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Lipids and Lipidomics in Brain Injury and Diseases

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ABSTRACT

Lipidomics is systems-level analysis and characterization of lipids and their interacting moieties. The amount of information in the genomic and proteomic fields is greater than that in the lipidomics field, because of the complex nature of lipids and the limitations of tools for analysis. The main innovation during recent years that has spurred advances in lipid analysis has been the development of new mass spectroscopic techniques, particularly the "soft ionization" techniques electrospray ionization and matrix-assisted laser desorption/ionization. Lipid metabolism may be of particular importance for the central nervous system, as it has a high concentration of lipids. The crucial role of lipids in cell signaling and tissue physiology is demonstrated by the many neurological disorders, including bipolar disorders and schizophrenia, and neurodegenerative diseases such as Alzheimer's, Parkinson's, and Niemann-Pick diseases, that involve deregulated lipid metabolism. Altered lipid metabolism is also believed to contribute to cerebral ischemic (stroke) injury. Lipidomics will provide a molecular signature to a certain pathway or a disease condition. Lipidomic analyses (characterizing complex mixtures of lipids and identifying previously unknown changes in lipid metabolism) together with RNA silencing, using small interfering RNA (siRNA), may provide powerful tools to elucidate the specific roles of lipid intermediates in cell signaling and open new opportunities for drug development.

Keywords: Acrolein, arachidonic acid, cerebral ischemia, cytidine-5′-diphosphocholine, CDP-choline, citicoline, docosahexaenoic acid, 4-hydroxynonenal, lipidomics, lipid peroxidation, MALDI-TOF, ESI-MS-MS, neuroprotectin D1, 10,17S-docosatriene, phospholipases, phospholipids, RNA silencing, small interfering RNA, siRNA, reactive oxygen species, stroke

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INTRODUCTION

Phospholipids are important components of all mammalian cells and have a variety of biological functions: (1) they form lipid bilayers that provide structural integrity necessary for protein function, (2) they function as an energy reservoir (eg, triglycerides), and (3) they serve as precursors for various second messengers such as arachidonic acid, docosahexaenoic acid, ceramide, 1,2-diacylglycerol, phosphatidic acid, and lyso-phosphatidic acid. A deeper knowledge of the complexity of phospholipid metabolism will elevate our understanding of the role of lipids in maintaining normal cell physiology and how alterations in lipid metabolism contribute to various disease states.

DEFINING A LIPID

Lipids comprise an enormous number of chemically distinct molecules arising from combinations of fatty acids with various backbone structures. Indeed, it is difficult to define what lipids are. Defining lipids as a class of biological molecules that share a high degree of solubility in organic solvents is considered at once too broad and too narrow, encompassing structurally and functionally unrelated molecules and excluding lipids, such as gangliosides and phosphoinositides, that have some water solubility.[1] More acceptable is the definition of lipids as fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds. Lipids are currently classified into 8 categories—fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides—containing distinct classes and subclasses of molecules.[2] Random permutation of fatty acids with various backbone positions and combination with different head groups would theoretically produce thousands of lipid species. However, the nonrandom distribution in biological systems generates a much smaller number of molecular species than is theoretically possible. Still, ~100 molecular species can be experimentally observed in

a given class of glycerophospholipids from crude tissue extracts.[3] It has been estimated that 300 to 400 distinct species of sphingolipids exist in human tissue.[4] Overall, mammalian cells may contain ~1000 to 2000 lipid species, not counting peroxidized or damaged lipids.[3]

Conventional Lipid Analyses

The "classical" methods of lipid analysis usually involve solvent extraction of the source material followed by separation of lipids by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC). Following TLC separation, lipid classes are identified in reference to a chromatography standard. Fatty acids of the various lipids can be converted to methyl esters and quantitated by gas chromatography with detection by either flame ionization or mass analysis. Phospholipids can also be quantitated by phosphorous assays, which determine the amount of phospholipid but do not provide information on the fatty acid composition. Detection of lipids following HPLC is typically accomplished by refractive index measurements or mass analysis. These methods have the advantage of being well established for many classes of lipids. Some of these aspects have been recently reviewed.[5] However, because of the complexity of lipids and the fact that many biologically active lipid species are present in very small amounts, conventional techniques of lipid analysis are often limited by lack of sensitivity and insufficient resolution. These methods are also labor-intensive, often requiring sample purification and/or derivatization prior to analysis, thus limiting sample throughput.

Lipidomics

The emerging field of lipidomics (Figure 1) tries to define the crucial role of lipids in the cell; the research is aimed at mapping the entire lipid population in a biological system, describing the composition and biological function.[3,6-8] Lipidomics will provide a molecular signature to a certain pathway or a disease condition. The role of lipids in the formation of cell membranes makes them both ligand and substrate for proteins, suggesting that advances in lipidomics could have far-reaching implications for genomics, proteomics, and metabolomics. In 2004 the National Institute on Drug Abuse sponsored a meeting entitled Targeted Lipidomics: Signaling Lipids and Drugs of Abuse (Washington, DC, 15 to 17 April), organized by Dr Rao Rapaka; the published proceedings from the meeting contain further information and references.[9] Advances have been made in genomics (5 building blocks) and proteomics (20 building blocks); however, similar advances have not occurred in lipidomics because of the complex nature of lipids and the limitations of tools for analysis. Despite these setbacks, there have been impressive achievements during the last few years.[10-15]

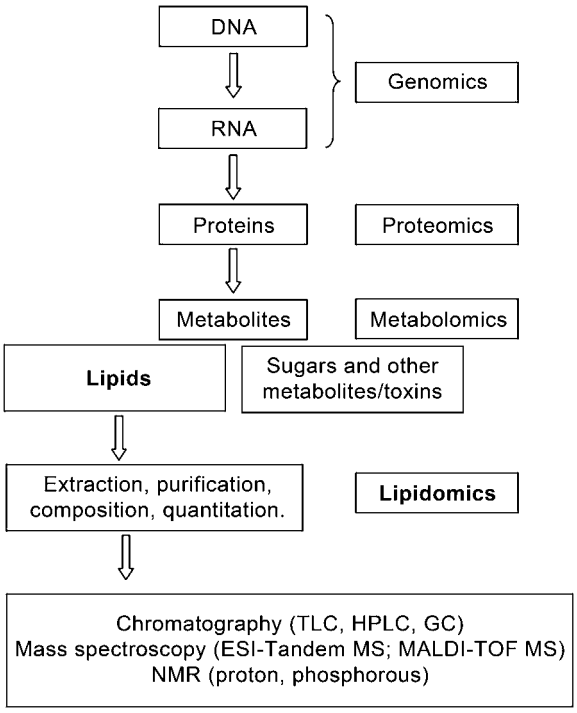

Figure 1. Evolution of genomics to lipidomics through proteomics and metabolomics. *Genomics*: mapping the entire genome (DNA and RNA sequences). *Proteomics*: identifying, sequencing and characterizing the functional protein network (the "proteome" or protein complement generated from the genome). *Metabolomics*: the comprehensive analysis of the whole metabolome under a given set of conditions. *Lipidomics*: the systems-level analysis and characterization of total lipids and their interacting moieties. TLC indicates thin-layer chromatography; HPLC, high-performance liquid chromatography; GC, gas chromatography; ESI-tandem MS, electrospray ionization–tandem mass spectroscopy; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy; NMR, nuclear magnetic resonance. Modified from Wenk.[3]

The main progress that has spurred advances in lipid analysis has been the development of new mass spectroscopic (MS) techniques, particularly the "soft ionization" techniques electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Conventional mass spectral analyses used electron impact ionization, a method widely employed for small, volatile molecules but not applicable to complex biological molecules. Other ionization techniques such as field desorption, chemical ionization, and fast atom bombardment have been introduced over the past 30 years but have had experimental limitations, including procedural complexity, poor reproducibility, the need for particular derivatization procedures, inherent matrix background signals, and only moderate sensitivity.[16] These techniques also can give rise to substantial fragmentation, so the molecular ion of the analyte is not detected. Both ESI and MALDI enable the analysis of high-molecular-weight

biomolecules without major degradation and, therefore, with a high probability of detecting the molecular ion. These methods also provide high sensitivity, and lipid analyses can be achieved with submicrogram samples. Comprehensive reviews of ESI-MS[16,17] and MALDI-TOF (time-of-flight) MS[18] and their application to lipidomics have been published.

LIPIDOMICS AND THE BRAIN

Central Nervous System Disorders

The crucial role of lipids in cell signaling and tissue physiology is demonstrated by the large number of diseases and neurological disorders in which lipid metabolism is altered.[3] Lipid metabolism may be of particular importance for the nervous system, as this organ has the second highest concentration of lipids, exceeded only by adipose tissue. Many neurological disorders, including bipolar disorders and schizophrenia, and neurodegenerative diseases such as Alzheimer's, Parkinson's and Niemann-Pick diseases, involve deregulated lipid metabolism.[3]

Focal Cerebral Ischemia (Stroke)

Stroke is the leading cause of long-lasting disability and the third leading cause of death in the United States. Approximately 3.9 million Americans are stroke survivors, and the aftereffects of stroke require more than $51 billion in health care costs annually. Presently, tissue plasminogen activator (tPA) is the only drug approved by the US Food and Drug Administration for the treatment of acute ischemic stroke; it needs to be administered within 3 hours of stroke onset.[19] However, there are some concerns that tPA has neurotoxic side effects in addition to its beneficial (thrombolytic) actions.[20] Thus, there is a pressing need to identify therapeutic targets and develop new stroke treatments. Altered lipid metabolism is believed to contribute to cerebral ischemic injury.[21-32] Lipidomics holds the promise of characterizing complex mixtures of lipids, identifying previously unknown changes in lipid metabolism, and opening new opportunities for drug development.[33]

Biochemical Changes in Cerebral Ischemia

Cerebral ischemia is characterized by an obstruction of blood flow to the brain, resulting in loss of glucose and oxygen supply. The energy needs of the brain are supplied by metabolism of glucose and oxygen for the phosphorylation of ADP to ATP. Most of the ATP generated is used in the brain in maintaining intracellular homeostasis and transmembrane ion gradients of sodium, potassium, and calcium. Energy failure results in rapid loss of ATP and uncontrolled leakage of ions across the cell membrane that results in membrane depolarization and release of the neurotransmitters such as dopamine and glutamate.[33,34] Glutamate stimulates neuronal receptors, resulting in elevated intracellular Ca^{2+} and activation of phospholipase A_2 (PLA_2) and phosphatidylinositol (PtdIns)-phospholipase C (PtdIns-PLC)[35] (Figure 2). Cerebral ischemia also results in activation of phosphatidylcholine (PtdCho)-phospholipase C (PtdCho-PLC) and PtdCho-phospholipase D (PLD).

In addition to serving as important structural components of cellular membranes, phospholipids are reservoirs of cellular messengers.[41] Activation of various phospholipases hydrolyzes membrane phospholipids to release second messengers 1,2-diacylglycerol (DAG), phosphatidic acid (PA), lyso-PA, and arachidonic acid (ArAc). ArAc enhances glutamate release as well as depolarization-evoked Ca^{2+} accumulation.[42,43] ArAc can also stimulate sphingomyelinase to produce ceramide.[44] Ceramide induces apoptosis[32,45-49] by inhibiting the mitochondrial electron transport[50] and releasing cytochrome c.[51] Cytochrome c initiates the apoptotic cellular death cascade by activation of caspase-3.[50-53]

Cardiolipin is an exclusive inner mitochondrial phospholipid enriched with unsaturated fatty acids and is essential for mitochondrial electron transport.[54] Studies have indicated that degradation of mitochondrial phospholipids also occurs and could affect the cellular energy metabolism via mitochondrial functions during ischemia/reperfusion.[13,55,56] Loss of cardiolipin has been implicated as a causative factor in mitochondrial dysfunction and release of cytochrome c.[13] Another lipid alteration implicated in initiation of apoptosis is translocation of phosphatidylserine (PtdSer) from the inner to the outer leaflet of the membrane lipid bilayer.[57,58] We have previously demonstrated significant loss of PtdCho, PtdIns, PtdSer, sphingomyelin, and cardiolipin, along with alterations in the fatty acid composition of PtdCho and PtdEtn, phosphatidyl ethanolamine, in transient cerebral ischemia.[30]

Alterations in Lipid Metabolism After Stroke

We have recently demonstrated activation of phospholipases and significant loss of PtdCho in a stroke model (transient focal cerebral ischemia).[25] PtdCho homeostasis is regulated by the balance between hydrolysis and synthesis. PtdCho is hydrolyzed by PLA_2, PtdCho-PLC, and PLD. PtdCho synthesis is rate-limited by CTP (cytidine triphosphate):phosphocholine cytidylyltransferase (CCT) that makes cytidine-5′-diphosphocholine (CDP-choline). The final step of PtdCho synthesis is catalyzed by CDP-choline:1,2-diacylglycerol choline phosphotransferase. In the brain, PtdCho synthesis is predominantly through the CDP-choline pathway. Inhibition of PtdCho synthesis through inactivation of CCT is sufficient to induce cell death.[41] Transient focal cerebral ischemia significantly

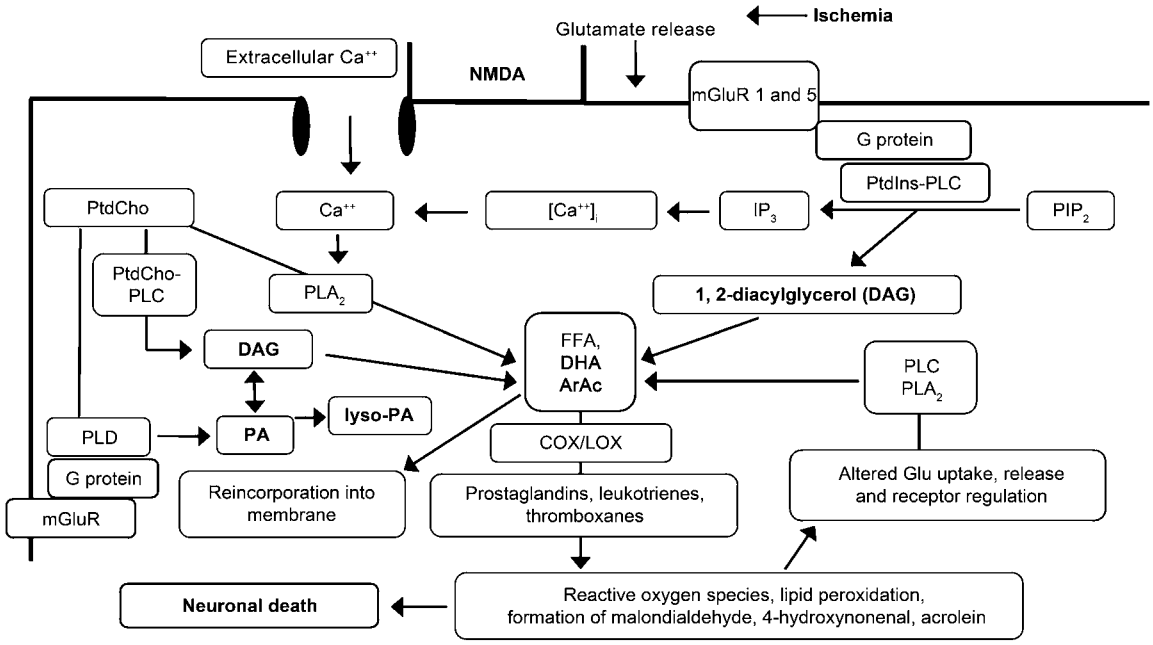

Figure 2. Lipid metabolism in ischemic neuronal death. Activation of phospholipases (PLA_2, PtdCho-PLC, PtdIns-PLC, and PLD) following cerebral ischemia results in release of lipid second messengers DAG, PA, lyso-PA, DHA, and ArAc. PA and DAG can be readily interconverted by phosphohydrolases and DAG-kinases. ArAc undergoes further metabolism by COX/LOX to generate important signaling and vasoactive eicosanoids. The DHA metabolite 10,17*S*-docosatriene serves as an endogenous neuroprotectant[15,36-40] by inhibiting the leukocyte infiltration. PLA_2 indicates phospholipase A_2; PtdCho-PLC, phosphatidylcholine-phospholipase C; PtdIns-PLC, phosphatidylinositol-phospholipase C; PLD, phospholipase D; ArAc, arachidonic acid; DAG, 1,2-diacylglycerol; PA, phosphatidic acid; lyso-PA, lyso-phosphatidic acid; DHA, docosahexaenoic acid; COX/LOX, cyclooxygenases/lipoxygenases; PIP_2, phosphatidylinositol-4,5-bisphosphate; IP_3, inositol trisphosphate; mGluR, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate; FFA, free fatty acids; Glu, glutamate.

increased PLA_2 activity, secretory PLA_2 ($sPLA_2$) IIA mRNA and protein levels, PtdCho-PLC activity, and PLD2 protein expression following reperfusion. CDP-choline treatment significantly attenuated PLA_2 activity, $sPLA_2$ IIA mRNA and protein levels, and PtdCho-PLC activity but did not affect PLD2 protein expression. Stroke also resulted in loss of CCT activity and CCTα protein that was partially restored by CDP-choline. No changes were observed in the $cPLA_2$ (cytosolic PLA_2) or calcium-independent PLA_2 protein levels. The upregulation of phospholipases (A_2, C, and D) and downregulation of CCTα collectively resulted in the loss of PtdCho, which was significantly restored by CDP-choline treatment. CDP-choline treatment significantly attenuated the infarction volume by 55% ± 5 after 1 hour of tMCAO (transient middle cerebral artery occlusion) and 1 day of reperfusion. Taken together, these results suggest that CDP-choline significantly restores PtdCho levels by differentially affecting $sPLA_2$ IIA, PtdCho-PLC, and CCTα after transient focal cerebral ischemia.

Reactive Oxygen Species and Lipid Peroxidation

ArAc released by the action of phospholipases is either reincorporated into membranes or metabolized by cyclooxygenases/lipoxygenases to form prostaglandins, leukotrienes, and reactive oxygen species (ROS) (Figure 2).[59] ROS formed by ArAc metabolism generate lipid peroxides and the cytotoxic byproducts malondialdehyde, 4-hydroxynonenal (HNE),[60] and acrolein,[61,62] which covalently bind to cellular proteins and alter their function. The time course of alterations in lipid metabolism and formation of lipid metabolites and lipid peroxidation products after transient cerebral ischemia is presented in Figure 3.[21,22,24,25,27,28,63,64]

Docosahexaenoic Acid and 10,17S-docosatriene (Neuroprotectin D1) in Stroke

Release of lipid second messengers after neuronal injury can promote either further neuronal injury or neuroprotection. A limited number of papers have been published applying lipidomic approaches to the analysis of brain lipid metabolism in stroke models or neurodegenerative diseases. Cerebral ischemia results in rapid accumulation of free fatty acids, including ArAc (20:4) and docosahexaenoic acid (DHA, 22:6), although the specific phospholipases involved in this have not been fully characterized. Free fatty acids can be released by the direct action of various PLA_2 isoforms (of which nearly 20 have been characterized[65]), by

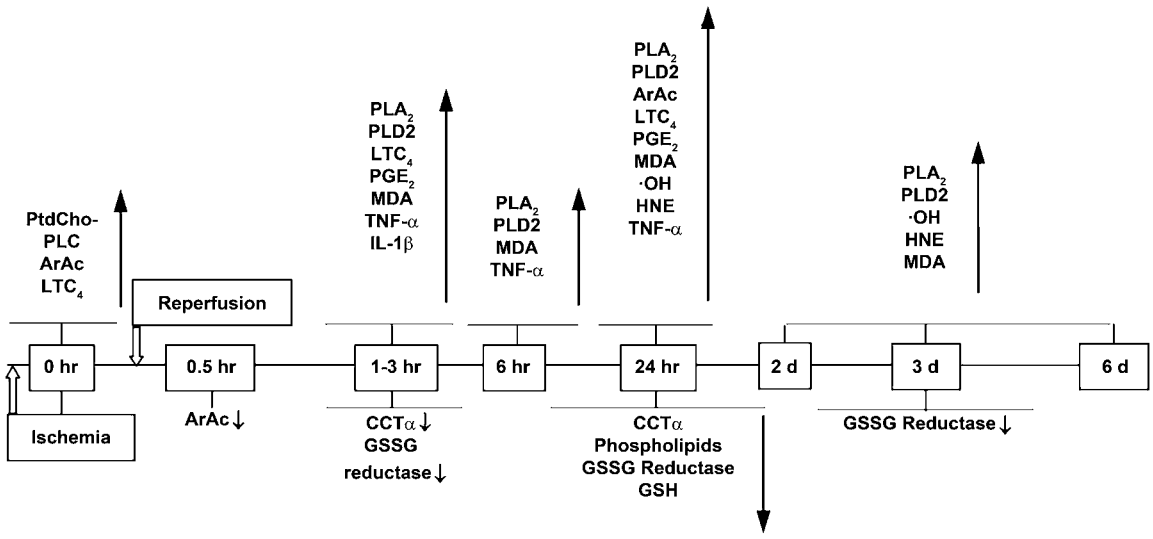

Figure 3. Time course of changes related to cytokines, lipid metabolism, and oxidative stress after transient brain ischemia.[21,22,24,25,27,28,63,64] PLA_2 enzyme activity, mRNA and protein expression, and PLD2 protein expression were increased after ischemia/reperfusion. CCT catalyzes the rate-limiting step in the biosynthesis of phosphatidylcholine. CCT activity and expression of CCTα protein decreased following ischemia/reperfusion. Activation of phospholipases and loss of CCT collectively resulted in loss of PtdCho. ↑ = increase, ↓ = decrease, compared with shams. ArAc indicates arachidonic acid; CCTα, cytidine triphosphate:phosphocholine cytidylyltransferase-α; GSSG, oxidized glutathione; HNE, 4-hydroxynonenal; IL-1β, interleukin-1β; LTC_4, leukotriene C_4; MDA, malondialdehyde; •OH, hydroxyl radical; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; PLD2, phospholipase D2; TNF-α, tumor necrosis factor-α.

hydrolysis of phospholipids by PtdCho-PLC and PtdIns-PLC to give DAG, followed by hydrolysis by DAG-lipases, or PLD to give phosphatidic acid, which can be hydrolyzed to DAG. This could also suggest that ArAc and DHA are liberated by phospholipases other than $cPLA_2$ (eg, $sPLA_2$, which has a preference for PtdEtn, which has a high content of ArAc and DHA in the brain).[30] Bazan's group has studied the role of PLA_2 and PLA_2 hydrolysis products and identified the DHA metabolite 10,17*S*-docosatriene (neuroprotectin D1 [NPD1]) following cerebral ischemia/reperfusion in mouse. NPD1 was found to serve an endogenous neuroprotective role by inhibiting apoptotic DNA damage, upregulating antiapoptotic proteins Bcl-2 and BclxL, and downregulating proapoptotic Bax and Bad expression. NPD1 also inhibited oxidative stress-induced caspase-3 activation and IL (interleukin)-1β-stimulated cyclooxygenase-2 expression.[15,36-40] Administration of albumin causes systemic mobilization of n-3 polyunsaturated fatty acids, including DHA, and provides substantial neuroprotection in models of brain ischemia and trauma. It was demonstrated that after DHA-albumin administration there was an increase in NPD1 in the ipsilateral brain at 20 hours of reperfusion after 2 hours of transient middle cerebral artery occlusion in rat.[10]

Mass Spectroscopy and Lipid Peroxidation Products

Several studies demonstrating increased lipid peroxidation, DNA oxidation, and protein oxidation in Alzheimer's disease provide an increasing amount of evidence supporting a role for oxidative damage in this disorder.[66] Beyond damage to membranes, lipid peroxides give rise to reactive α,β-unsaturated aldehydes, including HNE and acrolein. These aldehydes covalently bind to proteins through reaction with thiol groups. Recently, HPLC coupled with ESI/tandem mass spectroscopy was used to measure HNE and acrolein in several brain regions from patients affected by mild cognitive disorder and early Alzheimer's disease. These studies demonstrated increased levels of HNE and acrolein[66] in the brain tissue in both neurological disorders, indicating that lipid peroxidation occurs early in the pathogenesis of Alzheimer's disease.[66]

While generation of HNE has been demonstrated by our group[24,29] and others,[67,68] to the best of our knowledge no study has examined free acrolein formation in brain tissue after stroke. Recently, elevated levels of an acrolein-protein conjugate (N^{ϵ}-(3-formyl-3,4-dehydropiperidino)-lysine, FDP-lysine)[61,69] were demonstrated in plasma of stroke patients using ACR-Lysine Adduct ELISA System (NOF Corporation, Tokyo, Japan).[70]

In addition to its formation as a lipid peroxidation byproduct, acrolein is a ubiquitous environmental contaminant that is present in automobile exhaust and formed by the burning of fats during cooking.[71] Acrolein, by far the strongest electrophile among all α,β-unsaturated aldehydes, reacts with DNA bases including guanine, adenine, cytosine, and thymidine to form cyclic adducts, the major exocyclic adduct being acrolein-deoxyguanosine. These aldehyde-DNA

lesions may have a role in mutagenesis and carcinogenesis, and contribute to the pathogenesis of neurodegenerative diseases.[71] Analysis of adducts in DNA requires methods with high selectivity and sensitivity since the level of DNA adduction in biological samples is typically 1 adduct in 10^6 to 10^9 normal nucleotides. ^{32}P-postlabeling methods, in which γ-^{32}P-ATP of high specific activity is used to label adducted and normal nucleotides with a radioactive phosphorous group, provide high sensitivity but cannot provide structural information and require working with a high-energy isotope. Increased levels of acrolein-deoxyguanosine adducts were recently demonstrated in brain tissue from Alzheimer's disease patients using capillary HPLC coupled with nanoelectrospray isotope dilution tandem mass spectrometry.[71] This method was highly sensitive and had a detection limit of 50 adducts/10^9 nucleotides using 10 μg of DNA.

Conclusions: Lipidomics and RNA Silencing at a Crossroads

RNA interference[72,73] takes advantage of a naturally occurring process to degrade RNA, the intermediary translator between the DNA of genes and the protein molecules they encode. By degrading RNA, genes can be selectively and temporarily "turned off." RNA silencing provides a tool to analyze protein function and is a method of choice particularly for genes where transgenic knockouts are not possible because of lethality, for example CCTα[74] and PLD.[75] RNA interference may provide a powerful tool together with lipidomic analyses to elucidate the specific roles of lipid intermediates in cell signaling; already these approaches have provided crucial information that was previously unachievable.[76]

Acknowledgments

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References

1. Piomelli D. The challenge of brain lipidomics. *Prostaglandins Other Lipid Mediat*. 2005;77:23-34.

2. Fahy E, Subramaniam S, Brown HA, et al. A comprehensive classification system for lipids. *J Lipid Res*. 2005;46:839-862.

3. Wenk MR. The emerging field of lipidomics. *Nat Rev Drug Discov*. 2005;4:594-610.

4. Hannun YA, Luberto C, Argraves KM. Enzymes of sphingolipid metabolism: from modular to integrative signaling. *Biochemistry*. 2001;40:4893-4903.

5. Peterson BL, Cummings BS. A review of chromatographic methods for the assessment of phospholipids in biological samples. *Biomed Chromatogr*. 2006;20:227-243.

6. Balazy M. Eicosanomics: targeted lipidomics of eicosanoids in biological systems. *Prostaglandins Other Lipid Mediat*. 2004;73:173-180.

7. Hillard CJ. Lipids and drugs of abuse. *Life Sci*. 2005;77:1531-1542.

8. van Meer G. Cellular lipidomics. *EMBO J*. 2005;24:3159-3165.

9. Rapaka RS, Piomelli D, Spiegel S, Bazan N, Dennis EA. Targeted lipidomics: signaling lipids and drugs of abuse. *Prostaglandins Other Lipid Mediat*. 2005;77:223-234.

10. Belayev L, Marcheselli VL, Khoutorova L, et al. Docosahexaenoic acid complexed to albumin elicits high-grade ischemic neuroprotection. *Stroke*. 2005;36:118-123.

11. Forrester JS, Milne SB, Ivanova PT, Brown HA. Computational lipidomics: a multiplexed analysis of dynamic changes in membrane lipid composition during signal transduction. *Mol Pharmacol*. 2004;65:813-821.

12. Han X, Gross RW. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics. *J Lipid Res*. 2003;44:1071-1079.

13. Han X, Yang J, Cheng H, Yang K, Abendschein DR, Gross RW. Shotgun lipidomics identifies cardiolipin depletion in diabetic myocardium linking altered substrate utilization with mitochondrial dysfunction. *Biochemistry*. 2005;44:16684-16694.

14. Hunt AN, Fenn HC, Clark GT, Wright MM, Postle AD, McMaster CR. Lipidomic analysis of the molecular specificity of a cholinephosphotransferase in situ. *Biochem Soc Trans*. 2004;32:1060-1062.

15. Marcheselli VL, Hong S, Lukiw WJ, et al. Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. *J Biol Chem*. 2003;278:43807-43817.

16. Brugger B, Erben G, Sandhoff R, Wieland FT, Lehmann WD. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *Proc Natl Acad Sci USA*. 1997;94:2339-2344.

17. Pulfer M, Murphy RC. Electrospray mass spectrometry of phospholipids. *Mass Spectrom Rev*. 2003;22:332-364.

18. Schiller J, Suss R, Arnhold J, et al. Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry in lipid and phospholipid research. *Prog Lipid Res*. 2004;43:449-488.

19. Fisher M, Brott TG. Emerging therapies for acute ischemic stroke: new therapies on trial. *Stroke*. 2003;34:359-361.

20. Siao CJ, Fernandez SR, Tsirka SE. Cell type-specific roles for tissue plasminogen activator released by neurons or microglia after excitotoxic injury. *J Neurosci*. 2003;23:3234-3242.

21. Adibhatla RM, Hatcher JF, Dempsey RJ. Effects of citicoline on phospholipid and glutathione levels in transient cerebral ischemia. *Stroke*. 2001;32:2376-2381.

22. Adibhatla RM, Hatcher JF, Dempsey RJ. Phospholipase A_2, hydroxyl radicals and lipid peroxidation in transient cerebral ischemia. *Antioxid Redox Signal*. 2003;5:647-654.

23. Adibhatla RM, Hatcher JF. Citicoline decreases phospholipase A_2 stimulation and hydroxyl radical generation in transient cerebral ischemia. *J Neurosci Res*. 2003;73:308-315.

24. Muralikrishna Adibhatla R, Hatcher JF. Phospholipase A_2, reactive oxygen species, and lipid peroxidation in cerebral ischemia. *Free Radic Biol Med*. 2006;40:376-387.

25. Adibhatla RM, Hatcher JF, Larsen EC, Chen X, Sun D, Tsao FH. CDP-choline significantly restores phosphatidylcholine levels by differentially affecting phospholipase A_2 and CTP-phosphocholine cytidylyltransferase after stroke. *J Biol Chem*. 2006;281:6718-6725.

26. Lipton P. Ischemic cell death in brain neurons. *Physiol Rev*. 1999;79:1431-1568.

27. Rao AM, Hatcher JF, Kindy MS, Dempsey RJ. Arachidonic acid and leukotriene C_4: role in transient cerebral ischemia of gerbils. *Neurochem Res*. 1999;24:1225-1232.

28. Rao AM, Hatcher JF, Dempsey RJ. CDP-choline: neuroprotection in transient forebrain ischemia of gerbils. *J Neurosci Res*. 1999;58:697-705.

29. Rao AM, Hatcher JF, Dempsey RJ. Lipid metabolism in ischemic neuronal death. *Recent Res Develop Neurochem*. 1999;2:533-549.

30. Rao AM, Hatcher JF, Dempsey RJ. Lipid alterations in transient forebrain ischemia: possible new mechanisms of CDP-choline neuroprotection. *J Neurochem*. 2000;75:2528-2535.

31. Rao AM, Hatcher JF, Dempsey RJ. Does CDP-choline modulate phospholipase activities after transient forebrain ischemia? *Brain Res*. 2001;893:268-272.

32. Sastry PS, Rao KS. Apoptosis and the nervous system. *J Neurochem*. 2000;74:1-20.

33. Adibhatla RM, Hatcher JF. Cytidine 5′-diphosphocholine (CDP-choline) in stroke and other CNS disorders. *Neurochem Res*. 2005;30:15-23.

34. Ter Horst GJ, Korf J, eds. *Clinical Pharmacology of Cerebral Ischemia.* Totowa, NJ: Humana; 1997.

35. Choi DW. Methods for antagonizing glutamate neurotoxicity. *Cerebrovasc Brain Metab Rev*. 1990;2:105-147.

36. Bazan NG. Synaptic lipid signaling: significance of polyunsaturated fatty acids and platelet-activating factor. *J Lipid Res*. 2003;44:2221-2233.

37. Bazan NG. Neuroprotectin D1 (NPD1): a DHA-derived mediator that protects brain and retina against cell injury-induced oxidative stress. *Brain Pathol*. 2005;15:159-166.

38. Bazan NG, Marcheselli VL, Cole-Edwards K. Brain response to injury and neurodegeneration: endogenous neuroprotective signaling. *Ann N Y Acad Sci*. 2005;1053:137-147.

39. Lukiw WJ, Cui J-G, Marcheselli VL, et al. A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease. *J Clin Invest*. 2005;115:2774-2783.

40. Mukherjee PK, Marcheselli VL, Serhan CN, Bazan NG. Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proc Natl Acad Sci USA*. 2004;101:8491-8496.

41. Cui Z, Houweling M. Phosphatidylcholine and cell death. *Biochim Biophys Acta*. 2002;1585:87-96.

42. Freeman EJ, Terrian DM, Dorman RV. Presynaptic facilitation of glutamate release from isolated hippocampal mossy fiber nerve endings by arachidonic acid. *Neurochem Res*. 1990;15:743-750.

43. Ruehr ML, Zhang L, Dorman RV. Lipid-dependent modulation of Ca^{2+} availability in isolated mossy fiber nerve endings. *Neurochem Res*. 1997;22:1215-1222.

44. Jayadev S, Linardic CM, Hannun YA. Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to TNF-α. *J Biol Chem*. 1994;269:5757-5763.

45. Hannun YA, Obeid LM. Ceramide: an intracellular signal for apoptosis. *Trends Biochem Sci*. 1995;20:73-77.

46. Green DR, Reed JC. Mitochondria and apoptosis. *Science*. 1998;281:1309-1312.

47. Perry DK, Hannun YA. The role of ceramide in cell signaling. *Biochim Biophys Acta*. 1998;1436:233-243.

48. Kinloch RA, Treherne JM, Furness LM, Hajimohamadreza I. The pharmacology of apoptosis. *Trends Pharmacol Sci*. 1999;20:35-42.

49. Goswami R, Dawson G. Does ceramide play a role in neural cell apoptosis? *J Neurosci Res*. 2000;60:141-149.

50. Garcia-Ruiz C, Colell A, Mari M, Morales A, Fernandezcheca JC. Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species—role of mitochondrial glutathione. *J Biol Chem*. 1997;272:11369-11377.

51. Ghafourifar P, Klein SD, Schucht O, et al. Ceramide induces cytochrome c release from isolated mitochondria. Importance of mitochondrial redox state. *J Biol Chem*. 1999;274:6080-6084.

52. Cai J, Yang J, Jones DP. Mitochondrial control of apoptosis—the role of cytochrome c. *Biochim Biophys Acta*. 1998;1366:139-149.

53. Cai J, Jones DP. Superoxide in apoptosis—mitochondrial generation triggered by cytochrome c loss. *J Biol Chem*. 1998;273: 11401-11404.

54. Hoch FL. Cardiolipins and biomembrane function. *Biochim Biophys Acta*. 1992;1113:71-133.

55. Nakahara I, Kikuchi H, Taki W, et al. Degradation of mitochondrial phospholipids during experimental cerebral ischemia in rats. *J Neurochem*. 1991;57:839-844.

56. Nakahara I, Kikuchi H, Taki W, et al. Changes in major phospholipids of mitochondria during postischemic reperfusion in rat brain. *J Neurosurg*. 1992;76:244-250.

57. Rimon G, Bazenet CE, Philpott KL, Rubin LL. Increased surface phosphatidylserine is an early marker of neuronal apoptosis. *J Neurosci Res*. 1997;48:563-570.

58. Suzuki S, Furushiro M, Takahashi M, Sakai M, Kudo S. Oral administration of soybean lecithin transphosphatidylated phosphatidylserine (SB-tPS) reduces ischemic damage in the gerbil hippocampus. *Jpn J Pharmacol*. 1999;81:237-239.

59. Katsuki H, Okuda S. Arachidonic acid as a neurotoxic and neurotrophic substance. *Prog Neurobiol*. 1995;46:607-636.

60. Kruman I, Brucekeller AJ, Bredesen D, Waeg G, Mattson MP. Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J Neurosci*. 1997;17:5089-5100.

61. Uchida K, Kanematsu M, Sakai K, et al. Protein-bound acrolein—potential markers for oxidative stress. *Proc Natl Acad Sci USA*. 1998;95:4882-4887.

62. Calingasan NY, Uchida K, Gibson GE. Protein-bound acrolein: a novel marker of oxidative stress in Alzheimer's disease. *J Neurochem*. 1999;72:751-756.

63. Adibhatla RM, Hatcher JF. Citicoline mechanisms and clinical efficacy in cerebral ischemia. *J Neurosci Res*. 2002;70:133-139.

64. Adibhatla RM, Hatcher JF, Dempsey RJ. Cytidine-5′-diphosphocholine (CDP-choline) affects CTP:phosphocholine cytidylyltransferase and lyso-phosphatidylcholine after transient brain ischemia. *J Neurosci Res*. 2004;76:390-396.

65. Kudo I. Diversity of phospholipase A_2 enzymes. Foreword. *Biol Pharm Bull*. 2004;27:1157.

66. Williams TI, Lynn BC, Markesbery WR, Lovell MA. Increased levels of 4-hydroxynonenal and acrolein, neurotoxic markers of lipid peroxidation, in the brain in mild cognitive impairment and early Alzheimer's disease. *Neurobiol Aging*. In press.

67. Urabe T, Hattori N, Yoshikawa M, Yoshino H, Uchida K, Mizuno Y. Colocalization of Bcl-2 and 4-hydroxynonenal modified proteins in microglial cells and neurons of rat brain following transient focal ischemia. *Neurosci Lett*. 1998;247:159-162.

68. Urabe T, Yamasaki Y, Hattori N, Yoshikawa M, Uchida K, Mizuno Y. Accumulation of 4-hydroxynonenal-modified proteins in hippocampal CA_1 pyramidal neurons precedes delayed neuronal damage in the gerbil brain. *Neuroscience*. 2000;100:241-250.

69. Uchida K, Kanematsu M, Morimitsu Y, Osawa T, Noguchi N, Niki E. Acrolein is a product of lipid peroxidation reaction—formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins. *J Biol Chem*. 1998;273:16058-16066.

70. Tomitori H, Usui T, Saeki N, et al. Polyamine oxidase and acrolein as novel biochemical markers for diagnosis of cerebral stroke. *Stroke*. 2005;36:2609-2613.

71. Liu X, Lovell MA, Lynn BC. Development of a method for quantification of acrolein-deoxyguanosine adducts in DNA using isotope dilution-capillary LC/MS/MS and its application to human brain tissue. *Anal Chem*. 2005;77:5982-5989.

72. Cejka D, Losert D, Wacheck V. Short interfering RNA (siRNA): tool or therapeutic? *Clin Sci (Lond)*. 2006;110:47-58.

73. Dykxhoorn DM, Palliser D, Lieberman J. The silent treatment: siRNAs as small molecule drugs. *Gene Ther*. 2006;13:541-552.

74. Wang L, Magdaleno S, Tabas I, Jackowski S. Early embryonic lethality in mice with targeted deletion of the CTP:phosphocholine cytidylyltransferase alpha gene (Pcyt1a). *Mol Cell Biol*. 2005;25:3357-3363.

75. Klein J. Functions and pathophysiological roles of phospholipase D in the brain. *J Neurochem*. 2005;94:1473-1487.

76. Pettus BJ, Bielawski J, Porcelli AM, et al. The sphingosine kinase 1/sphingosine-1-phosphate pathway mediates COX-2 induction and PGE2 production in response to TNF-α. *FASEB J*. 2003;17:1411-1421.