – Master thesis (Chemistry) 2018
Robert Puschmann – Ph.D. thesis (Chemistry) 2020
Robert Harmel – Ph.D. thesis (Chemistry) 2020
Daniel Stoeppler – Ph.D. thesis (Biochemistry) 2019
Rashin Roshan Bin - BSc thesis (Biochemistry) 2017

ACQUISITION OF THIRD-PARTY FUNDS

2019-2022 *The inositol polyphosphate network unraveled by theory and experiment*
EXC UniSysCat (110k € to DF)
Inositol polyphosphate phosphatase activities will be characterized *in vitro* and *in cellulo* using ^{13}C-labeled compounds and kinetic models

2019-2022 *Controlling cellular pools of inositol phosphate messengers with light*
EXC UniSysCat (220k € to DF)
Photoswitchable kinase inhibitors will be developed, aided by NMR analysis of IP6K activity using ^{13}C-labeled inositol phosphates

2020-2023 *Molecular dissection of a dynamic inositol pyrophosphate signaling pathway controlling genome stability*
Sachbeihilfe (237k € to DF)
Inhibitors targeting the ATP grasp enzyme PPIP5K will be developed, aided by NMR analysis of PPIP5K activity using ^{13}C-labeled inositol phosphates

SCIENTIFIC EVENTS

2021 *Inositol phosphates: the more the merrier*
Virtual 4-day conference, DF co-organizer

4. Equal opportunities

The FMP is committed to equality. The institute aims to increase the proportion of women scientists in management positions - among other things through active recruitment. The equal opportunities officers are always involved in selection committees for the recruitment process from the start. FMP scientists are further supported in their development by encouraging them to participate in specific programs such as the Leibniz mentoring program. An FMP women scientist was already successful in the Leibniz female professor program and was appointed to a W2 professorship together with Charité.
The FMP records the gender-specific employee structure annually and sets concrete quota targets for future years based on the cascade model. In order to promote equality, various measures have also been implemented at the institute: a parent-child room, a re-entry position for scientists after family-related time off, work in part-time and mobile work, free advice on questions and services in the area of reconciliation as well as child care. Since 2013 the institute has been certified as a family-friendly employer by the work and family audit.

5. Quality assurance

The institute continuously records various performance parameters and communicates them in regular reports. The scientific advisory board of the FMP evaluates the institute's services and makes recommendations for future planning, as an external advisory body. The Board of Trustees of the Forschungsverbund Berlin (FVB), to which the FMP belongs, is responsible for overseeing all essential matters of the association.
For the employees of the FMP, the regulations of the FVB on good scientific practice apply, which take into account both the relevant guidelines of the Leibniz Association and the "Leitlinien zur Sicherung guter wissenschaftlicher Praxis" of the DFG. Two ombudspersons are regularly elected by the staff at the FMP in accordance with the election regulations. With regard to the long-term archiving of research data, the institute has established rules that are binding for all research groups. Recently, the FMP conducted a random check of publications, with regard to the quality of images and statistical evaluations. The FMP has joined the DEAL project so that its scientists can benefit from discounted conditions for open access publishing in Wiley and Springer Nature journals. In addition, the FMP has set up an in-house fund that supports Open Access publications in journals with an Impact Factor ≥ 7 with 2000 €.

6. Additional in-kind resources

The cell culture experiments in the Fiedler lab were supported by a technician (Lena von Oertzen), who spent ca. 50% of her time on the project. The solid state NMR experiments were supported by a permanent staff member in the Oschkinat group (Matthias Herrera-Glomm), who dedicated ca. 25% of his time to the

measurements. All three research groups are supported by administrative assistants that are financed by the institute.

7. Structures and collaboration

The research proposal was comprised of a collaboration between the Fiedler, Oschkinat, and Haucke groups, all situated at the FMP. For the analysis of the soluble InsPs and PP-InsPs by NMR in solution, access to the FMP NMR facility was instrumental and close guidance by Dr. Peter Schmieder, the head of the NMR facility, was greatly appreciated.

The Fiedler lab has initiated several new collaborations based on the labelled compounds described here, and include:

- Prof. Michael Hothorn (University of Geneva, Switzerland)
- Prof. Gabriel Schaaf (University of Bonn, Germany)
- Prof. Helma Wennemers (ETH Zurich, Switzerland)
- Prof. Adolfo Saiardi (UCL London, England)
- Prof. Henning Jessen (University of Freiburg, Germany)
- Prof. James Barrow (Johns Hopkins University School of Medicine, USA)
- Prof. Willem de Vos (University of Groningen, Netherlands)

The Oschkinat has achieved the required improvement of sensitivity in DNP spectra in collaboration with a group that synthesized new radicals:

- Prof. Snorri Sigurdsson (University of Iceland)

The Haucke lab has initiated a new collaboration regarding lipidomic analysis of cells depleted of the IP_3 5-phosphatase INPP5A with the group of:

- Prof. Britta Brügger, BZH, University of Heidelberg, Germany.

8. Outlook

Given the successful implementation of ^{13}C-labeled InsPs in biochemical assays and ^{13}C-labeled *myo*-inositol for metabolic labelling studies, the Fiedler lab will continue to use these tools to further annotate inositol polyphosphate metabolic pathways. While the small molecule kinases responsible for the biosynthesis of these highly phosphorylated messengers are in large part well characterized, relatively little is known about the dephosphorylation sequences by specific phosphatases, and how these dephosphorylation pathways are regulated. It is therefore planned to investigate these dephosphorylation sequences, both in biochemical settings, as well as in complex cellular environments, using ^{13}C-labeled *myo*-inositol and inositol phosphates, in combination with NMR spectroscopy and capillary electrophoresis electrospray ionization mass spectrometry. These analytical techniques will also be applied in the investigation of the metabolism of "other" inositols, such as *scyllo*-inositol and D-*chiro*-inositol, which are currently being prepared as ^{13}C-labeled compounds. These "other" inositols have functionally been linked to neural tube defects, polycystic ovary syndrome and neurodegeneration, but the mechanistic details on molecular interactions and metabolic conversion are not known to date.
Considering everything that has been learned from our work with the labelled InsPs/PP-InsPs, the Oschkinat group will continue the investigation of inositol-labeled membrane preparations obtained from mammalian cells, considering especially pH effects on spectra of inositol phosphate derivatives. As the major aim, it is still intended to distinguish inositol lipids where we will now investigate membrane 'patches' at different pH settings. Furthermore, the DNP device at the 800 MHz spectrometer will be used for this purpose, measuring also at higher temperatures, so that a better resolution is obtained.
Research in the Haucke laboratory has led to new insights into the role of $InsP_3$ in the regulation of membrane contact sites and the regulation of lysosome biogenesis. We speculate that these functions of $InsP_3$ and the enzymes that regulate its abundance play important roles in diseases as evidenced by the fact that the $InsP_3$-specific inositol 5-phosphatase INPP5A is downregulated in cancer and defective in spinocerebellar ataxia. We further anticipate that with the refinement of NMR-based methodologies to determine the intracellular concentrations of labeled InsPs/PP-InsPs, additional insights into the interplay between cellular InsPs and PP-InsP metabolism and their role in cell and tissue homeostasis will be gained.