REACTIVE CARBONYL SPECIES

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OBJECTIVES

(1) Know what are reactive carbonyl species (RCS)

(2) Know examples of enzymes that synthesize RCS

(3) Know enzymes that degrade RCS

(4) Know that RCS can generate ROS and form adducts on proteins
HEALTHY INDIVIDUALS

Oxidants

OH•  O₂•  ONOO•
H₂O₂  HOCl

Anti-oxidants

GSH  SOD
Catalase  GPx
Trx
- Alzheimer's, Parkinson's, ALS
• Cancers
• Hypertension,
• Heart Failure
• Stroke
• Diabetes
• Atherosclerosis

Oxidants

\[ \cdot O_2, \quad \text{ONOO}^-, \quad \cdot \text{OH}, \quad \cdot \text{HOCl}, \quad \cdot \text{H}_2\text{O}_2 \]

Anti-oxidants

Catalase
GSH
Trx
GPx
SOD
OXIDATIVE STRESS

- Reactive oxygen species
- Reactive carbonyl species
- ANTI-OXIDANTS

OXIDANTS
RCS are aldehydes are generated from oxidation of lipids, glucose and amino acids.
Lipid peroxidation derived RCS

Membrane Lipids

\[ \text{H}_2\text{O}_2 + \text{O}_2^- \]
\[ \text{Fe}^{2+} \]

\[ \text{OH}^- \]

Acrolein
Glyoxal
Malondialdehyde

4-Hydroxy-2-alkenals
EXAMPLES OF GLUCOSE-DERIVED REACTIVE CARBONYL SPECIES

(ii) Glycation-related aldehydes

Glyoxal

Methylglyoxal

3-Deoxyglucosone

Glucosone
ACROLEIN IS SYNTHESIZED MYELOPEROXIDASES

STEVENS JF AND MAIER CS
AMINE OXIDASES (VAP-1)

METHYLGLYOXAL
COPPER AMINE OXIDASES (VAP-1)

Glycine/Threonine

Aminoacetone

Physiology

- cECs
- Neurons
- Astrocytes
- Microglia

VAP-1

MG

H₂O₂

NH₄⁺
VPO1 can be secreted into the extracellular space to participate in extracellular matrix formation, suggesting that VPO1 may also play a role in cardiovascular remodeling (such as fibrosis).

\[ \text{Cl}^- + \text{H}_2\text{O}_2 + \text{H}^+ \xrightarrow{\text{VPO1}} \text{HOCl} + \text{H}_2\text{O} \]
RCS DEGRADING ENZYME

- Aldose reductase
- Glutathione-S-transferase
- Aldehyde dehydrogenase
- Glyoxalalases
- Cytochrome P450 enzymes
Figure 1.
Reaction of GSH with racemic HNE. 1,4-addition reaction, followed by an intramolecular cyclization.
Figure 4.
Human GSTA4-4 active site. GSTA4-4 is shown in complex with 3S,4R-GSDHN (PDB entry 3IK7) as a model for the ternary complex formed with GSH and HNE. The 4-hydroxyl group is in proximity of R15, whereas the aldehyde-derived oxygen is near Y212 at the bottom of the H-site. The alkyl chain extends into the hydrophobic groove lined with other key active site residues shown as spheres.
WHAT DOES RCS DO?

USE METHYLGLYOXAL AS AN EXAMPLE

(most potent)
METHYLGLYOXAL

Commerically available

Synthesized in OUR lab
MG PERTURBS INTRACELLULAR Ca^{2+} HOMEOSTASIS
RCS alter cytoplasmic Ca$^{2+}$ within seconds
MG IS A POTENT INDUCER OF ROS, BUT THIS TAKES TENS OF MINUTES

Green - Mitotracker Green
Red/Orange – MitoSox
RCS INDUCE MITOCHONDRIA ROS
Cardiac myocytes
MG INCREASE ROS in MITOCHONDRIA (cardiac progenitor cells)

30 min after addition of 24 µM MG

Mitotracker® Green

MitoSOX™ (488nm for ROS)

MitoSOX™/Mitotracker® Green overlay
MG INCREASE ROS in MITOCHONDRIA (endothelial cells)

Bright field | Mitotracker green | Mitosox | Mitotracker green/Mitosox overlay
---|---|---|---
before MG | | | |
7.5 min after 30 μM MG | | | |
MnTBAP (20 μM for 30 min), 7.5 min after 30 μM MG | | | |
IS MG’S EFFECT ON MITOCHONDRIA ROS DIRECT OR INDIRECT?
ELECTRON PARAMAGNETIC SPECTROSCOPY

**A**

- EPR amplitude (arbitrary units/min/μg mitochondria)
- [MGO] μM: 0, 1, 5, 10
- Bars with error bars

**B**

- EPR amplitude (arbitrary units/1st scan/μg mitochondria)
- Conditions: 0, 50 μM MGO, 50 μM MGO + 300 μM MnTBAP
- Bars with error bars**
OXIDATIVE STRESS

Reactive oxygen species

Reactive carbonyl species

OXIDANTS

ANTI-OXIDANTS
RCS are electrophile and can accept a pair of electrons to form a new covalent bond so they can form adducts.

Electrons flow from “electron rich” centers to “electron poor” centers.

Electron rich centers are called nucleophiles.

Electron poor centers are called electrophiles.
RCS ARE ELECTROPHILES AND CAN FORM ADDUCTS WITH SELECT AMINO ACIDS.

![Chemical Structures]

- **Imidazolone A**
- **Imidazolone B**
- **Lys-NH**
- **N-carboxymethyl lysine**
- **N-carboxyethyl lysine**
- **Argpyrimidine**
- In many diseases free RCS levels increased and the consequence can be devastating.

- They react with exposed lysine, arginines, histidines and cysteines to form adducts

- Increase ROS production in cells
Developing a Research Project Around RCS

Select the disease and really do some thinking
DIABETES CAUSES MANY COMPLICATIONS

- Kidney failure
- Impotency
- Nervous system damage
- Stroke
- Heart failure
- Cognitive deficits
- Periodontal diseases

Erectile dysfunction

Blindness

Lower limb amputation

Periodontal diseases

Loss of feeling?
Tingling?
Burning sensation?

Numbness?
The HOPE study. Ramipril lowered cardiovascular risk, but vitamin E did not.

Hoogwerf BJ, Young JB.
Department of Endocrinology, Cleveland Clinic, OH 44195, USA. hoogweb@ccf.org

Abstract
The Heart Outcomes Prevention Evaluation (HOPE) study found that the ACE inhibitor ramipril can lower the risk of atherosclerotic disease events and death in patients without heart failure but with known atherosclerosis or with diabetes plus at least one cardiovascular risk factor. This benefit was independent of ramipril’s effect on blood pressure. Additional benefits were a reduced risk of diabetic nephropathy in diabetic patients, and a lower likelihood of newly diagnosed diabetes. On the other hand, vitamin E in the doses and duration studied (400 IU/day for 4.5 years) did not lower risk significantly.

Effects of vitamins C and E and β-carotene on the risk of type 2 diabetes in women at high risk of cardiovascular disease: a randomized controlled trial

Yiqing Song, Nancy R Cook, Christine M Albert, Martin Van Denburgh, and JoAnn E Manson

Conclusion: Our randomized trial data showed no significant overall effects of vitamin C, vitamin E, and β-carotene on risk of developing type 2 diabetes in women at high risk of CVD. This trial was registered at clinicaltrials.gov as NCT00000541. Am J Clin Nutr 2009;90:429–37.
The 1027th target candidate in stroke: Will NADPH oxidase hold up?

Kim A Radermacher1, Kirstin Wingler1, Pamela Kleikers1, Sebastian Altenhöfer1, Johannes JR Hermans1, Christoph Kleinschnitz2 and Harald H HW Schmidt1*

DOI 10.1007/s00018-012-1107-1

LETTERS AND COMMENTS

...a promising strategy to treat ischemic stroke. As described in the review, we recently reported that NOX4-deficient mice are largely protected from brain damage caused by ischemic stroke, whereas we did not observe any effects by deleting NOX1 or NOX2. Thus, we believe that NOX4 is a highly promising target for stroke therapy. To further support our findings, we treated wild-type and NOX4 knockout mice with the NADPH oxidase inhibitor VAS2870 in a therapeutically relevant time window, i.e., post-stroke. Indeed, in wild-type mice, inhibition...
Failures of Clinical Trials……..

1. Intervention was too late, extensive pathobiology

2. Anti-oxidants used were not the best or not targeted

3. Incorrect doses or mixtures of antioxidants might have more benefit than higher doses of single agents

4. Animal models used were not the best

5. Biomarkers used were not adequate for assessing outcomes

6. ..............

7. ..............
HEART FAILURE IN DIABETES

Control

Diabetic 5-6 weeks

Diabetic 8 weeks

Diabetic 10 weeks

Diabetic 16 weeks

(M-MODE ECHOCARDIOGRAPHY)
Excitation-Contraction Coupling

- Ryanodine receptor
- L-type Ca\textsuperscript{2+} channels
- SERCA2

Diagram showing the interaction between excitation and contraction, highlighting key components like Ryanodine receptor (RyR2), SERCA2, and L-type Ca\textsuperscript{2+} channels.
Spontaneous
(Ca$^{2+}$ sparks)

Evoked

Diabetes alters intracellular Ca$^{2+}$ handling in myocytes

RyR2 and SERCA2 functions decreased but NOT steady-state protein levels

$[^3H] \text{Ryanodine binding}$

$[^3H] \text{Ryanodine bound (fmol/µg of RyR2 protein)}$

RyR2

$\text{Ca}^{2+}$ uptake

$\text{Ca}^{2+}$ uptake (nmol/mg membrane/30min)

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>STZ-diabetic (5-6 weeks)</th>
<th>STZ-diabetic (7-8 weeks)</th>
<th>insulin-treated diabetic (6D/2I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8D</td>
<td></td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6D/2I</td>
<td></td>
<td>*</td>
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</tr>
</tbody>
</table>

PLN level does not change
Question # 1

What is causing loss of function of these proteins without changing expression level?

The first thing that comes to mind is post-translational modifications (PTMs), but what kind
DEVELOPED A METHOD TO PURIFY RyR2
RyR2 FROM DIABETIC RAT HEARTS EXHIBITED FLUORESCENCE

Post-translational modification of SR Ca\textsuperscript{2+} cycling proteins in diabetes

**RyR2**
- N\textsuperscript{ε}(Carboxymethyl)lysine (CML)
- Pentosidine
- Argpyrimidine
- Pyrraline
- 3-Deoxyglucosone / hydroimidazolone
- GA-pyridine
- β-actin

**SERCA2**
- Argpyrimidine
- Pentosidine
- Carboxymethyllysine
- Pyrraline
- AGEs
- Imidazolone / 3-deoxyglucosone
- Carboxyethyllysine
- β-actin

Diabetes. 2003 Jul;52(7):1825-1836
*Diabetes. 2011, 60(3):947-959
*Mol Cell Biochem. 2013, 376(1-2):121-135*
DEVELOPED A METHOD TO PURIFY RyR2

Bilayer Cup

Purify protein (proteoliposomes)

Manipulate the channel by changing the voltage or current, drugs etc
Lipid Bilayer Set Up

Bilayer workstation

Amplifier and Digidata

Monitor

Bilayer Cups & Electrode
Question #2

Are RCS adducts formed on proteins functionally important?

(1) find the locations of the adducts
MASS SPECTROMETRY BASED METHOD TO SEARCH FOR ADDUCTS ON PROTEINS

Purify protein

Trypsin-Digestion

MALDI-TOF-MS

MALDI Data

Known Protein Sequence

digest. in silico

Theoretical Digest Table

INCLUDING MISCLEAVED PEPTIDES

use PERL algorithm To search for carbonyl adducts

CONFIRM SEQUENCE USING TANDEM MS

MASS SPECTROMETRY TO LOCATE ADDUCTS ON RyR2

Imidazolone B adduct on R4462

(CONTROL (C))

STZ-DIABETIC (D)

INSULIN-TREATED STZ-DIABETIC (Ins-D)

LOCATION OF ADDUCTS ON RyR2
(15,000 bp, 565 kDa)
Show that adduct impair the protein function
Cloned RyR2 into plasmid for mutation studies

A. Diagram illustrating the cloning of RyR2 into a plasmid.

B. Image showing expression of RyR2-pCMS-EGFP in HEK 293T cells after 48 hours.

C. Graph showing change in fluorescence 

\[
\frac{[F-F_0]}{F_0}
\]

over time for HEK 293T transfected with RyR2 and HEK 293T.

D. Bar graph showing triphenylenylamine (TMA) bound 

\[
\text{[H]}\text{yanodine bound (fmol/100\mu g protein)}
\]

for HEK 293T and HEK 293T transfected with RyR2.
PERFORM BINDING STUDIES TO ASSESS RyR2 ACTIVITY
CARBONYLATION INDUCES GAIN-OF AND LOSS-OF RyR2 FUNCTION
METHYLGLYOXAL IS SYNTHESIZED FROM SEVERAL SOURCES

- Protein → Aminoacetone
- Triacylglycerols → Fatty acids, Glycerol, Dihydroxyacetone Phosphate
- Polyol Flux → Glyceraldehyde-3-phosphate
- VAP-1 / SSAO → Aldehyde dehydrogenase
- Pyruvate → Reduced GSH, D-lactoyl glutathione, Glyoxalase-II
- 2-oxoaldehyde dehydrogenase
- NAD, NADP
- Aldose Reductase → Glyoxalase-I
- NADPH
- Acetol → Propanediol

METHYLGLYOXAL (MG)
INSERTED FUNCTIONAL GLYOXALASE 1 INTO pZAC 2.1

**2.0mM Methylglyoxal**

![Graph showing the relationship between cell extract (µg/ml) and glyoxalase I activity (ΔA/min) at 2.0mM Methylglyoxal for different clones.]

**5.0µg cell extract**

![Graph showing the relationship between methylglyoxal (mM) and glyoxalase I activity (ΔA/min) for 5.0µg cell extract for Clone #4.]

Clone #3
Clone #4
Clone #6
Clone #8
PROTOCOL USED FOR STUDY

Inject STZ
Inject IV AAV2/9

Measure Cardiac function

1 week
8 weeks
AAV2/9-Endo-Glo-I blunt cardiac function loss in diabetes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>eGFP-treated control (n = 8)</th>
<th>eGFP-treated STZ-diabetic (n = 8)</th>
<th>AAV2/9-Endo-Glo-1-treated STZ-diabetic (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate</td>
<td>367.1 ± 10.1</td>
<td>289.1 ± 8.1*</td>
<td>320.1 ± 5.1**</td>
</tr>
<tr>
<td>% Fractional Shortening</td>
<td>49.1 ± 1.7</td>
<td>41.9 ± 1.9*</td>
<td>47.5 ± 0.7**</td>
</tr>
<tr>
<td>% Ejection Fraction</td>
<td>79.1 ± 1.9</td>
<td>70.9 ± 2.3*</td>
<td>77.4 ± 0.7**</td>
</tr>
<tr>
<td>Left ventricular end diastolic diameter (mm)</td>
<td>6.6 ± 0.3</td>
<td>7.3 ± 0.2*</td>
<td>7.0 ± 0.1**</td>
</tr>
<tr>
<td>Left ventricular end systolic diameter (mm)</td>
<td>3.4 ± 0.2</td>
<td>4.3 ± 0.2*</td>
<td>3.8 ± 0.1**</td>
</tr>
</tbody>
</table>

* denotes significantly from control
** denotes significantly from control
Methylglyoxal modification of Na\textsubscript{v}1.8 facilitates nociceptive neuron firing and causes hyperalgesia in diabetic neuropathy


Affiliations | Contributions | Corresponding author

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Corrigendum (September, 2012)
SUMMARY

(1) You should know what are reactive carbonyl species (RCS)

(2) You should know examples of enzymes that synthesize RCS

(3) You should know enzymes that degrade RCS

(4) You should know that RCS can generate ROS and form adducts on proteins
QUESTIONS?

Supported in part by NIH, ADA, PEN, Nebraska Redox Biology Center, GTRP