Biological Protein Nitration: Mechanisms and Significance

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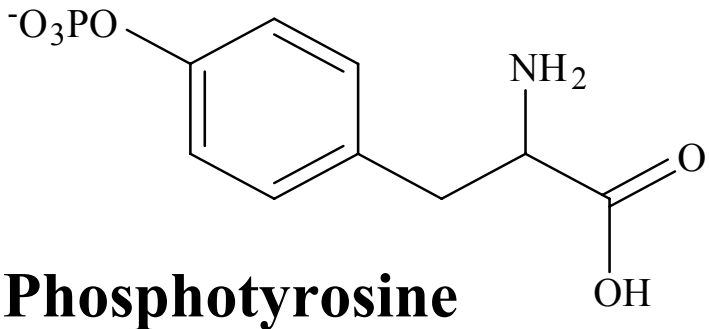
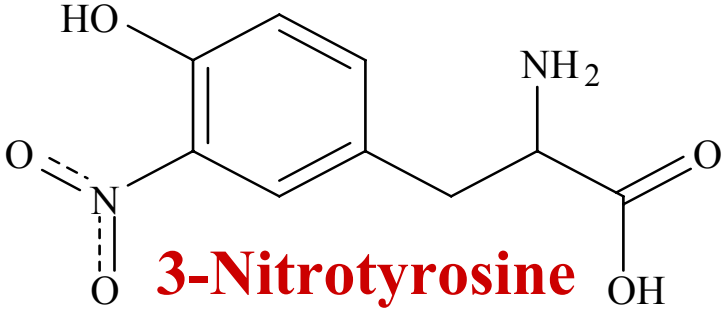
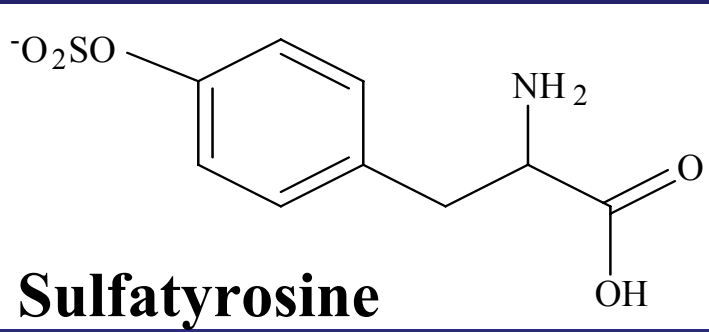
Post-translational Modifications of Protein Tyrosine Residues



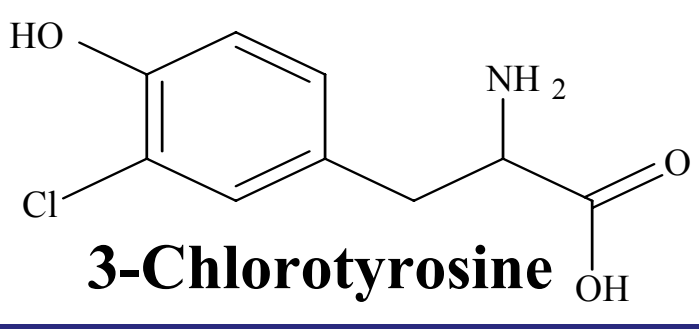
Phosphotyrosine



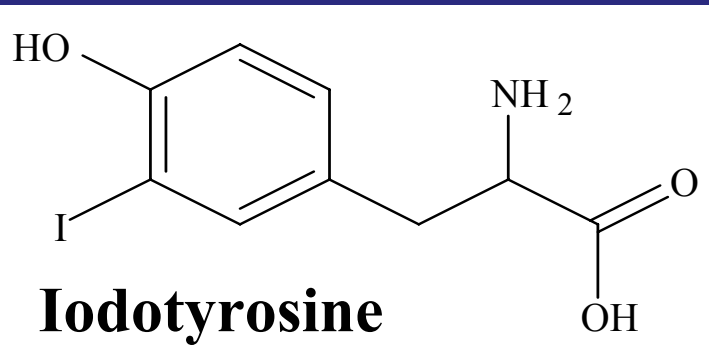
3-Nitrotyrosine



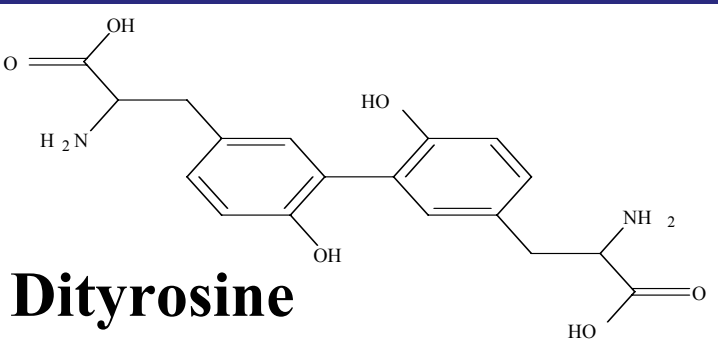
Sulfatyrosine



3-Chlorotyrosine



Iodotyrosine



Dityrosine

Biological Protein Nitration: Outline

- In search of the *in vivo* nitrating agents
- Detection of 3-nitrotyrosine and nitrated proteins
- Specific protein targets
- Consequences in protein function and in the pathogenesis of disease
- Metabolism of nitrated proteins
- Possible role in signal transduction - comparison with other tyrosine signaling pathways and with S-nitrosocysteine

In search of the *in vivo* nitrating agents: Possible nitration pathways

Oxidation state (n)	2	3	4	5
Element	$^{\cdot}NO$	NO_2^-	$^{\cdot}NO_2$	$ONOO^-$
Intermediates/ catalysts	$Tyr^{\cdot}$	HNO_2 H_2O_2, HOCl Hemeproteins **MPO/EPO**	$Tyr^{\cdot}$	***CO_2*** Me^{n+} ROH, RCO_2 MPO

Tyrosyl Radical/•NO: Prostaglandin H Synthase-2, Ribonucleotide Reductase
Peroxidases: Catalysts of both nitrite and peroxynitrite-mediated nitration: *In vivo* contribution has been confirmed by the use of MPO or EPO knock-out mice
Hypochlorous acid/NO_2^-: Likely not involved in peroxidase-mediated nitration
Nitrogen Dioxide: Inefficient in the absence of tyrosyl radical
$ONO(O)CO_2^-$: More efficient nitrating agent than $ONOO^-$ in some but not all proteins

Methods for Quantification and Detection of Nitrotyrosine

1. **Analytical Methods:**

   HPLC (UV, Electrochemical Detection)
   Gas Chromatography/Mass Spectrometry
   LC/Mass Spectrometry

   Major concern: artificial formation during acid hydrolysis
   Remedy: base hydrolysis, inclusion of uniformly labeled tyrosine

2. **Immunological Methods (Antibodies)**

   Western Blotting, Direct or in conjunction with IP
   Immunocytochemistry/Immunohistochemistry
   ELISA

   Major concern: antibody specificity
   Remedy: raise specific monoclonal antibodies to target protein, controls, controls, controls…

Immunohistochemical Methods for Nitrotyrosine Detection

Specificity of Antibody Binding

1) Blocking primary antibody with 3-nitrotyrosine (1 mM)
2) Reduce nitrotyrosine to aminotyrosine with repeated washes in dithionite
3) Omit primary antibody
4) Generate a positive control by treatment with a nitrating agent

<u>Recommendations:</u> Use $F(ab)_2$ fragment of secondary antibody or direct labeling of primary. Raise primary antibodies against specific nitrated proteins and use synthetic peptides rather than chemically ($ONOO^-$, TNM, MPO + H_2O_2 + NO_2^-) treated proteins or peptides as antigens.

Protein Tyrosine Nitration

- Specific proteins modified by nitration have been detected in more than 50 human disorders
- Associated with oxidative stress, most of the nitrating agents require the formation of reactive nitrogen and oxygen species
- Localized at site(s) of injury and in selective cell types
- Only a selective number of proteins are modified by nitration *in vivo*
- Only specific tyrosine residues in proteins are targets for nitration:
  Selectivity is derived from protein structure and folding

Selectivity of Tyrosine Nitration *in vivo*

Presumed factors driving selectivity:

Proteins in close proximity to the site of generation of nitrating agents

Proteins contain tyrosine residue(s) in environments that promotes nitration

Factors that do not predict selectivity:

Abundance of protein and/or number of tyrosine residues

There is no apparent requirement for specific primary sequence

Apparent structural requirements for the *in vivo* selectivity of protein tyrosine nitration

Rank in the presumed order of most importance:

- **Paucity of reactive cysteine residues in the vicinity of the tyrosine**
- **Proximity to a negatively charged residue**
- **Absence of steric hindrances**
- **Surface exposure**
- **Preference for tyrosine residues in loop structures**

Nitration of plasma proteins in ARDS patients

Protein	Activity in Controls	Activity in ARDS	Activity *in vitro* (% of control)	Mole nitrotyr/ molecule
Ceruloplasmin	10.06 ± 1.64	1.92 ± 0.54	50.3 ± 1.6	2.80
Transferrin	nd	nd	~100	4.0
α1-Anti-chymotrypsin	547 ± 165	350 ± 38	91.3 ± 6.1	1.72
α1-Protease inhibitor	9.42 ± 0.49	5.23 ± 0.62	60.3 ± 5.3	0.97
Fibrinogen	nd	nd	193.4 ± 8.5	1.13

Protein function is unaffected (transferrin, α1 anti-chymotrypsin), decline or increase (fibrinogen) upon nitration. nd: not determined

Possible role in signal transduction and immune response

In order for tyrosine nitration to function as a signal transduction event it must meet two requirements:

1) must be a selective process

2) must be reversible

Fine Print: **The first requirement appears to be fulfilled whereas the second is a possibility waiting further characterization and isolation of the putative denitrase enzyme**

Nitrated proteins:

1) May induce antibody production

2) May serve as chemotactic factor(s)

3) May be phagocytized by macrophages and other cells

Fine Print: **The first is a safe bet, the other two are attractive and testable hypotheses**

Pathways to remove and/or repair nitrated proteins

- ✓ 3-Nitrotyrosine is not reduced by bacterial and mammalian nitroreductases
- ✓ Nitrated tyrosine residues are not resistant but significantly retard cleavage by chymotrypsin
- ✓ Nitration of a single tyrosine residue is sufficient to accelerate the degradation of certain proteins by the proteasome
- ✓ Human and rodent tissues are able to repair/remove nitrated proteins by specific “denitrase” or unique proteolytic pathways

A Repair mechanism "Denitrase"

- Loss of antigenic binding without apparent protein degradation
- Exhibit different kinetics towards different nitrated protein substrates
- Does not function when 3-nitrotyrosine or 3-nitrotyrosine peptides are used as substrates
- The activity in rat tissues appears to be in the soluble fractions of lung and spleen, is heat and trypsin labile and is induced by endotoxin
- The products of the reaction are not known but it does not appear to be aminotyrosine

Protein tyrosine nitration is not important *in vivo*

- ✓It represents another marker of oxidative stress
- ✓Yield is low
- ✓Other modifications may contribute to loss of function
- ✓It is effectively removed or repaired

Protein tyrosine nitration is important *in vivo*

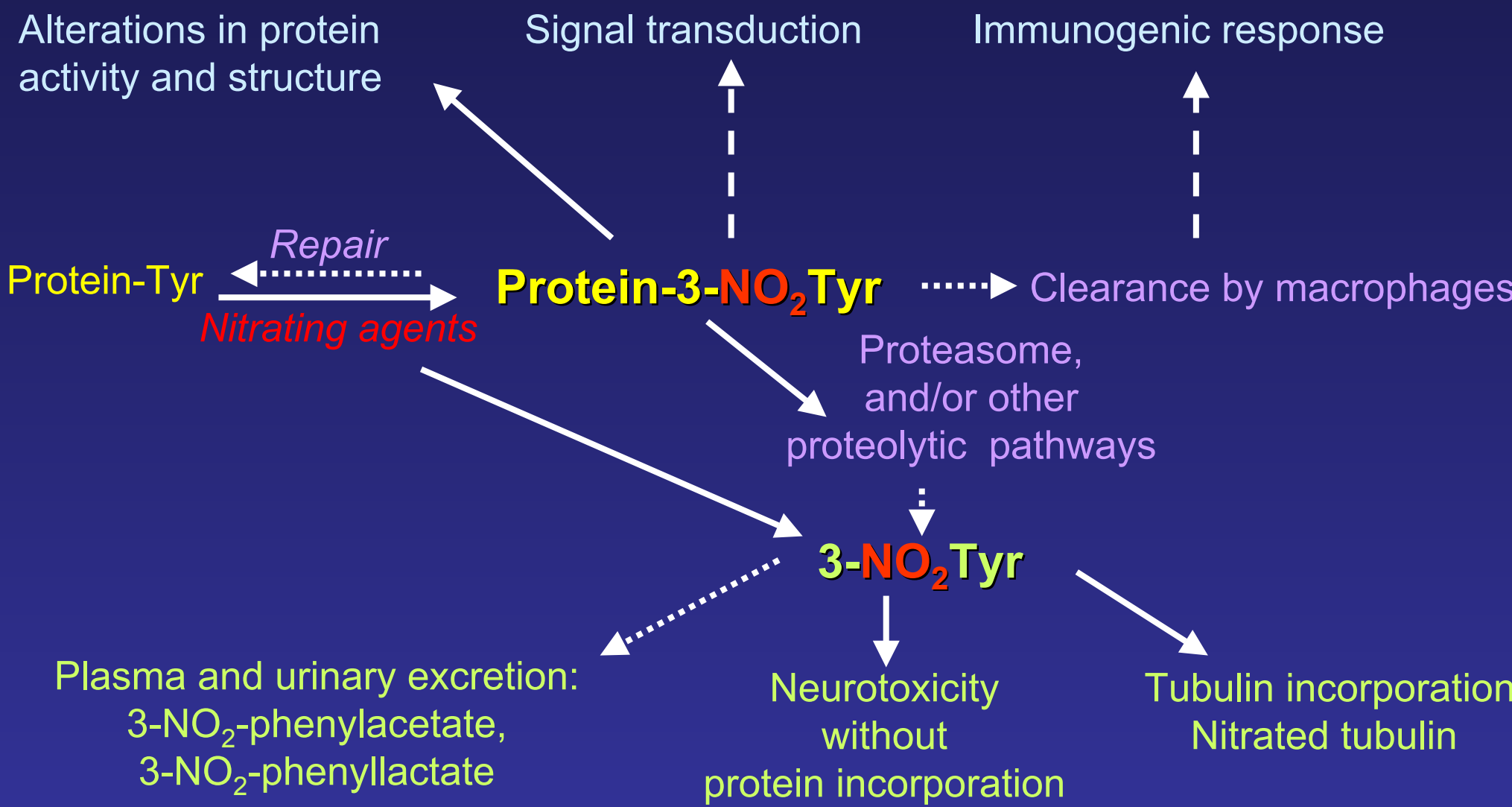
- ➢Selective, not all proteins are modified
- ➢Yield of specific proteins is sufficient to alter activity
- ➢Alter function in some but not all proteins, could serve as a signaling pathway
- ➢Alter protein turn-over
- ➢Induce immune responses

Post-translational modification	Enzyme	Consensus Sequence	Reversibility	Function
Phosphorylation	Tyrosine Kinases	[Lys-Arg]-X-X-X-[Glu-Asp]-X-X-X-**Tyr**	Tyrosine Phosphatases	Signal transduction
Sulfation	Tyrosylprotein sulfotransferase	None, but other structural requirements	Irreversible, resistant to Chymotrypsin	Protein targeting/ processing
Oxidation/ chlorination	MPO/EPO and non-enzymatic	Not known	Degradation by 20S Proteasome	Covalent cross linking
Nitration	MPO/EPO and non-enzymatic	None, but other structural requirements	Potential "denitrase" Chymotrypsin sensitive Proteasomal degradation	Alterations in function/ turn-over Signal transduction

NO-mediated post-translational protein modifications: S-nitrosocysteine vs 3-nitrotyrosine

Post-translational modification	Chemistry of formation	Consensus Sequence Secondary structure	Reversibility	Function
Cysteine *S-nitrosylation* -SNO	Ceruloplasmin Heme protein and non-enzymatic	X-[Lys,Arg,His]-Cys-[Glu-Asp] and Hydrophobic motifs	SNO-transferases: Anion exchanger-1 Metabolism: Alcohol dehydrogenase III	Regulation of protein function, on/off signal, NO transport
Tyrosine *Nitration* -YNO_2	MPO/EPO, Heme protein and non-enzymatic	None, but some structural requirements have been defined	Potential "denitrase" Degradation by Proteasome, Chymotrypsin sensitive	Alterations in function, Immune responses Signal transduction?

Tyrosine Nitration Subway Map: best documented data is represented by solid lines, dashed lines represent work in progress or working hypotheses



Protein Tyrosine Nitration: some relevant references

Reviews:

Olah GA, Malhotra R, Narang SC. (1989) In: *Nitration, Methods and Mechanisms. Organic Nitro-Chemistry Series*, VCH Publishers, Inc.

Ischiropoulos H. (1998) Biological tyrosine nitration: A pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Biophys.* **356**: 1-11.

Greenacre SAB., Ischiropoulos H. (2001) Tyrosine Nitration: Localisation, quantification, consequences for protein function and signal transduction. *Free Rad Res.* **34**:541-581.

Specific Proteins:

MacMillan-Crow LA, Crow JP, Kirby JD, Beckman JS, Thompson JA. (1996) Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc Natl Acad Sci USA.* **93**:11853-8.

Giasson BI, Duda JE, Murray I, Chen Q, Souza JM, Hurting HI, Ischiropoulos H, Trojanowski JQ, Lee M-Y. (2000) Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleiopathy lesions. *Science.* **290**:985-989.

Souza JM, Daikhin E, Yudkoff M, Raman CS, Ischiropoulos H. (1999) Factors determining the selectivity of protein tyrosine nitration. *Arch Biochem Biophys.* **371**:169-178.

Reversibility:

Kamisaki Y, Wada K, Bian K, Balabanli B, Davis K, Martin E, Behbod F, Lee Y-C, Murad F. (1998) An activity in rat tissues that modifies nitrotyrosine containing proteins. *Proc Natl Acad Sci USA*. **95**:11584-11589.

Gow A, Duran D, Malcolm S, Ischiropoulos, H. (1996) Effects of peroxynitrite induced modifications to signal transduction and protein degradation. *FEBS Lett.* **385**:63-66.

Signal Transduction:

Kong S-K, Yim MB, Stadtman ER, Chock PB. (1996) Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism: lymphocyte-specific tyrosine kinase fails to phosphorylate nitrated cdc2(6-20)NH_2 peptide. *Proc Natl Acad Sci USA.* **93**:3377-3382.

Brito C, Naviliat M, Tiscotina AC, Vuillier F, Gualco G, Dighiero G, Radi R, Cayota AM (1999) Peroxynitrite inhibits T lymphocyte activation and proliferation by promoting impairment of tyrosine phosphorylation and peroxynitrite-driven apoptotic death. *J Immunol.* **162**:3356-3366.