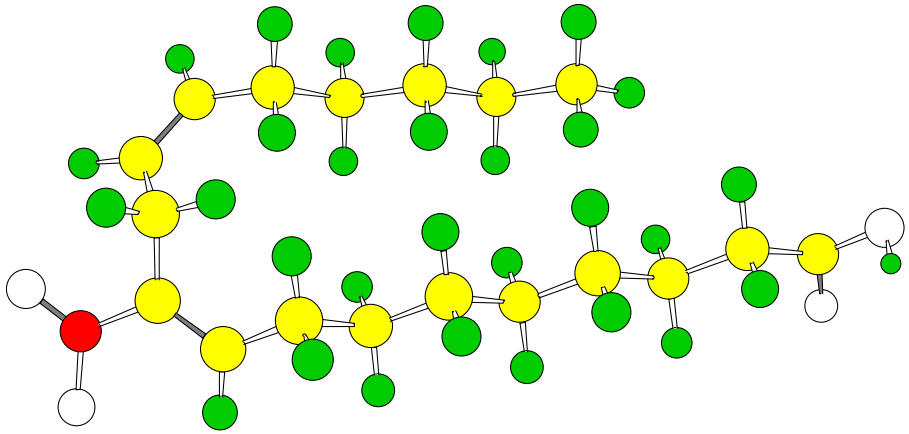
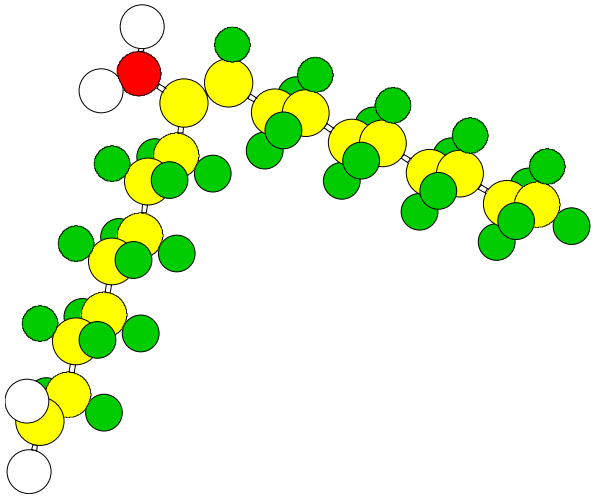
Nitrated Fatty Acids
Formation and Actions

Bruce Freeman, PhD
Department of Pharmacology
University of Pittsburgh School of Medicine



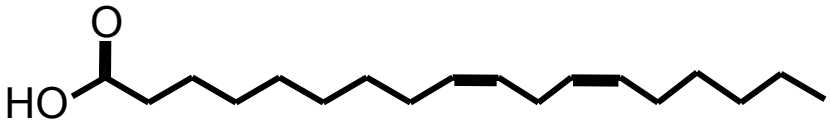


Objectives of Course Lecture and Supplied Materials

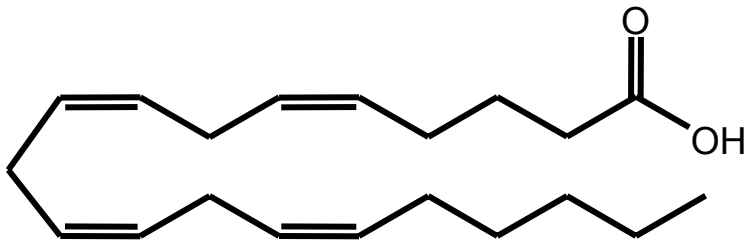
- **Discuss the biological formation and structural characterisitics of nitrated fatty acid derivatives**
- **Teach how to synthesize, purify, quantify and handle nitrated fatty acids**
- **Convey present knowledge about the cell signaling actions of nitrated fatty acids**

Fatty Acids

Fatty acids have common names, IUPAC names and acronyms



Linoleic Acid
Octadeca-9,12-dienoic acid
18:2 LA



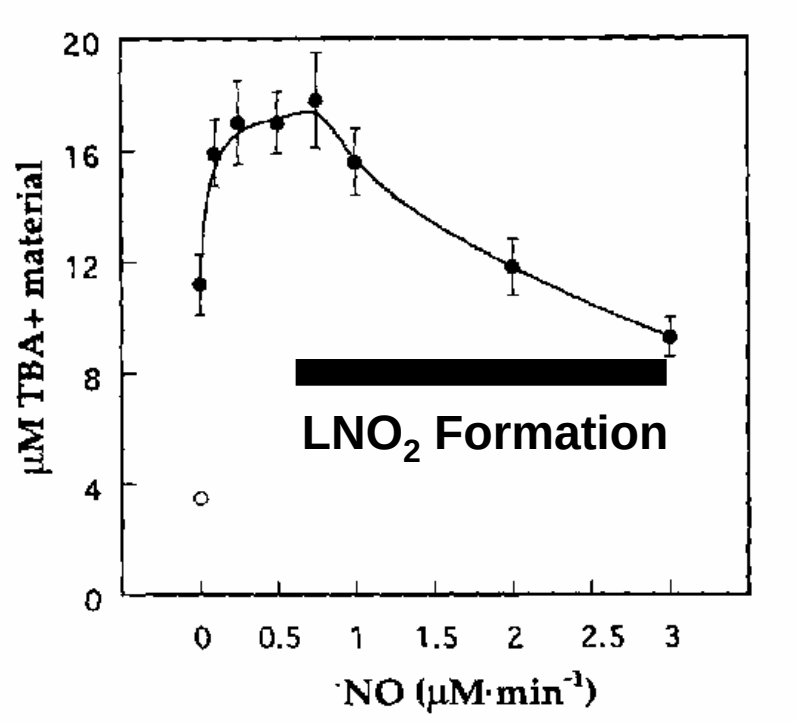
Arachidonic Acid
Eicosatetraenoic Acid
Dodeca-5,8,11,14-tetraenoic acid
20:4
AA

Myristic Acid (14:0)
Palmitic Acid (16:0)
Stearic Acid (18:0)
Oleic Acid (18:1)

Linolenic Acid (18:3)
Arachidic Acid (20:0)
Docosahexanoic acid (22:6)

Endogenous fatty acids have the *cis*-stereochemical orientation

A Crucial Experiment



- **NO manifests both pro-oxidant and antioxidant actions**
- **Reactions of NO-derived species with oxidizing unsaturated fatty acids yields NO_2 derivatives**

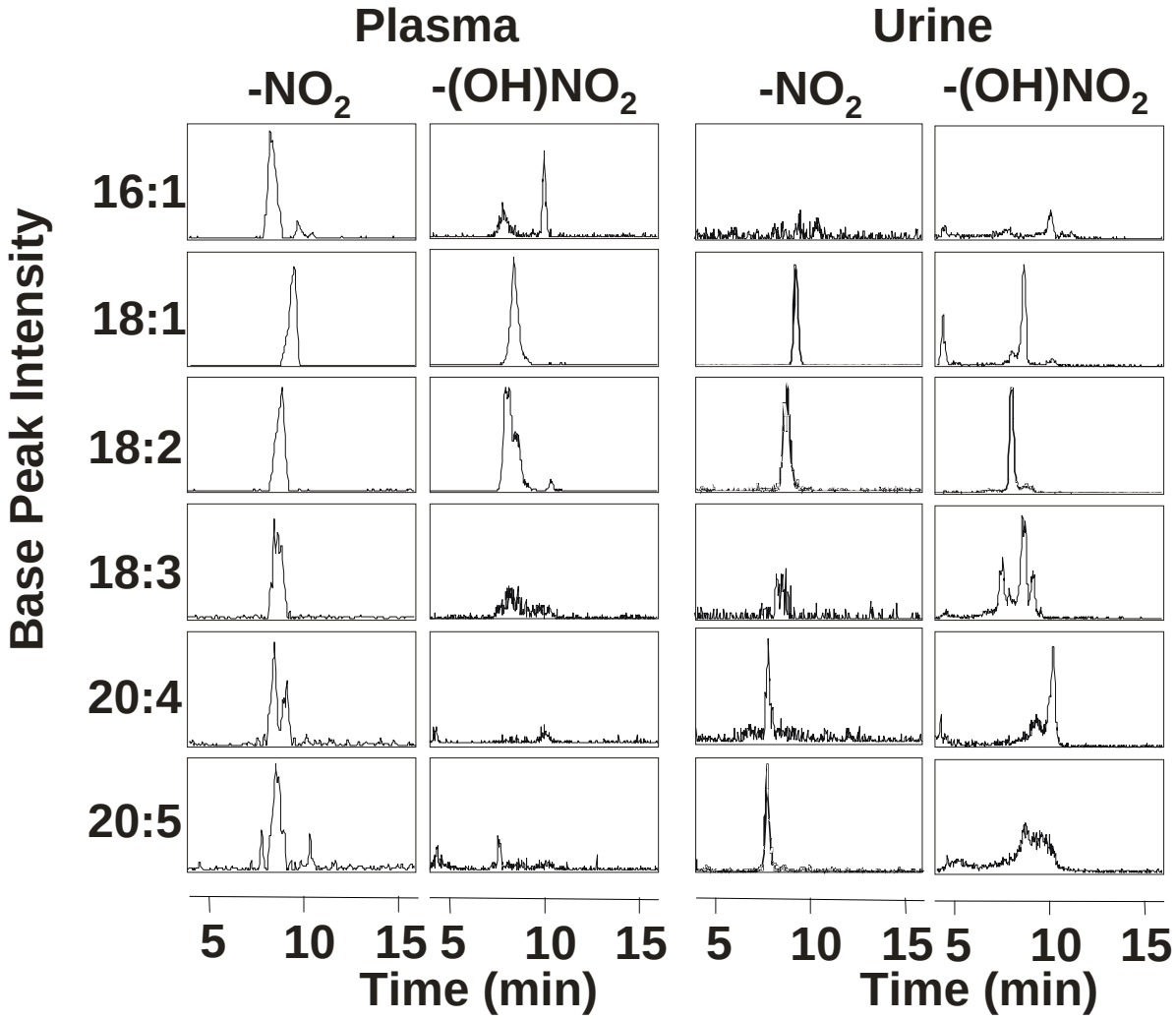
J Biol Chem 269:28066-28075, 1994

Nitrated Fatty Acids – Most Abundant Bioactive Oxides of Nitrogen in the Vascular Compartment

Species	Compartment	Fraction	Concentration (nM)
NO_2^-	Plasma	Total	205 ± 21
RSNO	Plasma	Total	7.2 ± 1.1
3-Nitro-tyrosine	Plasma	Total	0.7 ± 0.3
Hb-NO	Blood	Total	< 50
Hb-SNO	Blood	Total	< 50
LNO_2	Plasma	Free	79 ± 35
		Esterified	550 ± 275
		Total	630 ± 240
$OANO_2$	Plasma	Free	619 ± 52
		Esterified	302 ± 369
		Total	921 ± 421
LNO_2	Packed red cells	Free	50 ± 17
		Esterified	199 ± 121
		Total	249 ± 104
ONO_2	Packed red cells	Free	59 ± 11
		Esterified	155 ± 65
		Total	214 ± 76
LNO_2	Blood	Total	477 ± 128*
$OANO_2$	Blood	Total	639 ± 366*

PNAS 101:11577-11582, 2004
J Biol Chem, 2005

All Unsaturated Fatty Acid Species Display Nitrated Derivatives In Healthy Human Plasma and Urine



J Biol Chem, in press 2005

Inflammatory Conditions Induce Oleic Acid Nitration
Fatty Acid Micellar Emulsion

Condition	NO_2-oleic acid (nM)
Control	0.12
NO_2^-, pH 3.0	0.76*
MPO/H_2O_2/ NO_2^-	1.45*
$ONOO^-$	3.31*

MPO- 50 nM, H_2O_2- 600 µM, NO_2^-- 100 µM, $ONOO^-$- 1.5 mM

Linoleic acid nitration results can be found in: O'Donnell *et al.* Chem Res Tox 12: 83-92, 1999.

* p< 0.05

J Biol Chem, in press 2005

Inflammatory Conditions Induce Fatty Acid Nitration

Intraperitoneal Injection of LPS in Mice

Condition	NO_2-oleic acid	NO_2-linoleic acid
Plasma (nM)		
Control	38	29
LPS	323*	120*
Lung (nmol/g)		
Control	0.017	0.006
LPS	0.066*	0.030*
Heart (nmol/g)		
Control	0.017	0.014
LPS	0.060*	0.044*
Kidney (nmol/g)		
Control	0.030	0.009
LPS	0.215*	0.069*

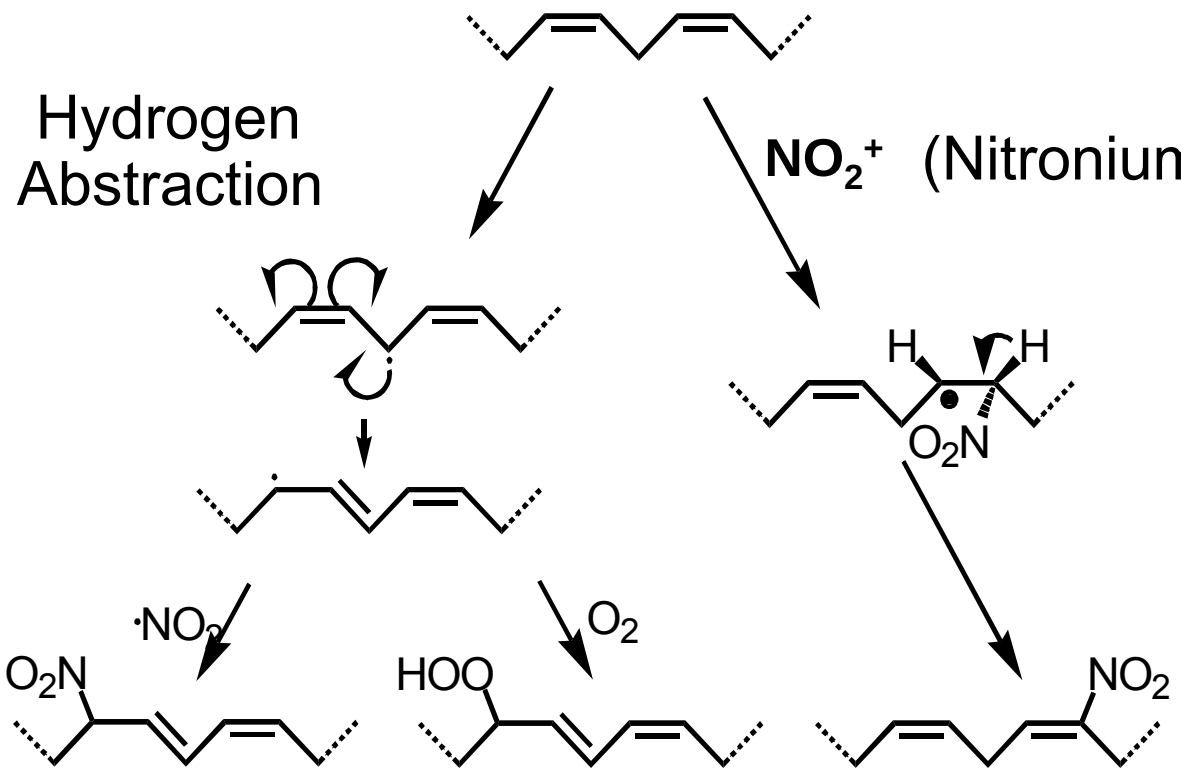
* p< 0.05, 3-4 animals

Inflammatory Conditions Induce Fatty Acid Nitration
Acute Respiratory Distress Syndrome Patients

Condition	NO_2-oleic acid	NO_2-linoleic acid
Matched plasma (nmol/mg prot)	-	3.0 ± 0.7
Pulmonary edema fluid	-	45 ± 6*

* p< 0.05

Mechanisms of Fatty Acid Nitration

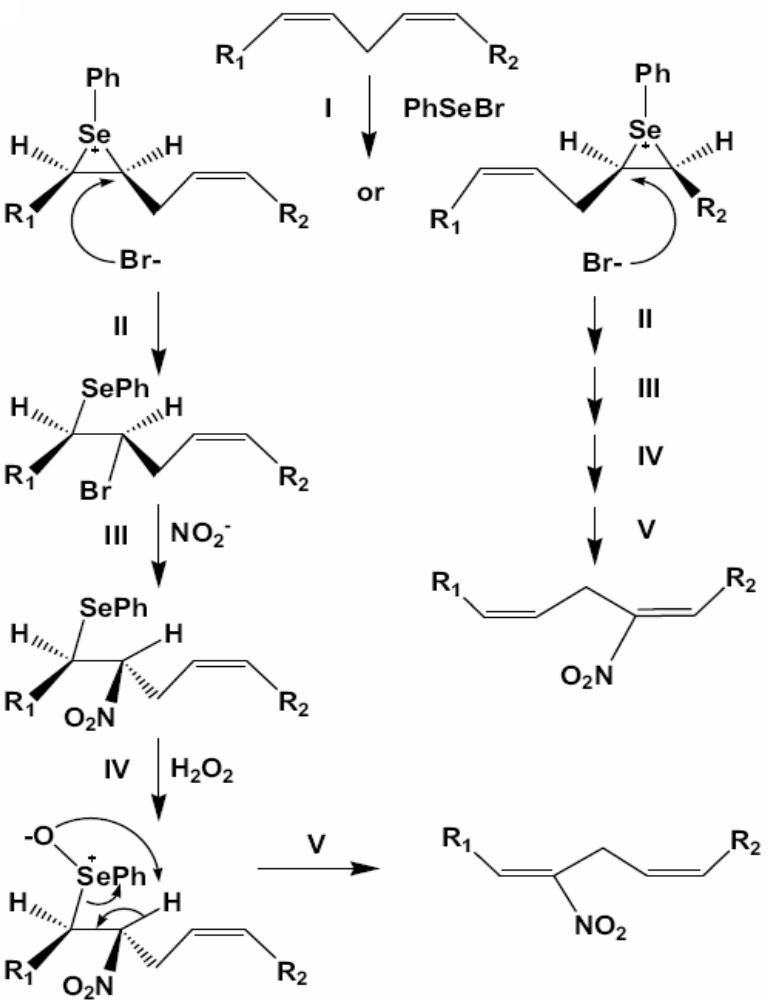


- **Dietary sources**
- **Acidic nitration**
- **Enzymatic?**

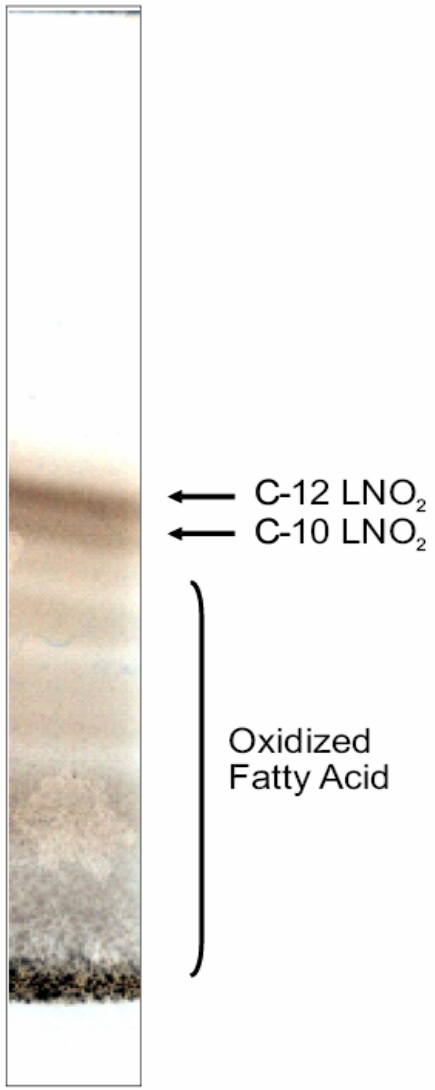
Why Were Nitrated Unsaturated Fatty Acids (Nitroalkenes) Not Identified and Studied Sooner?

- First identified in 1912 (Zelinsky), 1991 (Finlayson-Pitts)
- To observe clinically-occurring nitrated fatty acids, required development of sensitive MS capabilities
- Decay rapidly with traditional lipid extraction and FA derivatization techniques used for GC-MS
- NO_2 group leaves peptides as *m/z* = 45 in positive ion mode, leaves NO_2-FA adducts as *m/z* = 47
- NO_2-FA adducts of proteins “lost” with typical thiol-based protein reduction and electrophoresis strategies
- NO_2-FA adducts of proteins are retained on column with typical acetonitrile gradients used for peptide separation

Synthesis of Nitrated Linoleic Acid



PNAS 101:11577-11582, 2004



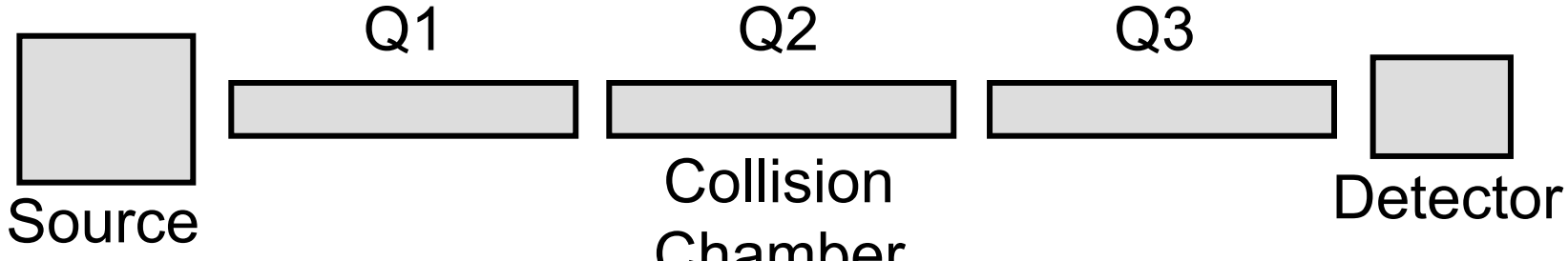
Detection/Characterization of LNO_2

Method	Results	Comments
GC MS (EI)	**Structural data from standard**	1. Very low sensitivity 2. Derivatization
GC MS (NICI)	***In vivo* detection of LNO_2**	1. Good sensitivity 2. Extensive sample preparation 3. Sample degradation
3-D Ion Trap ESI MS/MS	**Structural data from standards**	1. Low sensitivity 2. MS3 and above, not realistic

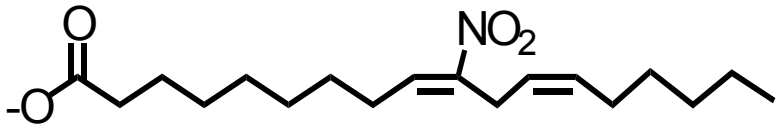
→ Unable to characterize/quantitate *in vivo* samples

Triple Quadrupole MS

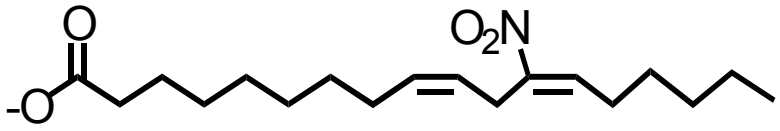
- **MS** – Scan specific mass range in either Q1 or Q3
- **Product Ion Analysis** – Set Q1 for specific mass; Q3 for product range
- **Precursor Ion Analysis** – Set Q3 for a specific product mass; Q1 for precursor range
- **Multiple Reaction Monitoring** (MRM) – Set Q1 for a specific precursor; set Q3 for a specific product



HPLC Elution Profile for LNO_2 Regioisomers

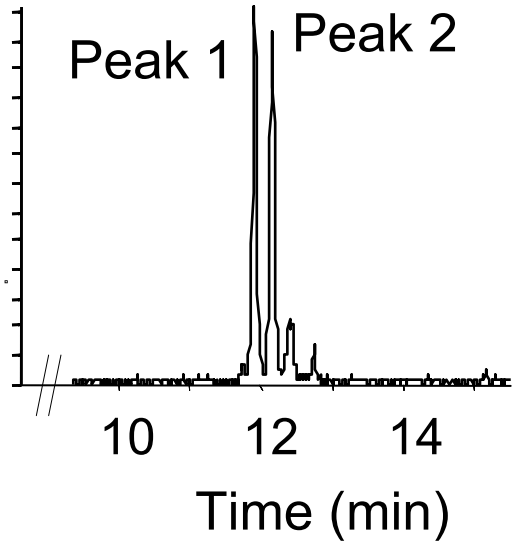


Peak 2

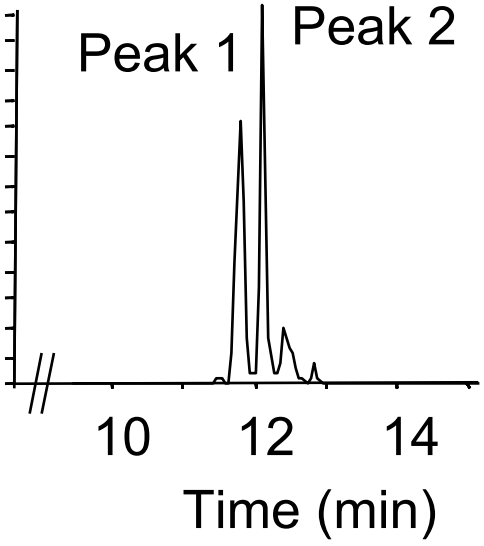


Peak 1

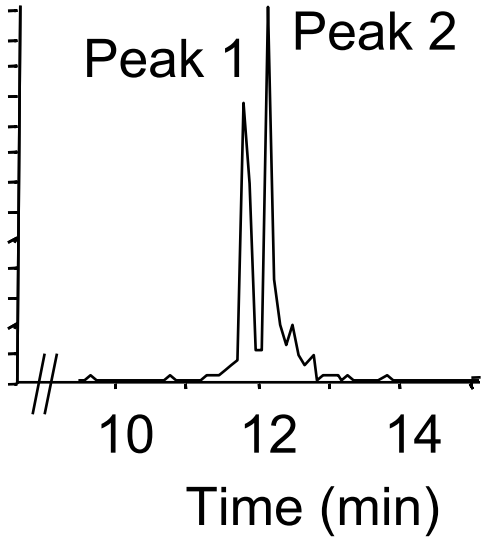
Synthetic LNO_2



Red Cell



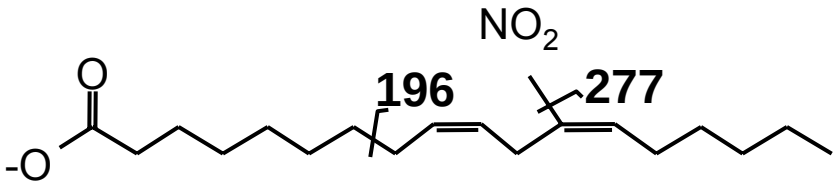
Plasma

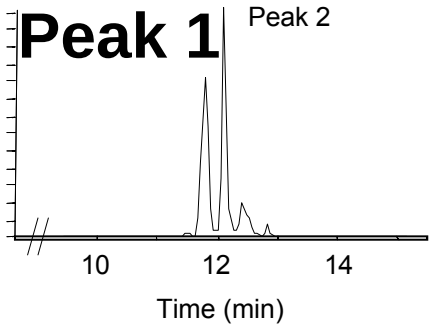


MRM transition: 324 $[M-H]^-$ / 277 $[M-HNO_2]^-$

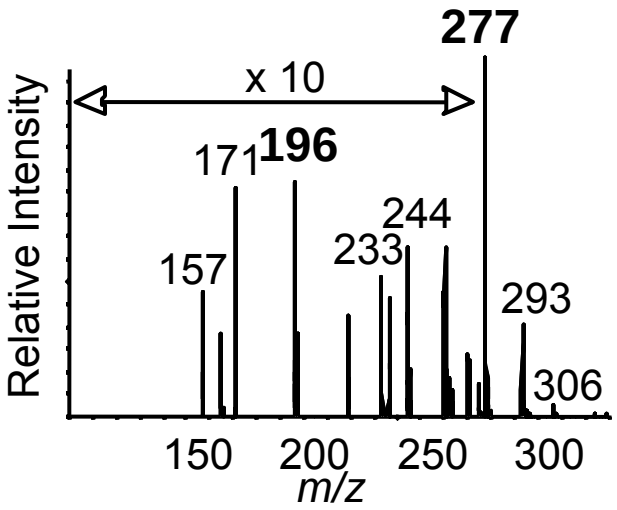
Product Ion Analysis of LNO_2 Regioisomers Peak 1

PNAS 101:11577-11582, 2004

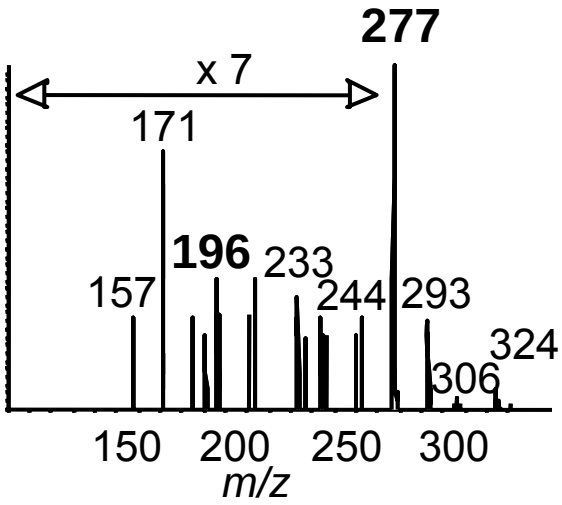




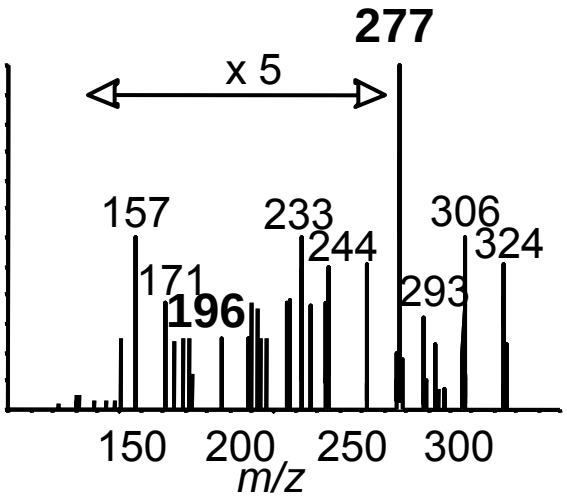
Standard



Red Blood Cell

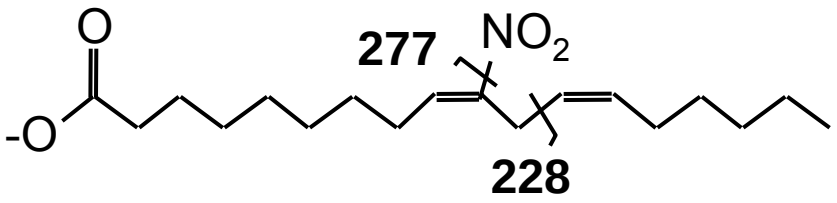


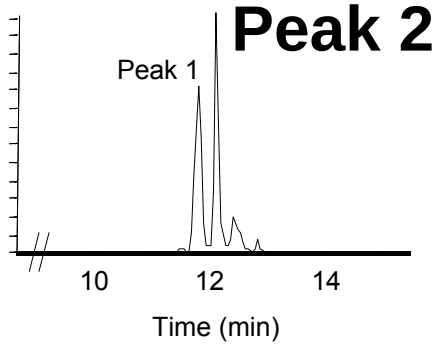
Plasma



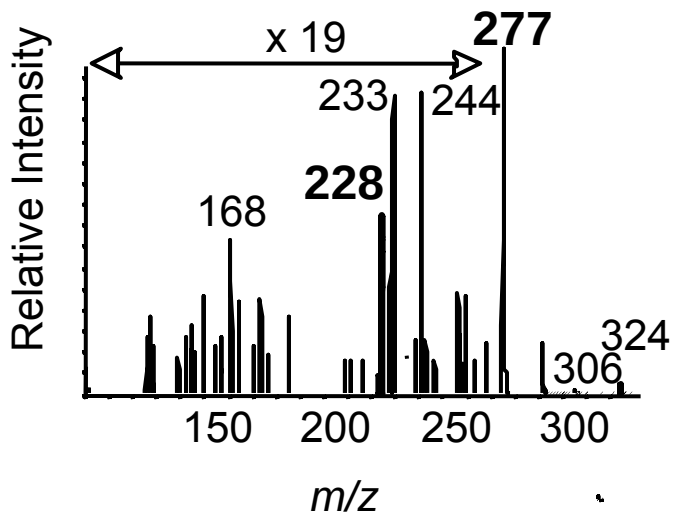
Product Ion Analysis of LNO_2 Regioisomers Peak 2

PNAS 101:11577-11582, 2004

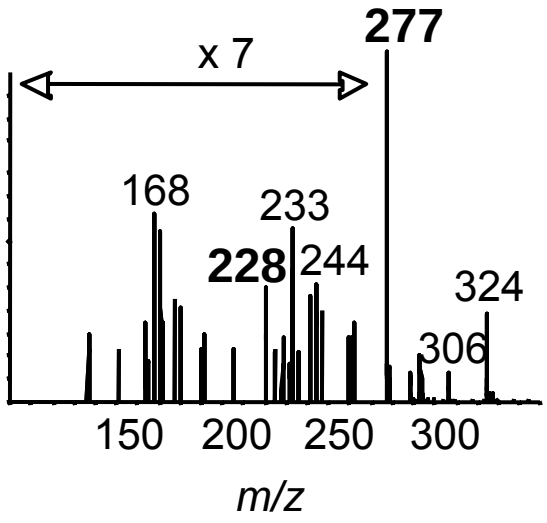




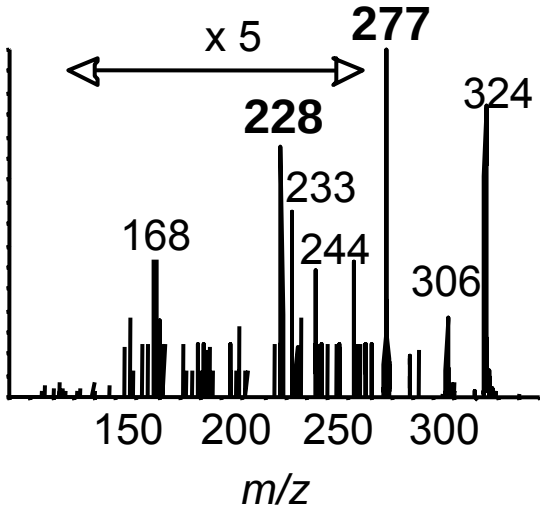
Standard



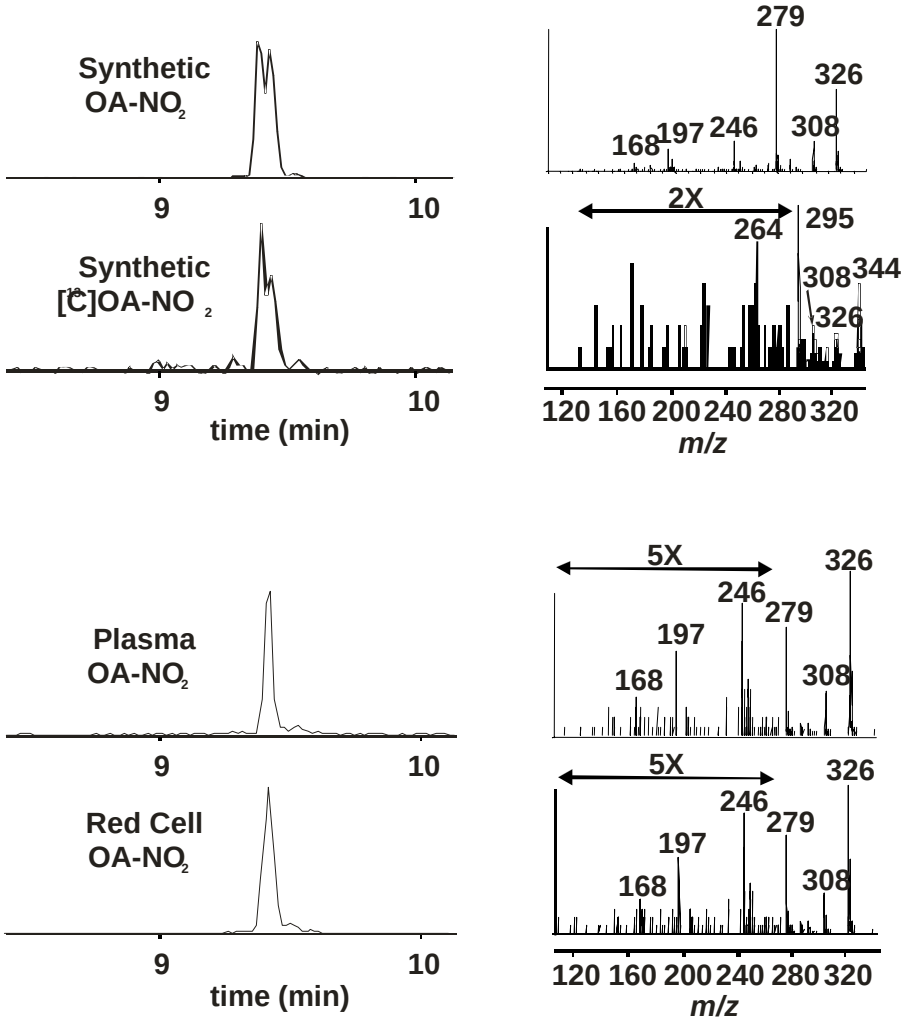
Red Blood Cell



Plasma



Nitrated Oleic Acid ($OA\text{-}NO_2$) is Also Present in Human Blood

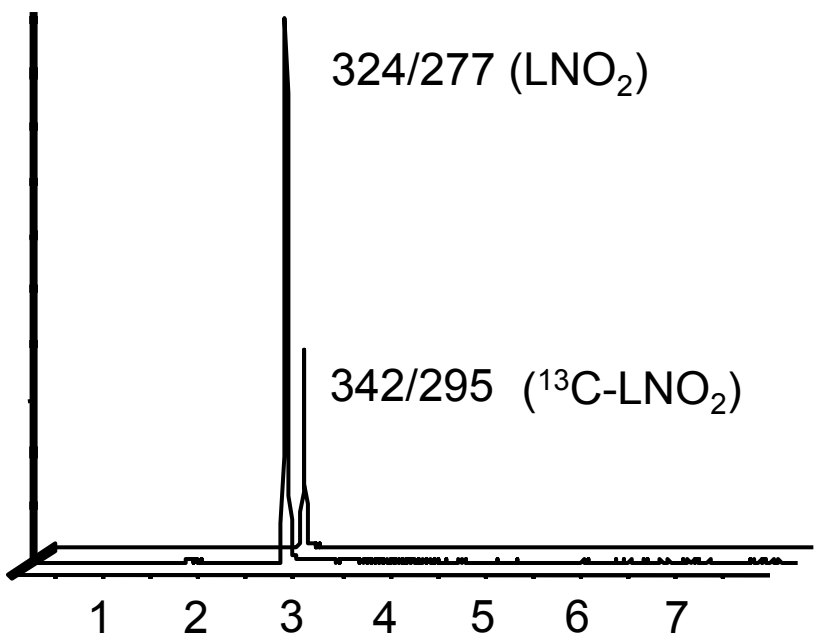


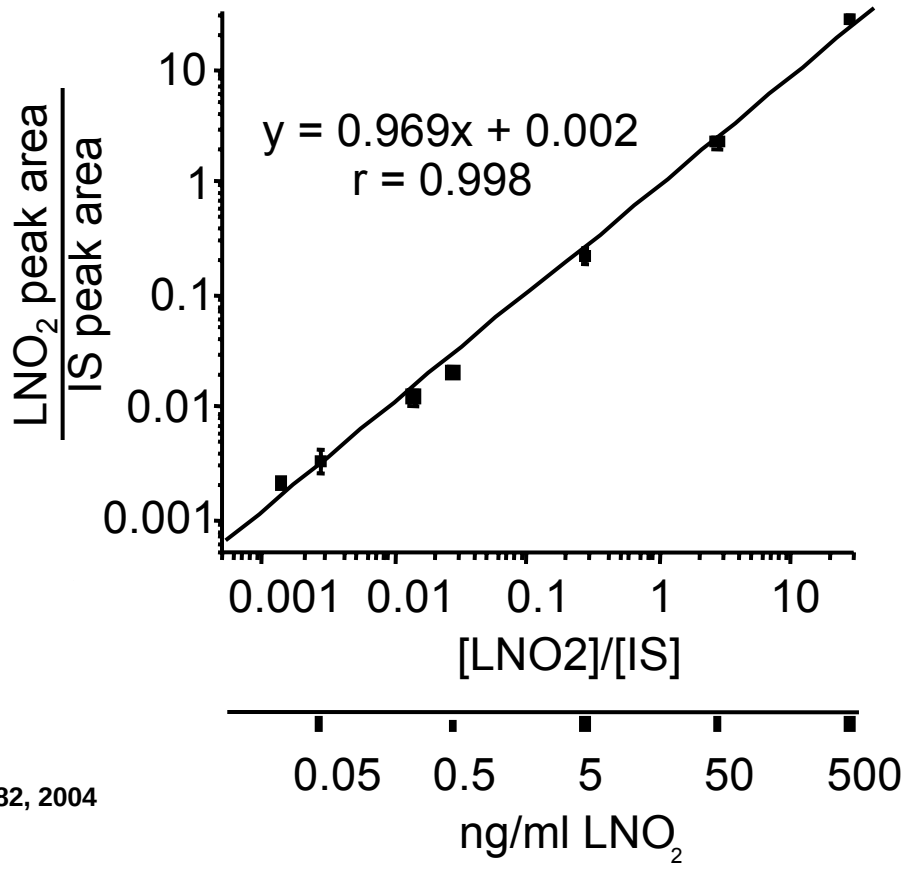
J Biol Chem, in press 2005

MS-Based Quantitation of NO_2-FA

- Include [^{13}C]-fatty acid and [^{15}N]-nitrite during sample processing and analysis to detect artifactual fatty acid nitration
    - Evaluate ranges of pH and adventitious NO_2^-
    - Evaluate impact of oxidized lipid derivatives
- Use [^{13}C]LNO_2 and [^{13}C]NO_2-OA as internal standards to correct for sample preparation loss
- Internal standard curve linear over 5-orders of magnitude; limit of quantitation = 300 amol on column

Quantitative Analysis of LNO_2 in Clinical Samples



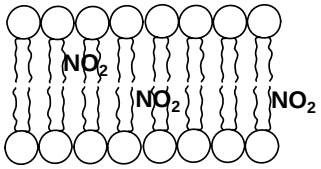


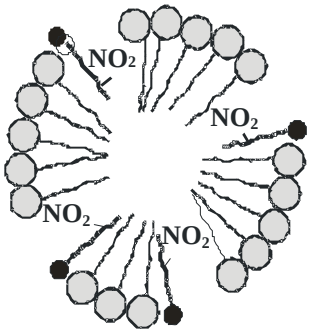
PNAS 101:11577-11582, 2004

LOQ ~ 300 amol on column

Standard curve linear over 5-orders of magnitude

Complex Lipid Nitro-Fatty Acid Derivatives Membrane and Lipoprotein Reservoirs?

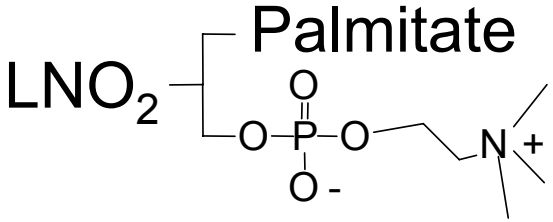




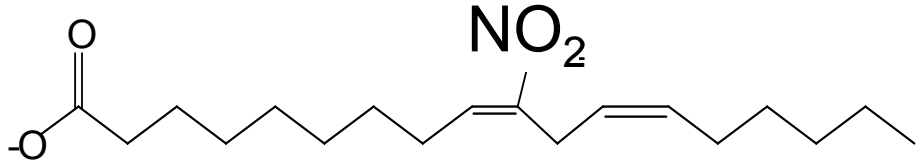
"Stable" LNO_2 Storage

Linoleic acid represents ~10% of net fatty acids in cells, with ~0.1-1.0% of net linoleate nitrated

~10-100,000 molecules LNO_2/cell

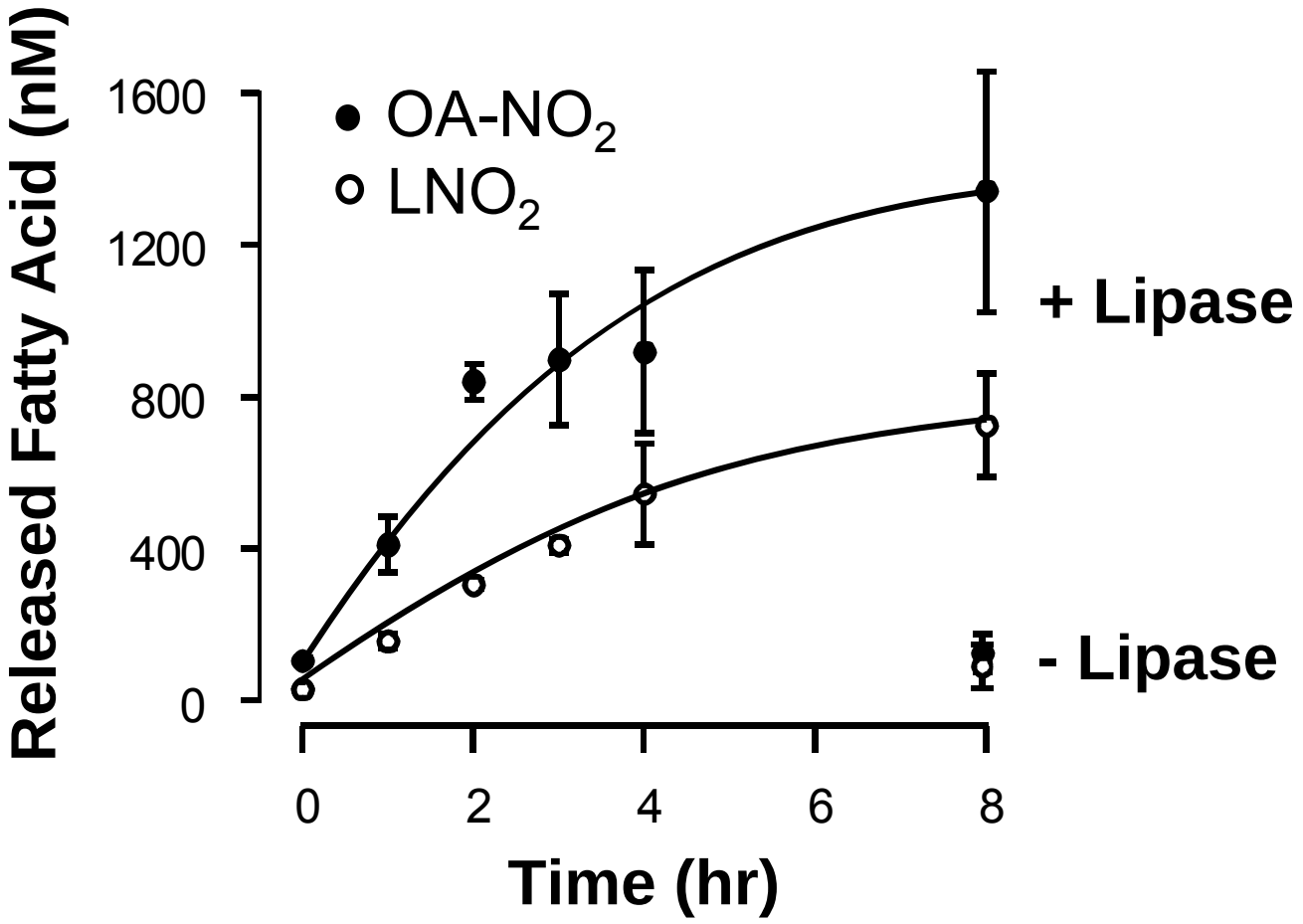


PLA_2

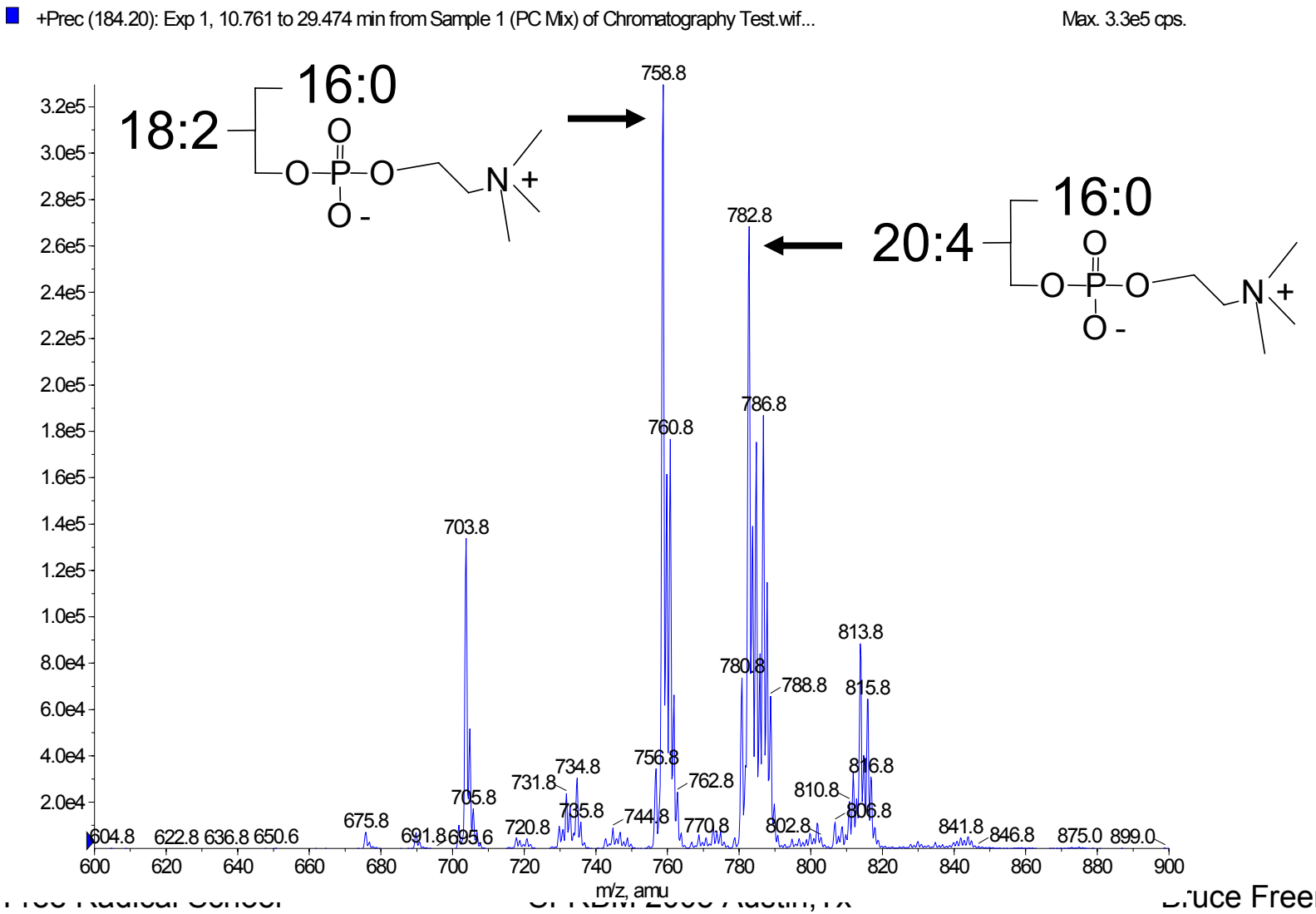


Cell Signaling

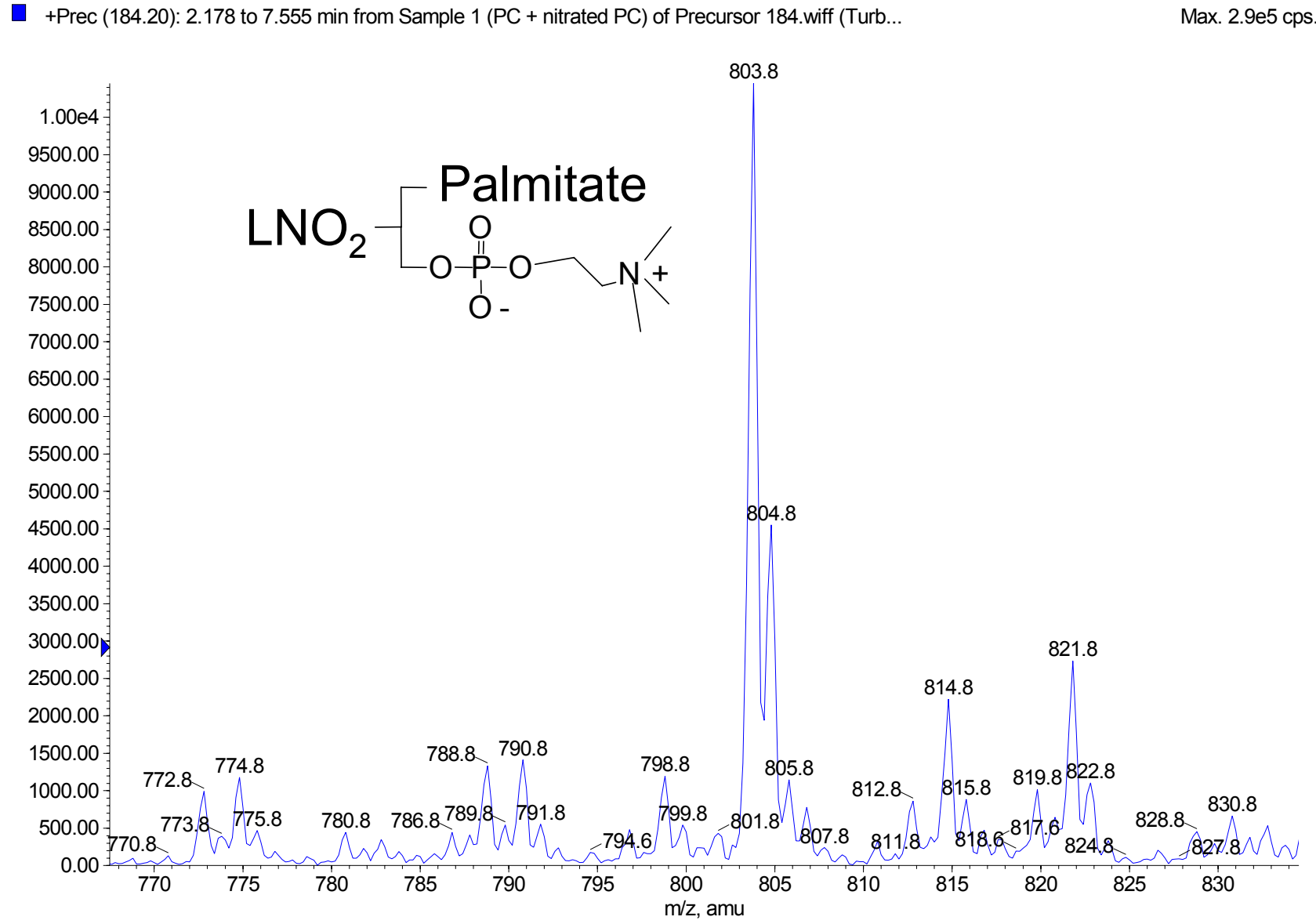
Lipase-Treated Complex Biological Lipids Release Nitrated Fatty Acids

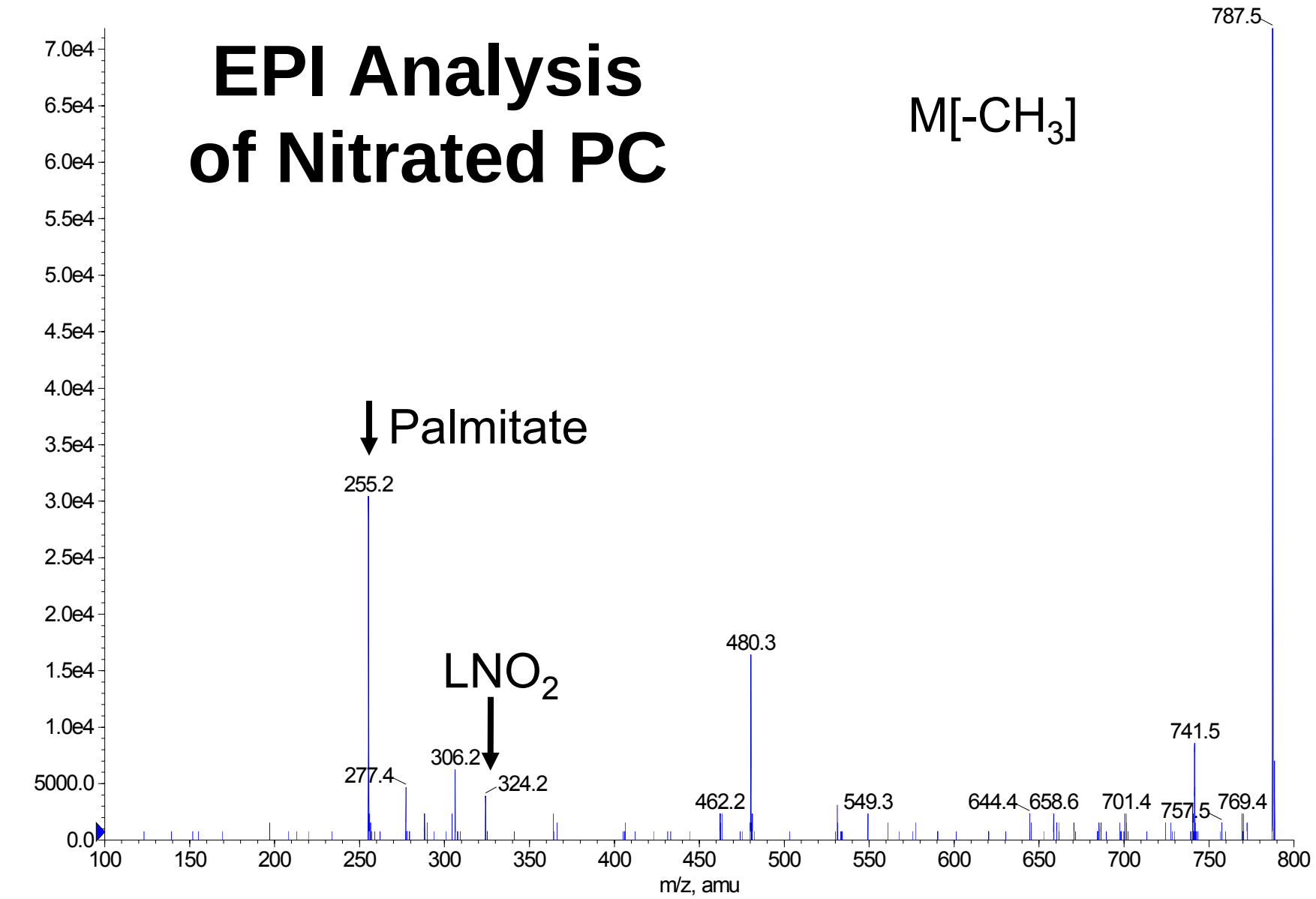


Precursor scan *m/z* 184—molecular species of phosphatidylcholine



Precursor Analysis of Nitrated Phosphatidylcholine (*m/z* 184)




-EPI (787.50) CE (-45): 0.227 to 0.692 min from Sample 8 (EPI of 787) of nitrated PC.wiff (Turbo ...
Max. 7.2e4 cps.
EPI Analysis of Nitrated PC
M[-CH3]
Palmitate
LNO2
787.5
255.2
480.3
306.2
277.4
324.2
741.5
462.2
549.3
644.4
658.6
701.4
757.5
769.4
7.0e4
6.5e4
6.0e4
5.5e4
5.0e4
4.5e4
4.0e4
3.5e4
3.0e4
2.5e4
2.0e4
1.5e4
1.0e4
5000.0
0.0
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
m/z, amu

Nitroalkene Derivatives

Redox-derived fatty acid derivatives that display unique signaling reactivities

- **Acidic hydrogen on C-N bond of nitroalkene capable of acid-base chemistry (Nef reaction), leading to NO release and cGMP-dependent signaling**

- **In "native" form, function as high affinity ligands for the nuclear receptor family PPAR**

- **C-N bond undergoes Michael addition reactions with nucleophiles (Cys, His)**

Agilent Array Analysis of Cell Gene Expression Responses to LNO_2
Human Monocytes, Endothelium

- **Transcription and initiation factors**
- **Adhesion molecules, cytokines**
- **Enzymes, receptors**

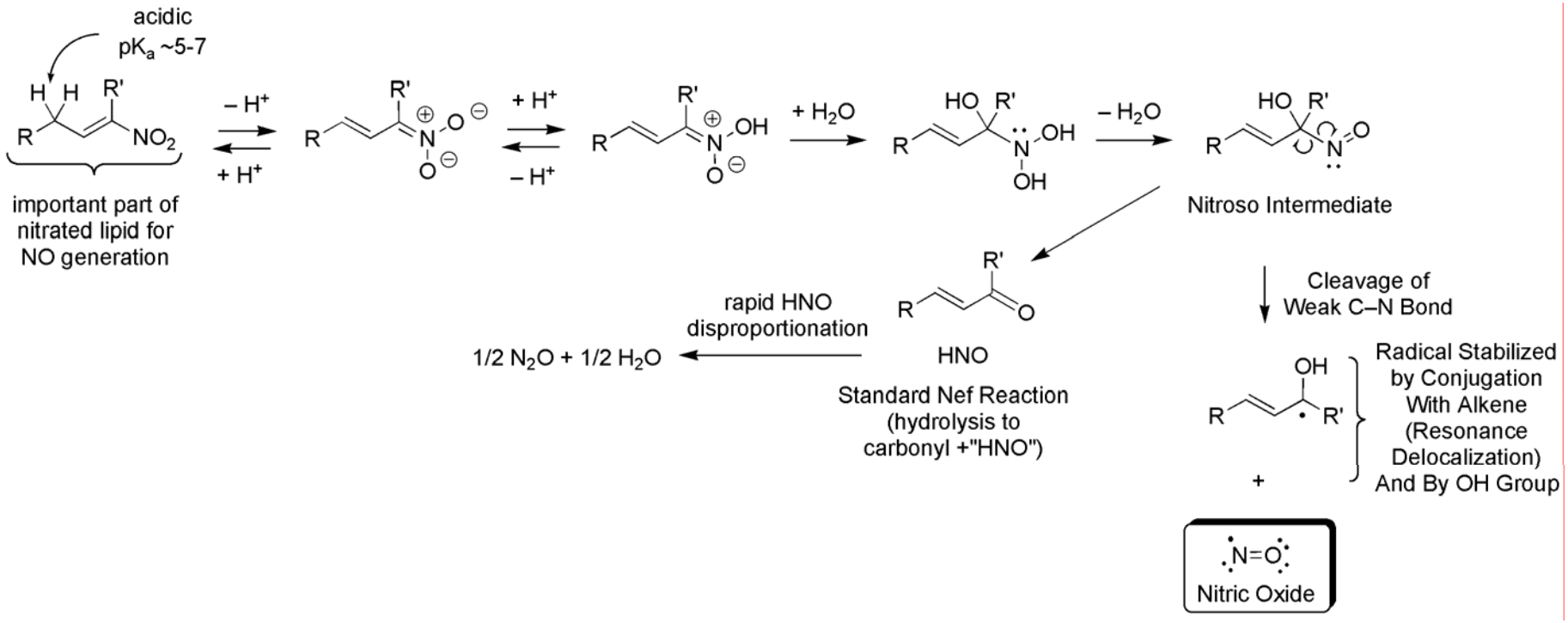
HO-1, PPAR-linked genes

Nitrolinoleic acid regulates 5504 genes

Up 2376 genes
Down 3128 genes

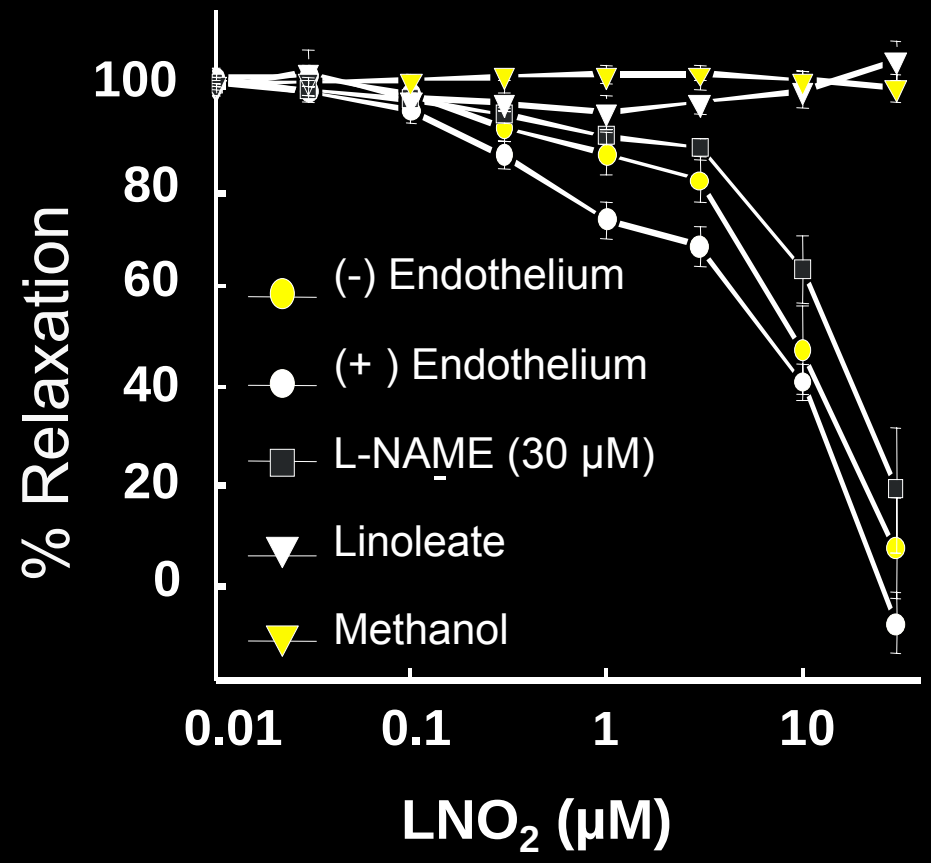
Linoleic acid regulates 14 genes – 5 up, 9 down

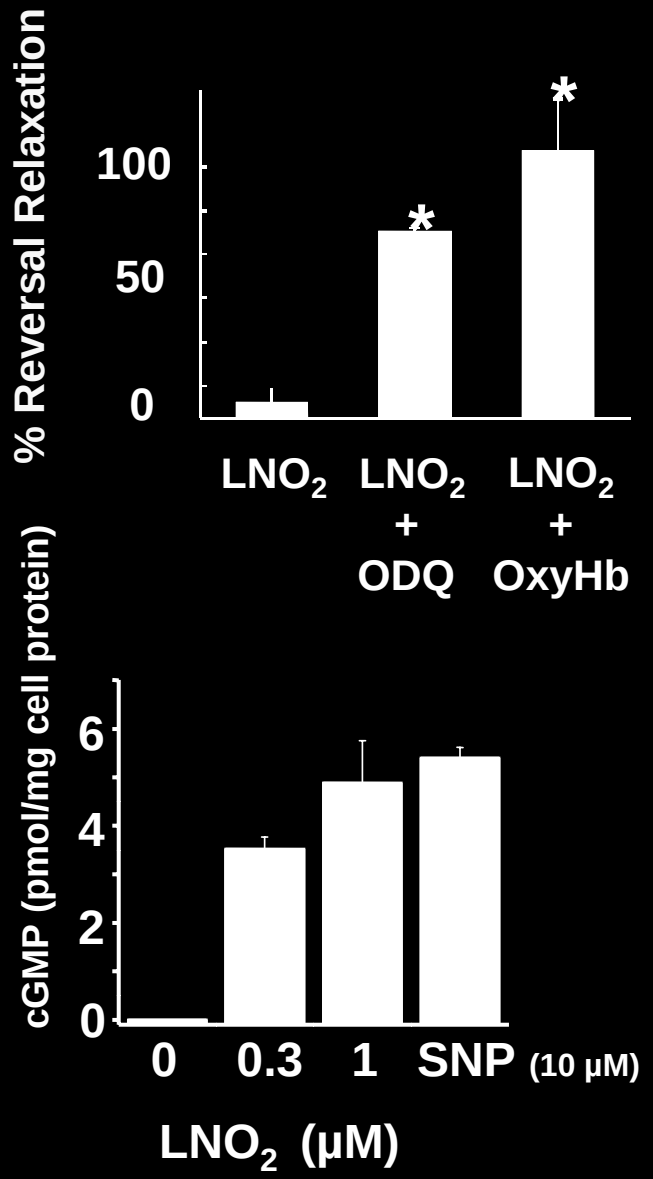
NO Release From Nitroalkenes Via Modified Nef Reaction



JBC 280:19289-19297, 2005

Nitrolinoleate Mediates NO-Like Vascular Signaling



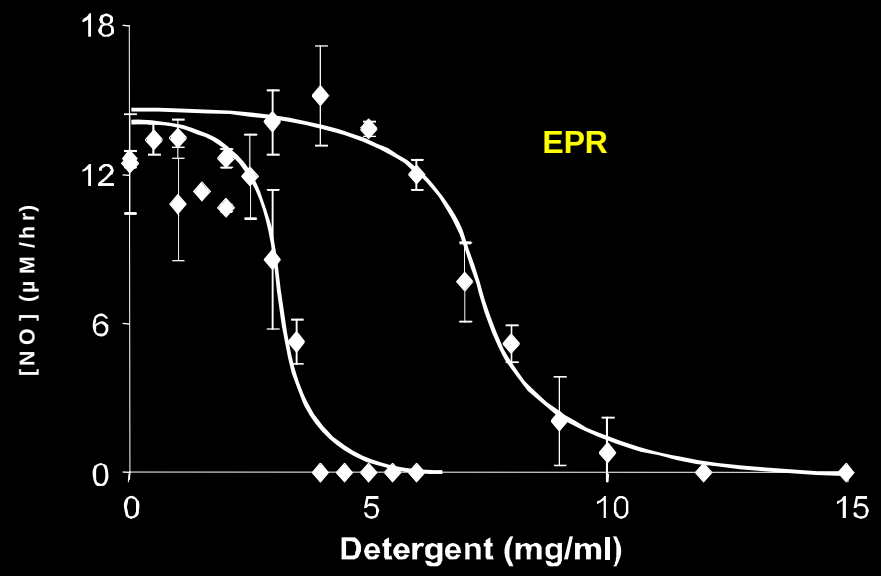


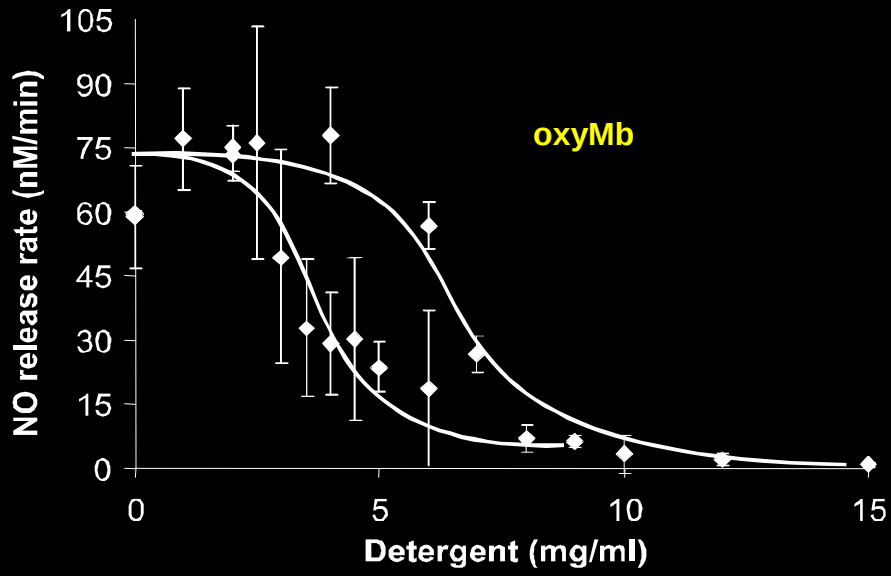
PNAS 99:15941-46, 2002

Nitroalkenes are Hydrophobically Stabilized

Octyl-thio-β-glucopyranoside CMC=2.8 mg/ml

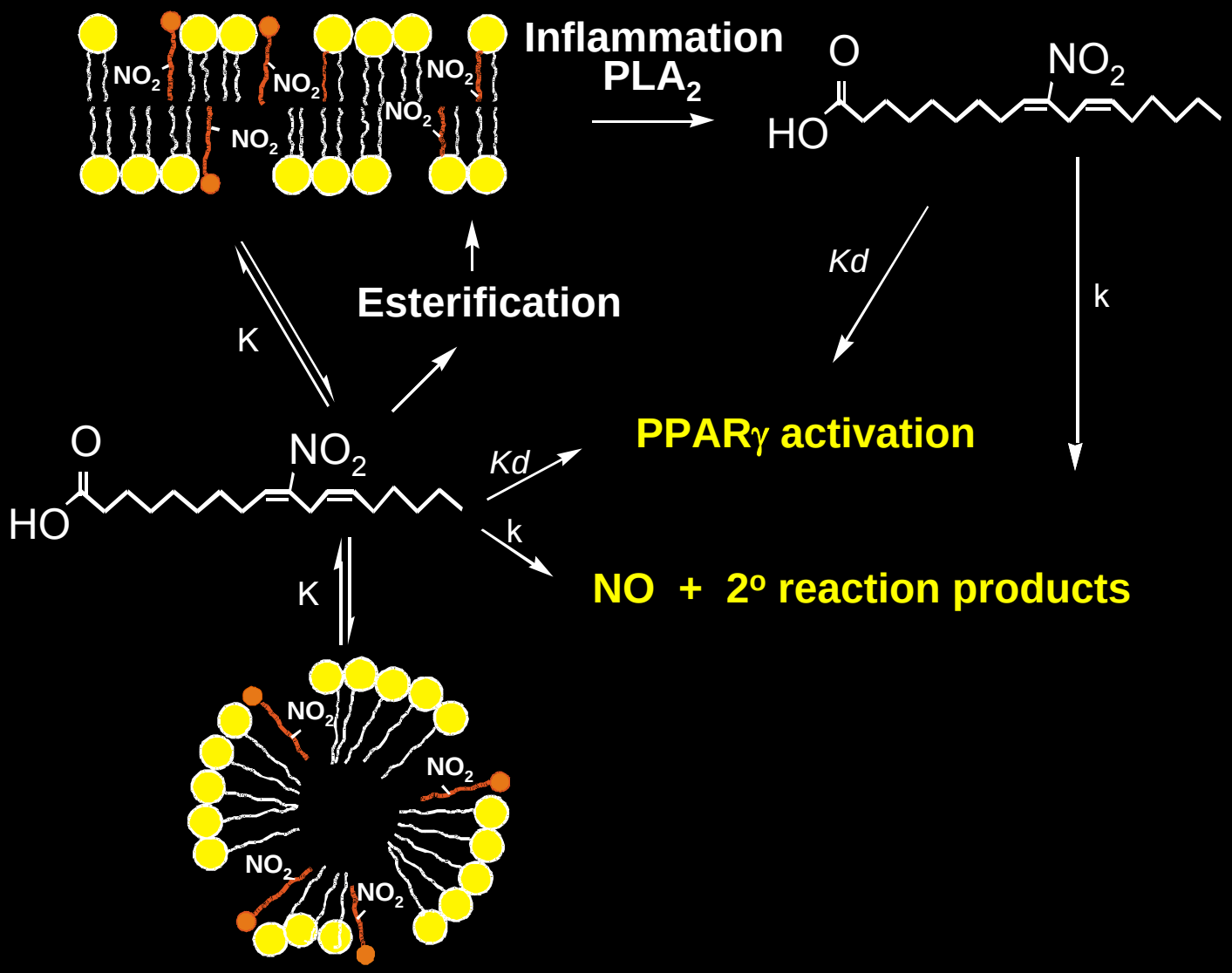
Octyl-β-glucopyranoside CMC=7.3 mg/ml





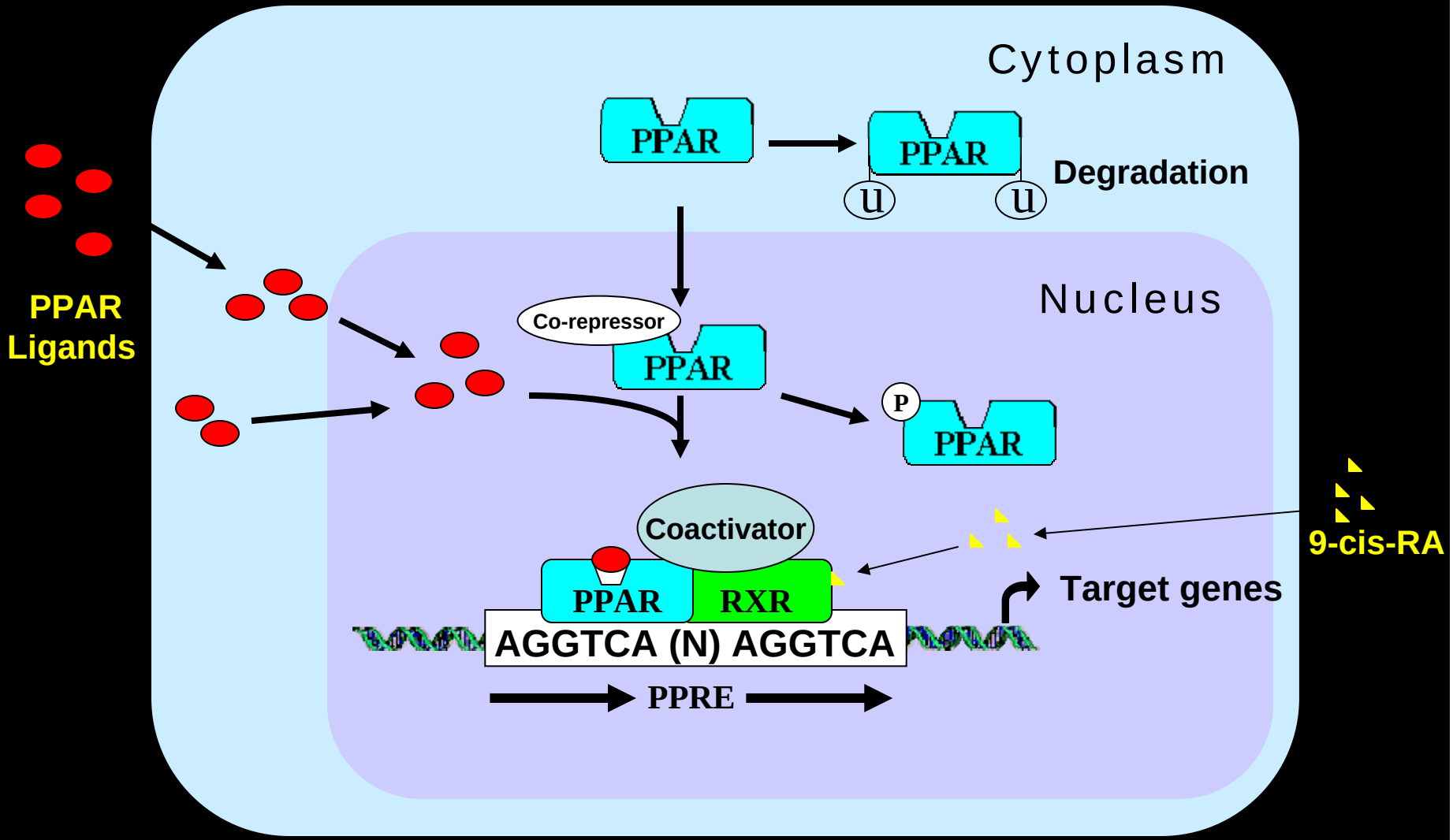
JBC 280:19289-19297, 2005

Hydrophobic Stabilization of LNO_2



JBC 280:19289-19297, 2005

PPAR-Regulated Gene Expression



Nitroalkenes Induce PPARγ-Dependent Cell Responses

Monocytes

- Induce CD36 expression
- Inhibit TNF, IL-1, MCP expression, NFκB
- Inhibit vascular adhesion

Adipocytes

- Induction of adipogenesis
- Induce PPARγ and aP2 expression
- Increased glucose uptake

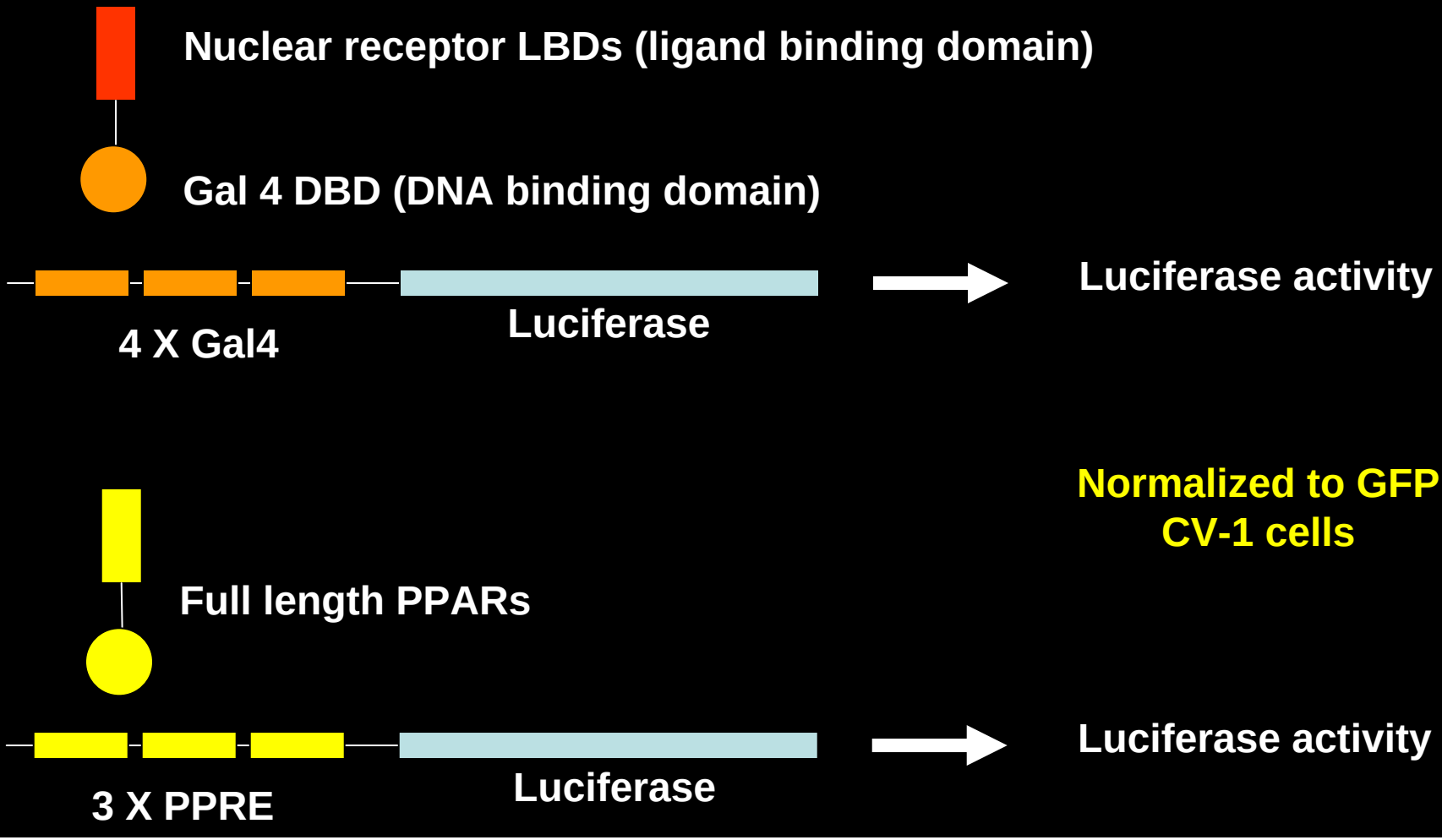
Pulmonary Endothelium

- Inhibition of TNF-induced VCAM-1 expression

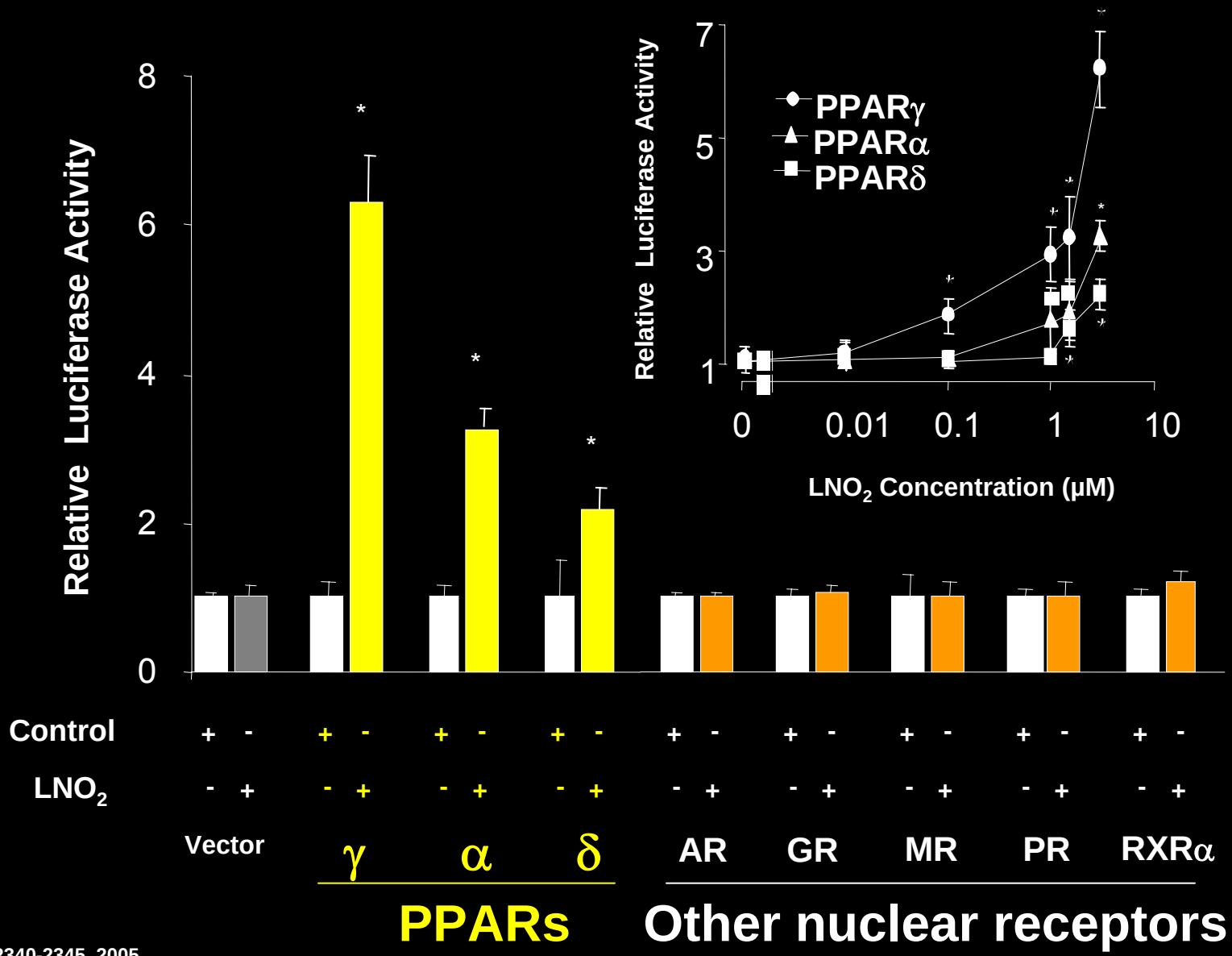
Alveolar Epithelium

- Lipid metabolism, apoptosis, redox signaling

Reporter Constructs for Analyzing PPAR Ligand Activity



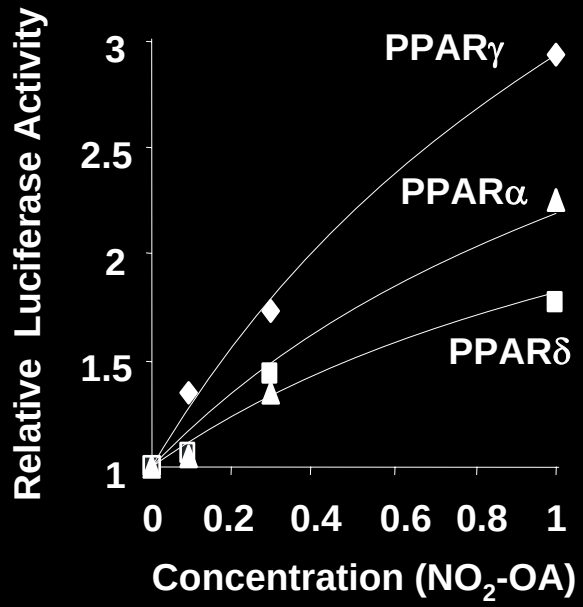
LNO_2 is a Specific PPAR Agonist

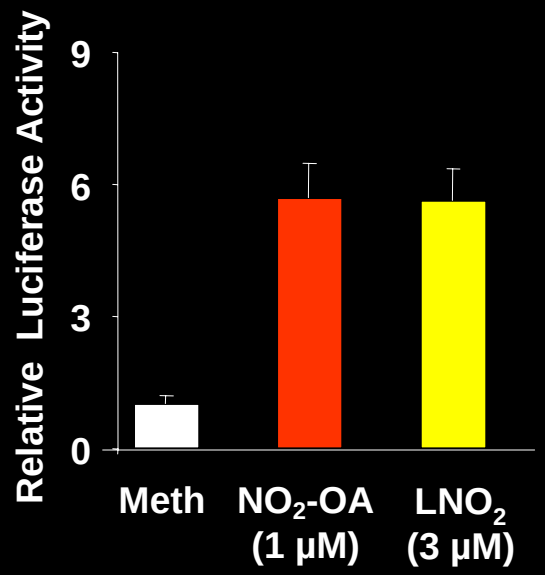


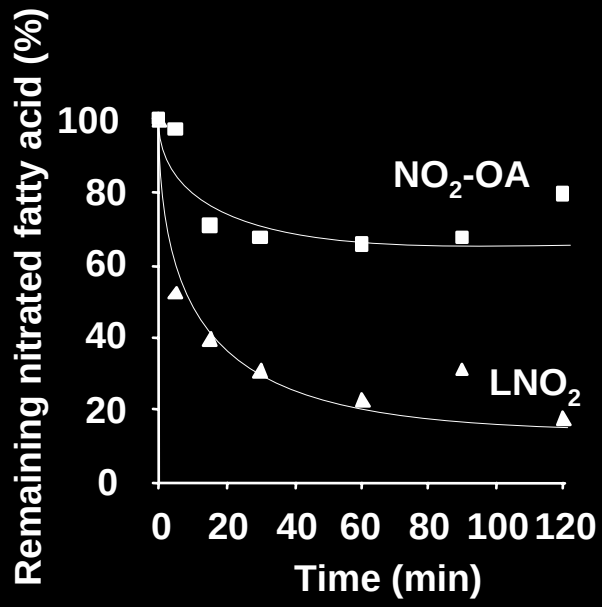
PNAS 102:2340-2345, 2005

NO_2-OA is a More Potent PPARγ Ligand

HO-1 expression
Inhibition of VCAM-1 expression
Adipocyte differentiation, glc uptake



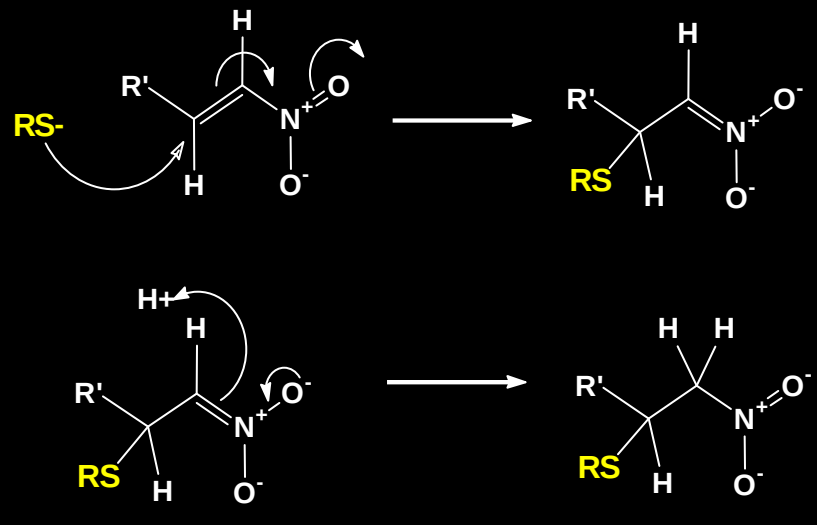




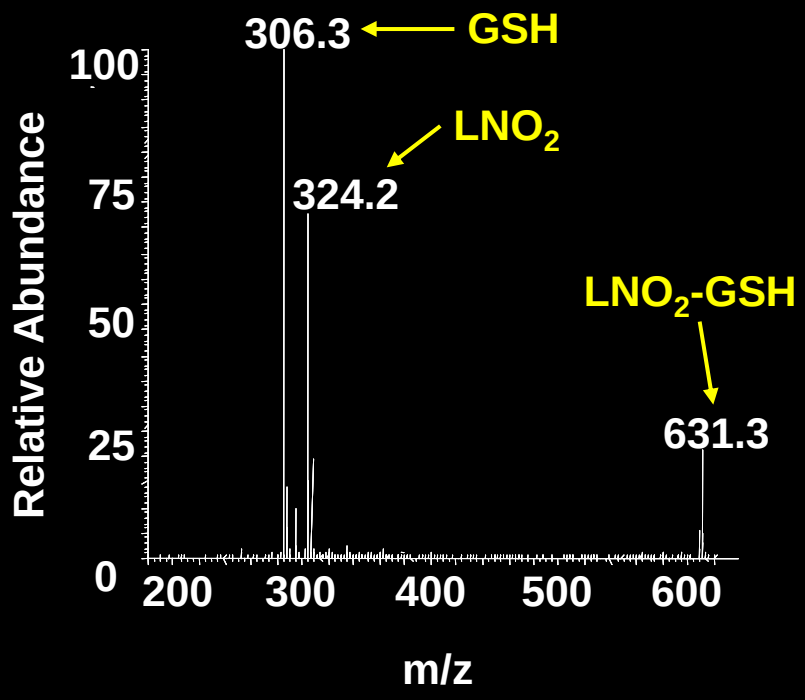
NO_2-OA > LNO_2 "potency" may be due to stability

PNAS 102:2340-2345, 2005

Nitroalkenes Undergo Reversible Michael Addition Reactions with Nucleophiles - Thiols

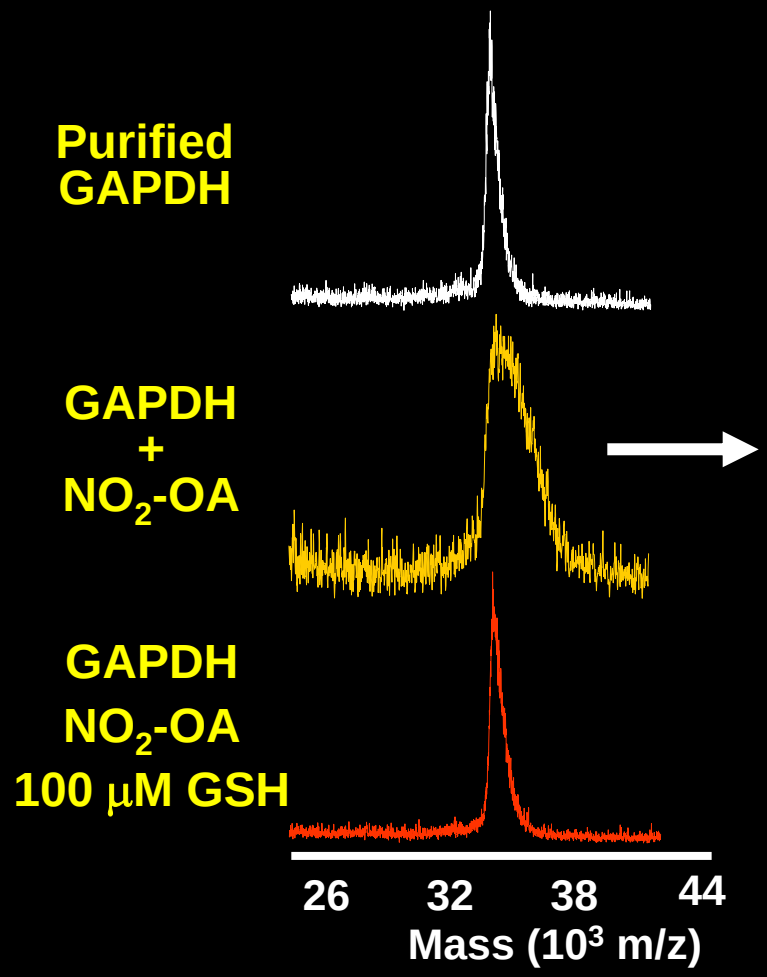


Nitroalkylation

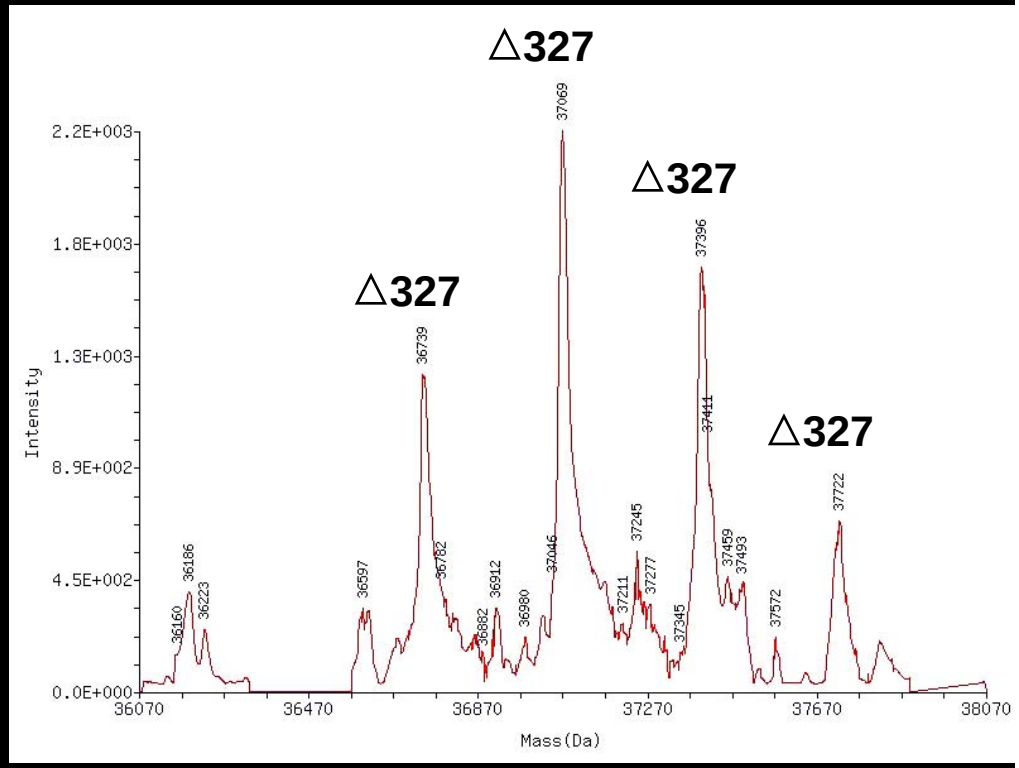


Submitted

Protein Nitroalkylation



MALDI-TOF-MS

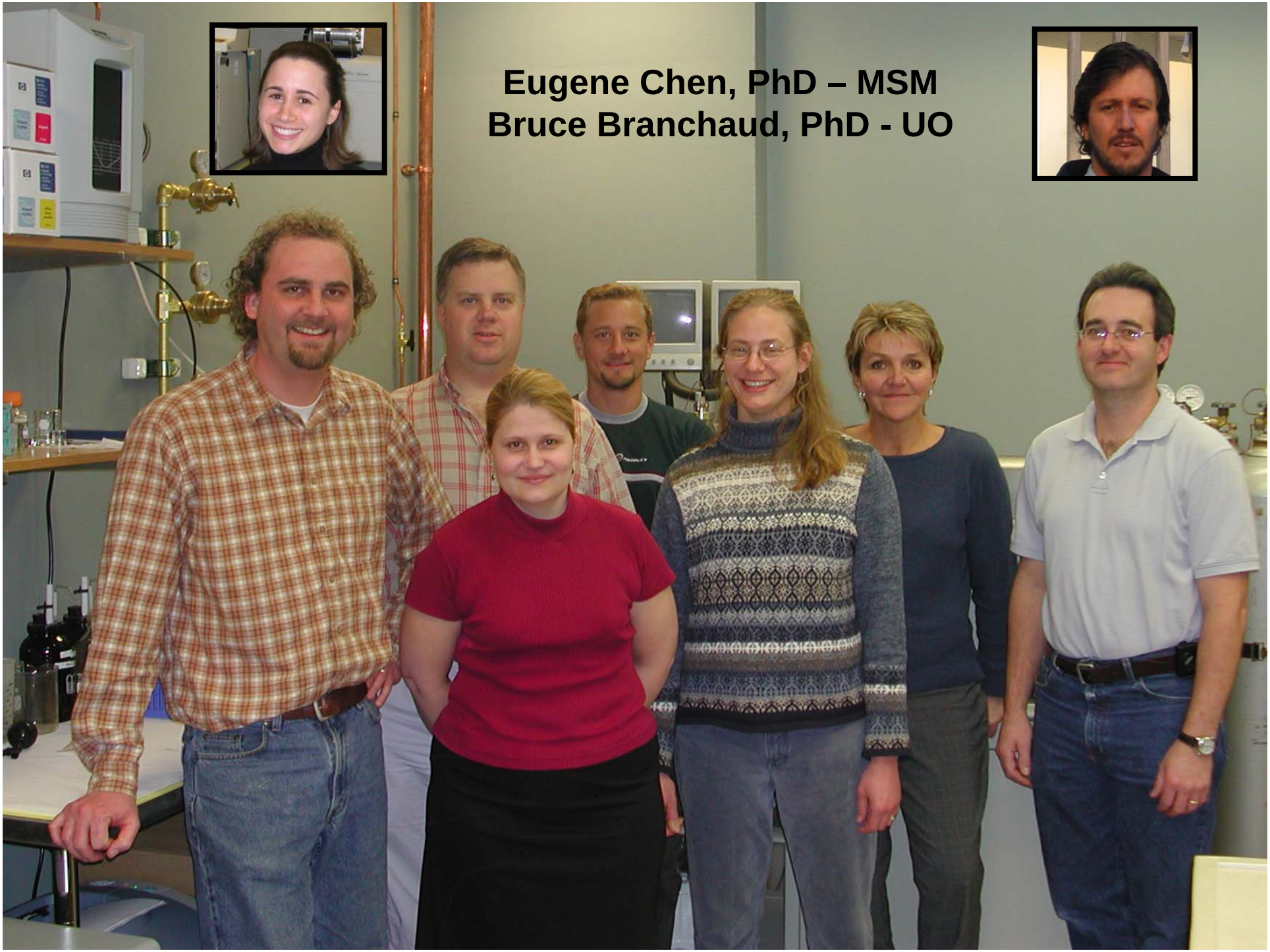


2-D Ion Trap

Submitted

NO and Fatty Acid Signaling Converge

- NO terminates lipid radicals, inhibiting propagation reactions and preserving lipophilic antioxidants
- NO-derived species either stimulate or inhibit lipid and lipoprotein oxidation, depending on basal oxidative and inflammatory conditions
- NO is consumed by reactions of eicosanoid biosynthesis, gene expression and catalytic activity of eicosanoid biosynthetic enzymes regulated by NO
- Nitrated fatty acids transduce NO and NO_2^- signaling
  - → formed by reactions of NO and NO_2^- derived species
  - → can be misidentified as other NO_x derivatives
  - → signal via receptor-dependent mechanisms and unique chemical reactivities


Eugene Chen, PhD – MSM
Bruce Branchaud, PhD - UO