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Slide 1

S-Nitrosation and denitrosation of proteins by variation of superoxide/nitric oxide ratio

implications for prevention of sulfoxidation-dependent enzyme inactivation during ischemia/reperfusion

Ksenija Vujacic-Mirski[1], Andreas Daiber[1], Sanela Kalinovic[1], Volker Ullrich[2], Stefan Schildknecht[2]

[1] Center for Cardiology, Department of Cardiology 1, Laboratory of Molecular Cardiology, University Medical Center of the Johannes Gutenberg University, 55131 Mainz, Germany;
[2] In Vitro Toxicology and Biomedicine, Department of Biology, University of Konstanz, 78457, Konstanz, Germany.

PhD student Ksenija Vujacic-Mirski
Center for Cardiology, Department of Cardiology 1, Laboratory of Molecular Cardiology,
University Medical Center of the Johannes Gutenberg University,
55131 Mainz, Germany;

Slide 2 – Full apstract

Oxidative stress plays a key role for the development of cardiovascular, metabolic and neurodegenerative disease. This concept was never widely proven by the therapeutic use of classical antioxidants in large scale clinical trials. However, we know from numerous (pre)clinical studies that the formation of reactive oxygen and nitrogen species (RONS) as well as stable oxidative stress markers are a hallmark of most forms of diseases and higher oxidative stress markers are frequently associated with higher disease risk and mortality. Besides the detrimental role of RONS, they are also involved in cellular functions via redox signaling. We have previously identified an efficient mechanism of S-nitrosation by low levels of nitric oxide and superoxide (3:1 ratio) with potential formation of N2O2 [Daiber, Schildknecht et al. and Ullrich, FRBM 2009]. Here we, elucidated whether S-nitrosation in the presence of higher nitric oxide than superoxide concentrations (as observed under hypoxic conditions) could prevent sulfoxidation and thereby oxidative inactivation of enzymes in the presence of higher superoxide than nitric oxide concentrations (as observed during reoxygenation). We found that increasing concentrations of xanthine oxidase/hypoxanthine caused conversion of S-nitrosoglutathione (GSNO) to reduced glutathione (GSH) up to a certain concentration of xanthine oxidase, indicating that superoxide can induce denitrosation of GSNO. This finding was unexpected since in the presence of excess superoxide one would not expect regeneration of reduced GSH from GSNO. This was even more surprising since we observed substantial dihydrorhodamine oxidation that was prevented by uric acid and tyrosine nitration of albumin during denitrosation of GSNO by superoxide, all of which points to intermediary formation of peroxynitrite. In summary, we propose that S-nitrosation of (mitochondrial) proteins during ischemia represents a protective mechanism to prevent irreversible overoxidation of thiols during the reperfusion phase and to re-establish reduced thiol state in (mitochondrial) key enzymes of energy metabolism and cell survival. Wide-spread mitochondrial protein S-nitrosation may represent a central feature of the protective
preconditioning effects of nitric oxide.

Keywords: S-nitros(yl)ation; denitrosation; superoxide and nitric oxide; ischemia/reperfusion.

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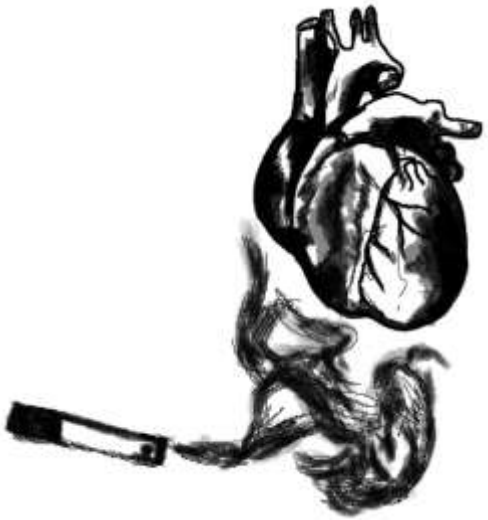
Slide 3 – Introduction

Oxidative stress plays a key role in:

1. Cardiovascular
2. Metabolic
3. Neurodegenerative

} diseases



But it is also involved in cellular function via redox signaling.

S-nitrosation represents a highly important redox-regulatory mechanism as it is involved in the process of apoptosis, as well as metabolic (mitochondrial) pathways via S-nitrosation of caspase-3, isocitrate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase.
S-nitrosation confers protection against ischemia/reperfusion (I/R) damage via regulation of the mitochondrial permeability transition pore (mPTP) opening probability through S-nitrosated cyclophilin D, as well as direct suppression of mitochondrial RONS formation during I/R by S-nitrosation of complex I. S-nitrosation also contributes to regulation of gene expression.

Slide 4 - Specific terminology

S-nitrosation

Nitrosation is a process of converting organic compounds into nitroso derivates (R-NO functionality).
S-nitrosation (of proteins) is a posttranslational modification in which a cystein residue is modified by nitric oxide.
It may impact many biological systems since S-nitrosation is an important regulatory machanism of protein function in both physiological and pathological pathways.

Sulfoxidation of cysteine residues

Sulfoxidation is a chemical reaction in which a sulfoxide is formed by oxygen transfer. Cysteine sulfoxides are chemical compounds containing a -SOH, $-SO_2H$ or $-SO_3H$ group attached to one carbon atom. For example, sulfoxidation can occur in mitochondrial thiol-dependent proteins and cause irreversible damage.

$R\text{-}SO_XH$

Sulfenic, sulfinic, sulfonic acid

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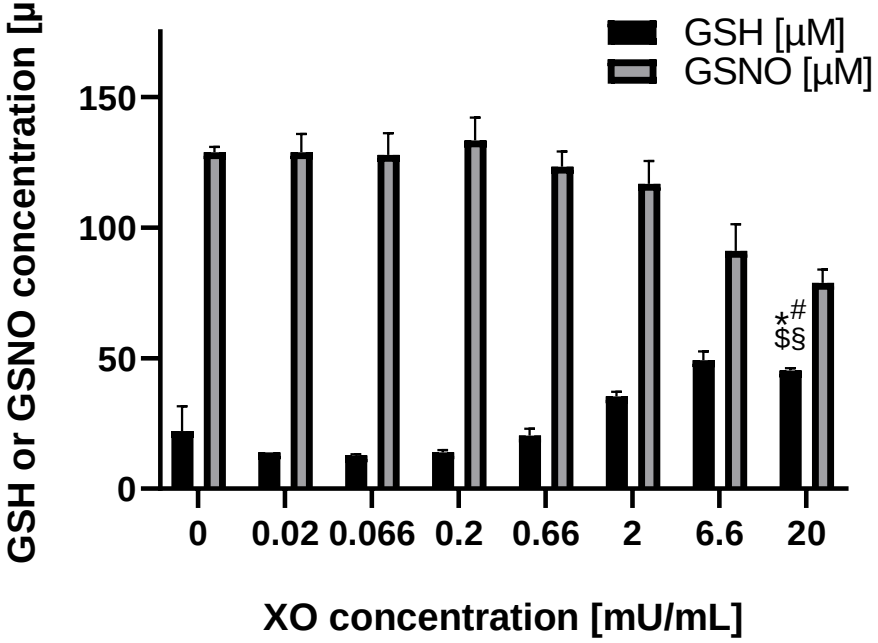
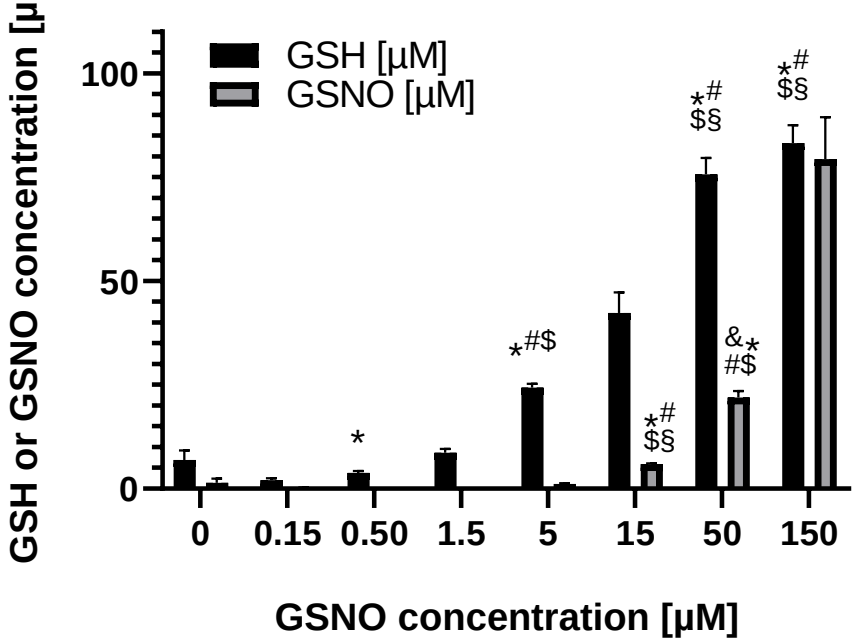
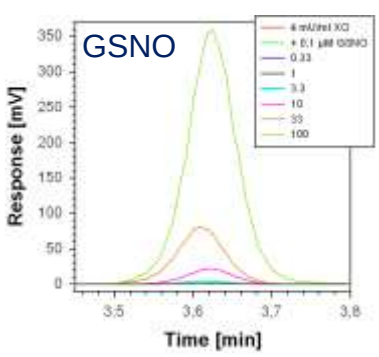
Slide 5 – Preliminary work with GSNO as a model compound

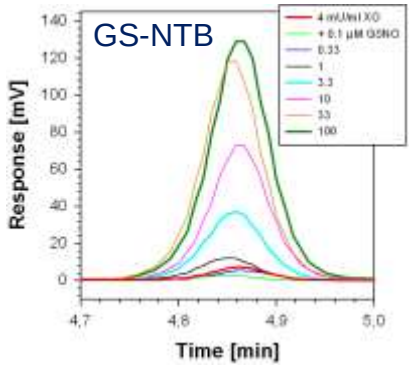
HPLC assay: GSNO fix, XO variable



HPLC assay: XO fix, GSNO variable







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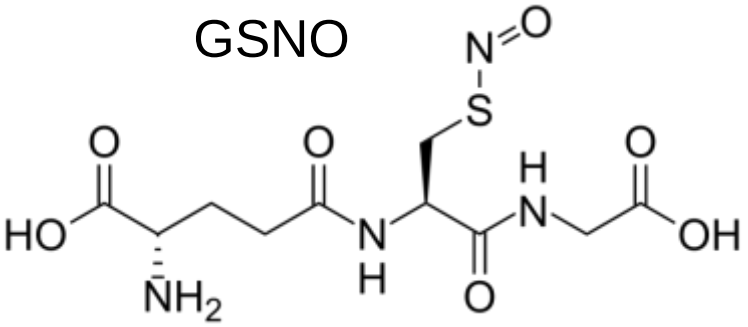
GSNO



Slide 6 - Aim

Could S-nitrosation (as observed under hypoxic (ischemic) conditions) prevent sulfoxidation and thereby oxidative irreversible inactivation of enzymes by superoxide/hydrogen peroxide (as observed during reoxygenation (reperfusion))?

S-nitrosation as a posttranslational modification occurs predominantly through transfer of the nitrosonium cation (NO^+) by low molecular weight „NO“ carriers such as S-nitrosoglutathion (GSNO), sodium nitroprusside (SNP) or N_2O_3 gas. S-nitrosation by radical recombination between nitric oxide ($^{\bullet}NO$) and thiyl ($-S^{\bullet}$) radicals is rather unusual as this reaction is thermodynamically unfavorable.

We nitrosated the enzyme aldehyde dehydrogenase (ALDH) with the NO donor (GSNO) and tried to regain activity (via denitrosation) by further incubation with small amounts of the superoxide producing system xanthine oxidase (XO).

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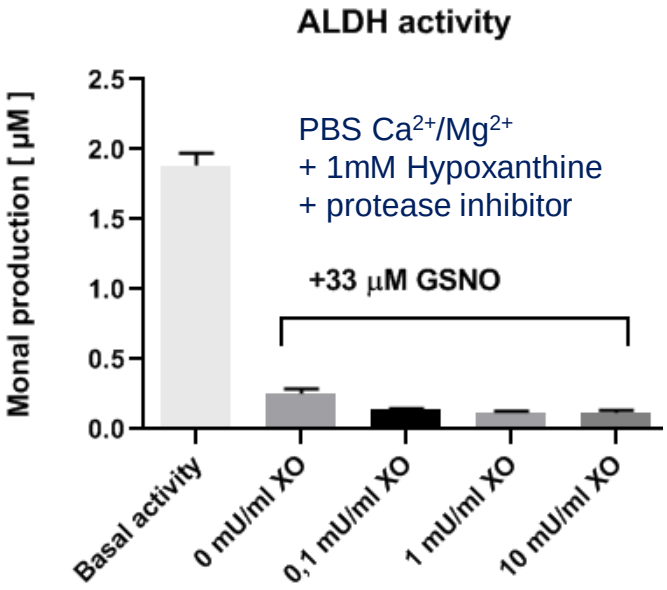
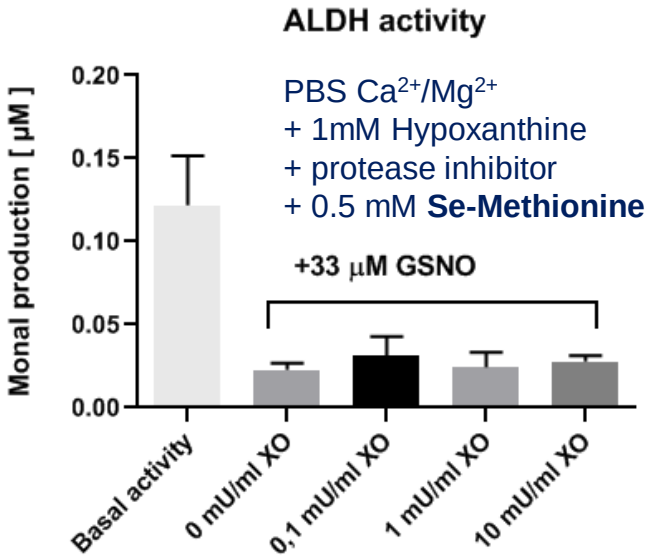
Slide 7 - Methods

The activity of ALDH was determined by measuring the conversion of 6-methoxy-2-naphthylaldehyde (Monal 62) to the fluorescent naphthoic acid product in the absence and presence of 33 µM GSNO and superoxide producing enzyme (XO in 1mM hypoxanthine buffer). Monal 62 (100 µM) was added 5 U/ml ALDH solution and incubated for 60 min at 37 °C, then stopped with the ALDH inhibitor benomyl (10 µM). Samples were centrifuged 10 min at 18,000 x g and 50 µl supernatant were subjected to HPLC analysis. The system consisted of a Jasco HPLC system and a C_{18}-Nucleosil 125x4mm 100-3 reversed phase column from Macherey & Nagel (Düren, Germany). The Monal oxidation product was detected by fluorescence (excitation 310 nm / emission 360 nm), and quantified using an internal and external standard. The typical retention time for the Monal62 oxidation product was 5.2 min.

Incubation with GSNO and with XO with increasing concentrations 0.1 mU/ml; 1 mU/ml and 10 mU/ml for 30min was done prior to the incubation with Monal 62.

Slide 8 - Results





There does not seem to be reagaining of the ALDH-2 activity with addition of small amounts of XO in HX buffer, on the contrary; the activity was decresed further more. The reason for this may be production of peroxynitrite by the reaction of NO and superoxide.

By adding Se-methione, which can act as a peroxynitrite scavenger, total basal ALDH activity was decreased by 10-fold, but addition of XO did not decreased activity further more, as it was detected without Se-methione, which points to the contribution of peroxynitrite in the loss of ALDH-2 activity.

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Slide 9 - Conclusion

There were difficulties in optimizing conditions for the hypothesis that was proposed : that small amount of superoxide can trigger reactivation of nitrosated enzymes as observed when using GSNO, which was reactivated by low concentrations of XO/HX.

With addition of Se-methionine which is scavenger for peroxynitrite there was no further drop in ALDH-2 activity and but rather a slight increase was detected with the smallest concentration of superoxide procuding enzyme (0.1 mU/ml XO) but unfortunatley there was no significant change.

We conclude that ALDH-2 may not be the optimal enzyme to test the above hypothesis (e.g. potentially by overoxidation or disulfide bridge formation in the active site). Other mitochondrial enzymes should be tested.

Of note, adding Se-methionine decreased basal activity by 10-fold.

We conclude that more stable conditions (e.g. better peroxynitrite scavengers, maybe missing cofactors) are needed to prove the hypothesis.

Slide 10 - Acknowledgments

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