Reactive Oxygen Species

- The Earth was originally anoxic
- Metabolism was anaerobic
- O_2 started appearing ~2.5 x 10^9 years ago

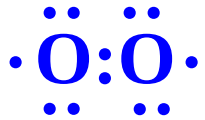
Anaerobic metabolism-glycolysis

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i \longrightarrow \text{Lactate} + 2\text{ATP} + 2\text{H}_2\text{O}$$

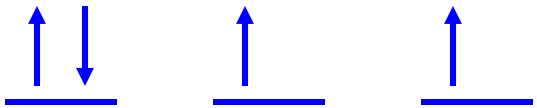
O_2 an electron acceptor in aerobic metabolism

$$\text{Glucose} + 6\text{O}_2 + 36\text{ADP} + 36\text{P}_i \longrightarrow 6\text{CO}_2 + 36\text{ATP} + 6\text{H}_2\text{O}$$

- **Ground-state oxygen has 2-unpaired electrons**



- **The unpaired electrons have parallel spins**



- **Oxygen molecule is minimally reactive due to spin restrictions**

Basics of Redox Chemistry

Term	Definition
Oxidation	Gain in oxygen Loss of hydrogen Loss of electrons
Reduction	Loss of oxygen Gain of hydrogen Gain of electrons
Oxidant	Oxidizes another chemical by taking electrons, hydrogen, or by adding oxygen
Reductant	Reduces another chemical by supplying electrons, hydrogen, or by removing oxygen

Prooxidants

Free Radicals:

- Any species capable of independent existence that contains one or more unpaired electrons
- A molecule with an unpaired electron in an outer valence shell

$R_3C^{\cdot}$	Carbon-centered
$R_3N^{\cdot}$	Nitrogen-centered
$R\text{-}O^{\cdot}$	Oxygen-centered
$R\text{-}S^{\cdot}$	Sulfur-centered

Non-Radicals:

- Species that have strong oxidizing potential
- Species that favor the formation of strong oxidants (e.g., transition metals)

H_2O_2	Hydrogen peroxide
$HOCl^{-}$	Hypochlorous acid
O_3	Ozone
$^1O_2$	Singlet oxygen
$ONOO^{-}$	Peroxynitrite
Me^{n+}	Transition metals

Reactive Oxygen Species (ROS)

Radicals:	
$O_2^{\cdot -}$	Superoxide
$OH^{\cdot}$	Hydroxyl
$RO_2^{\cdot}$	Peroxyl
$RO^{\cdot}$	Alkoxyl
$HO_2^{\cdot}$	Hydroperoxyl

Non-Radicals:	
H_2O_2	Hydrogen peroxide
$HOCl^-$	Hypochlorous acid
O_3	Ozone
$^1O_2$	Singlet oxygen
$ONOO^-$	Peroxynitrite

Reactive Nitrogen Species (RNS)

Radicals:

$NO^{\cdot}$	Nitric Oxide
$NO_2^{\cdot}$	Nitrogen dioxide

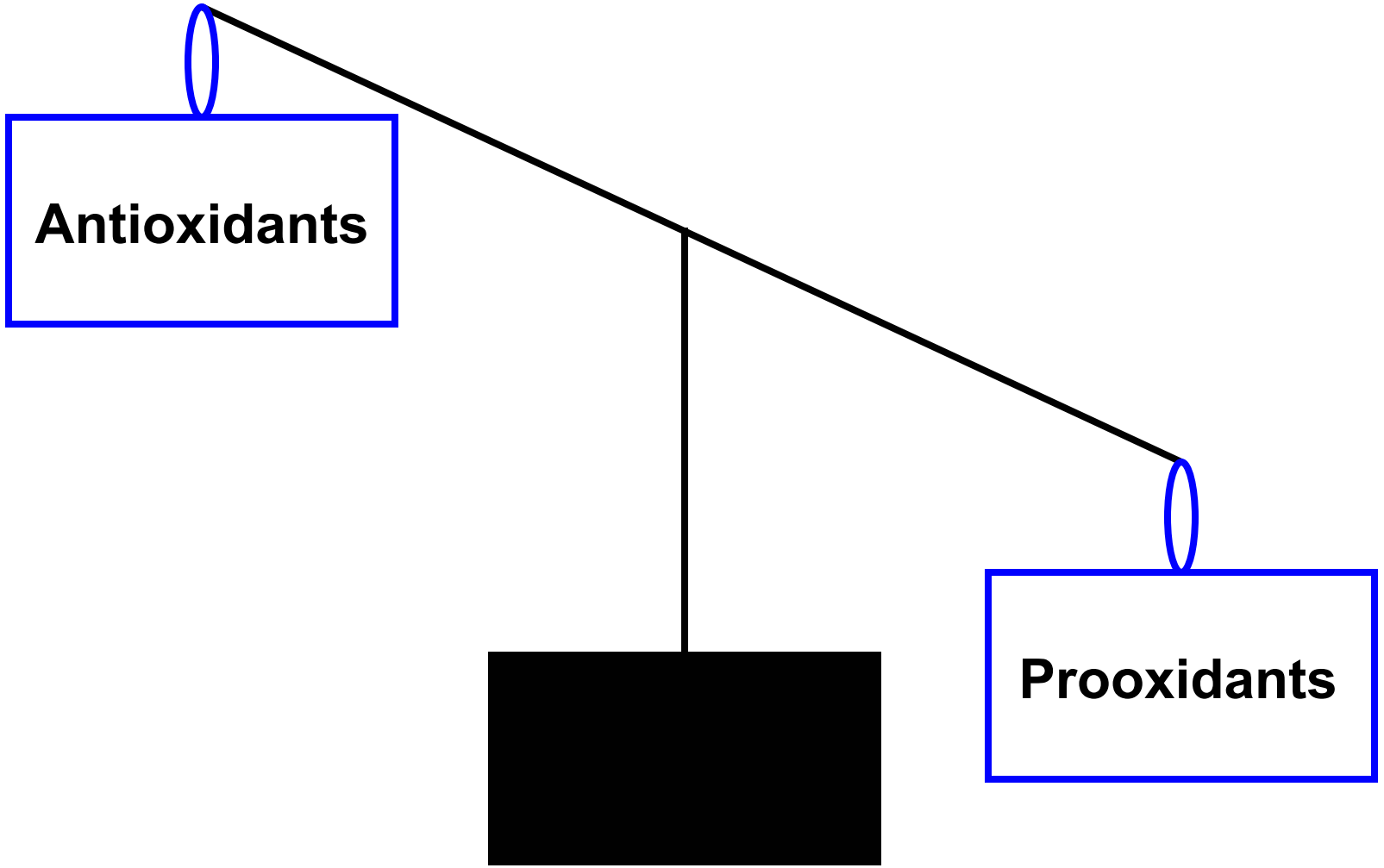
Non-Radicals:

$ONOO^-$	Peroxynitrite
ROONO	Alkyl peroxynitrites
N_2O_3	Dinitrogen trioxide
N_2O_4	Dinitrogen tetroxide
HNO_2	Nitrous acid
NO_2^+	Nitronium anion
NO^-	Nitroxyl anion
NO^+	Nitrosyl cation
NO_2Cl	Nitryl chloride

"Longevity" of reactive species

Reactive Species	Half-life
Hydrogen peroxide Organic hydroperoxides Hypohalous acids	~ minutes
Peroxyl radicals Nitric oxide	~ seconds
Peroxynitrite	~ milliseconds
Superoxide anion Singlet oxygen Alcoxyl radicals	~ microsecond
Hydroxyl radical	~ nanosecond

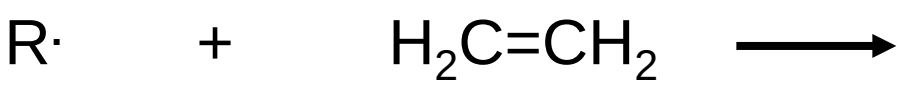
Oxidative Stress



"An imbalance favoring prooxidants and/or disfavoring antioxidants, potentially leading to damage" -H. Sies

Radical-mediated reactions

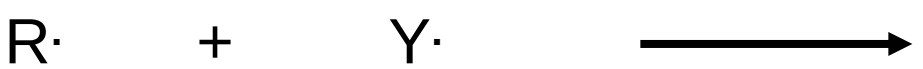
Addition



Hydrogen abstraction

Electron abstraction

Termination



Disproportionation

Hydroxyl radical (·OH)

$$O_2^{\cdot -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

Fenton $$H_2O_2 + Fe^{2+} \longrightarrow OH^- + {\cdot}OH + Fe^{3+}$$

Haber-Weiss $$O_2^{\cdot -} + H_2O_2 \longrightarrow OH^- + O_2 + {\cdot}OH$$

- **Transition metal catalyzed**
- **Other reductants can make Fe^{2+}** (e.g., GSH, ascorbate, hydroquinones)
- **Fe2+ is an <u>extremely</u> reactive oxidant**

Important Enzyme-Catalyzed Reactions

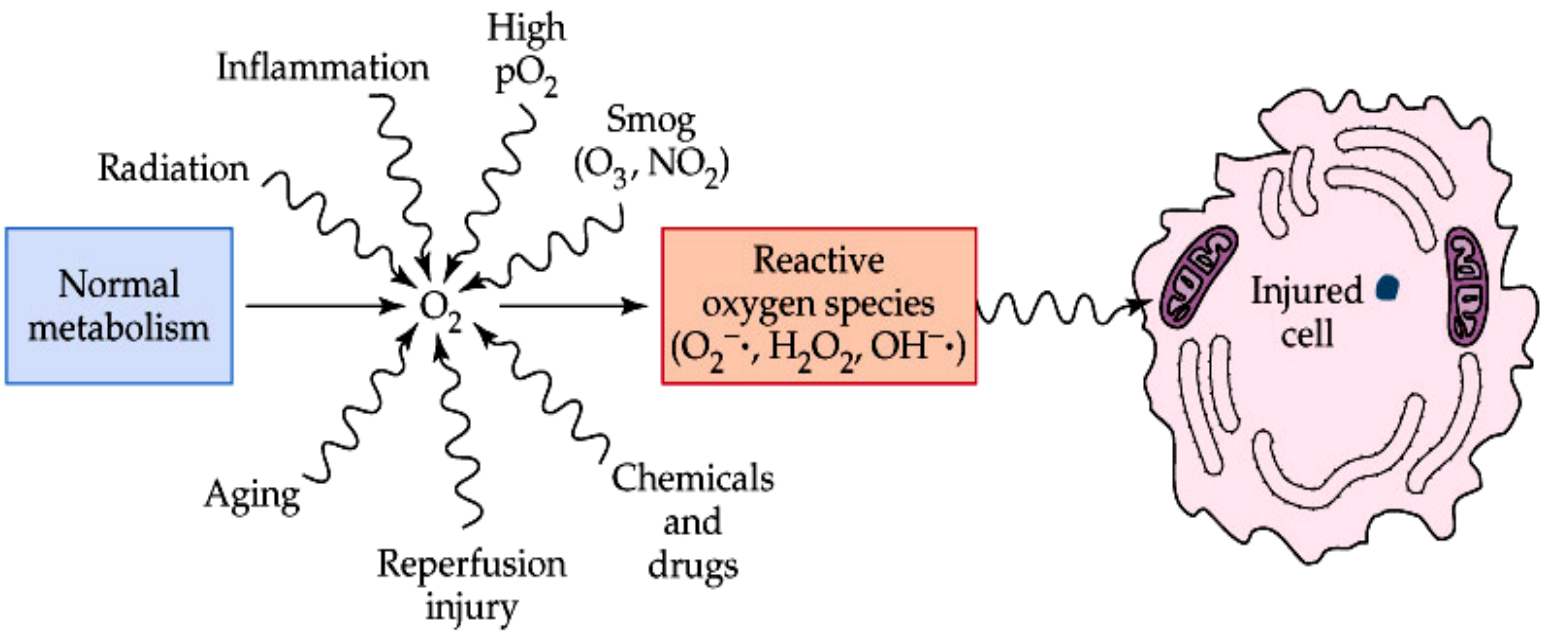
$$O_2^{\bullet -} + O_2^{\bullet -} + 2\,H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

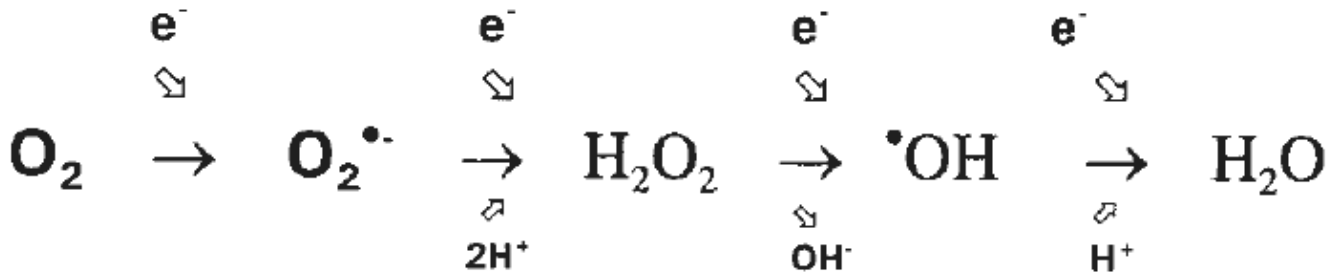
$$2\,H_2O_2 \xrightarrow{CAT} O_2 + 2\,H_2O$$

$$ROOH + 2\,GSH \xrightarrow{GPx} ROH + H_2O + GSSG$$

$$H_2O_2 + 2\,GSH \xrightarrow{GPx} 2\,H_2O + GSSG$$

Biological Pathways for Oxygen Reduction





$O_2^{\cdot-}$ = superoxide, H_2O_2 = hydrogen peroxide, $^{\bullet}OH$ = hydroxyl radical

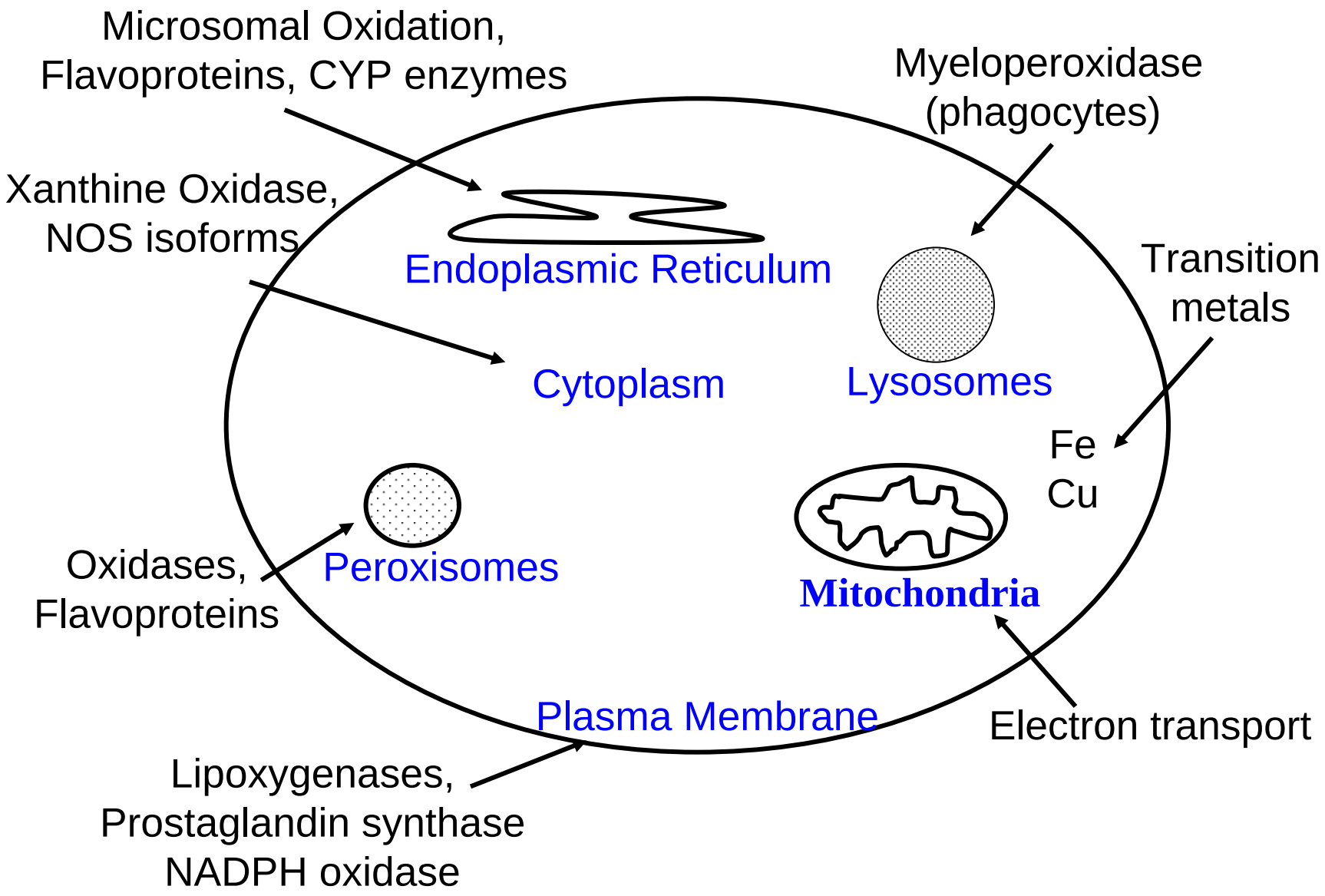
From: McMurry and Castellion "Fundamentals of general, organic and biological chemistry"

Important physiological functions that involve free radicals or their derivates

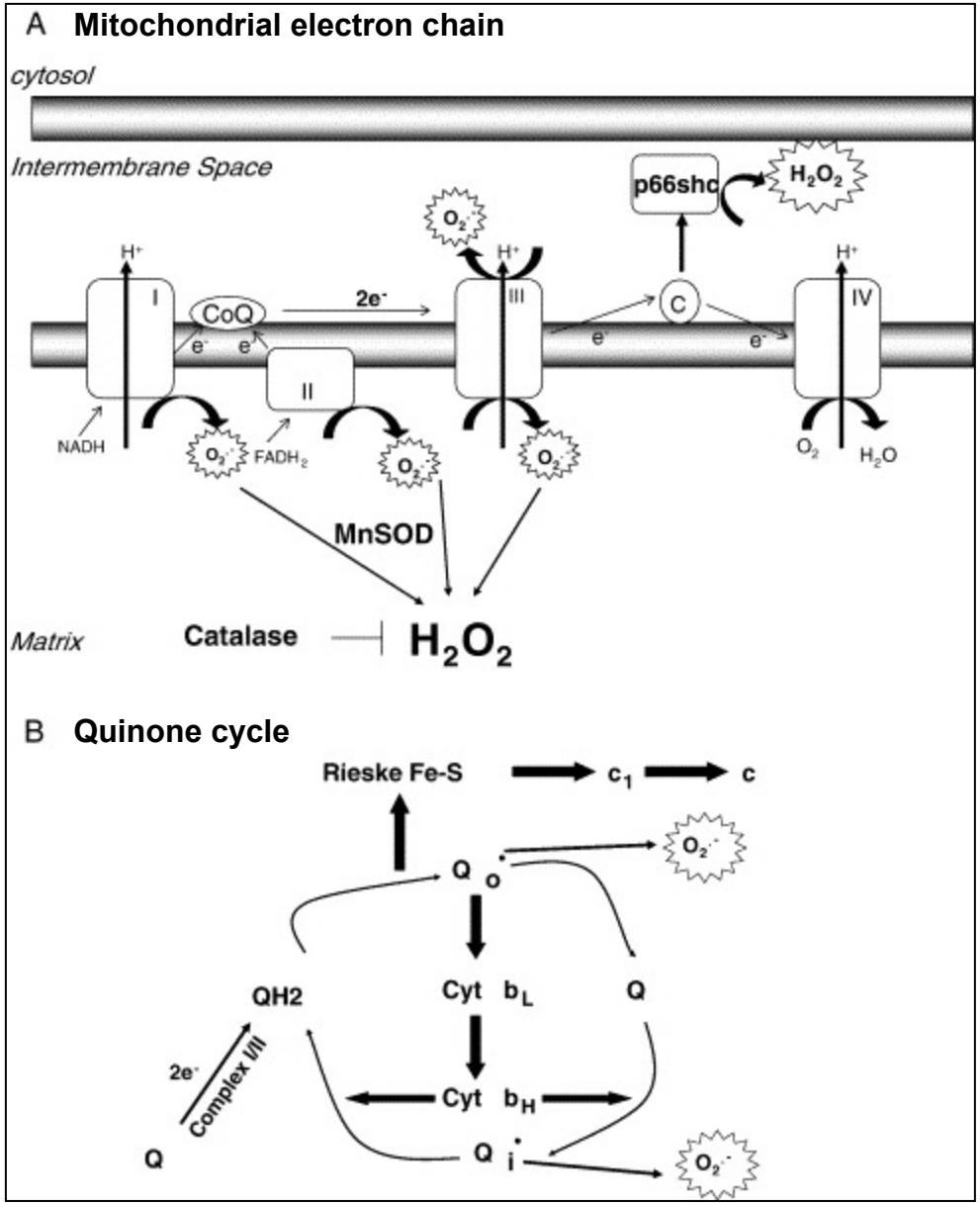
Type of Radical	Source of Radical	Physiological Process
Nitric oxide ($NO^{\cdot}$)	Nitric oxide synthase	Smooth muscle relaxation (control of vascular tone) and various other cGMP-dependent functions
Superoxide ($O_2^{-}\cdot$) and related ROS	NAD(P)H oxidase	Control of ventilationControl of erythropoietin production and other hypoxia-inducible functionsSmooth muscle relaxationSignal transduction from various membrane receptors/enhancement of immunological functions
Superoxide ($O_2^{-}\cdot$) and related ROS	Any source	Oxidative stress responses and the maintenance of redox homeostasis

From Droge W (2002) Physiol Rev 82: 47-95

Endogenous sources of ROS and RNS



Mitochondria as a source of ROS



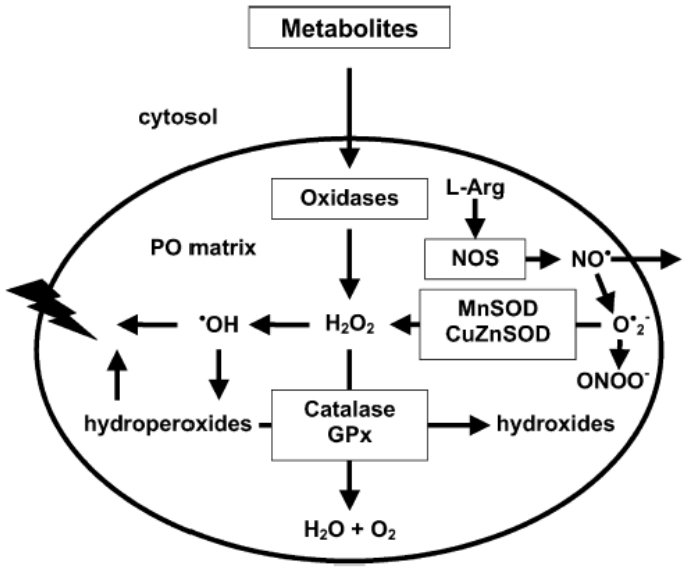
Chandel & Budinger, Free Radical Biol Med, 2007

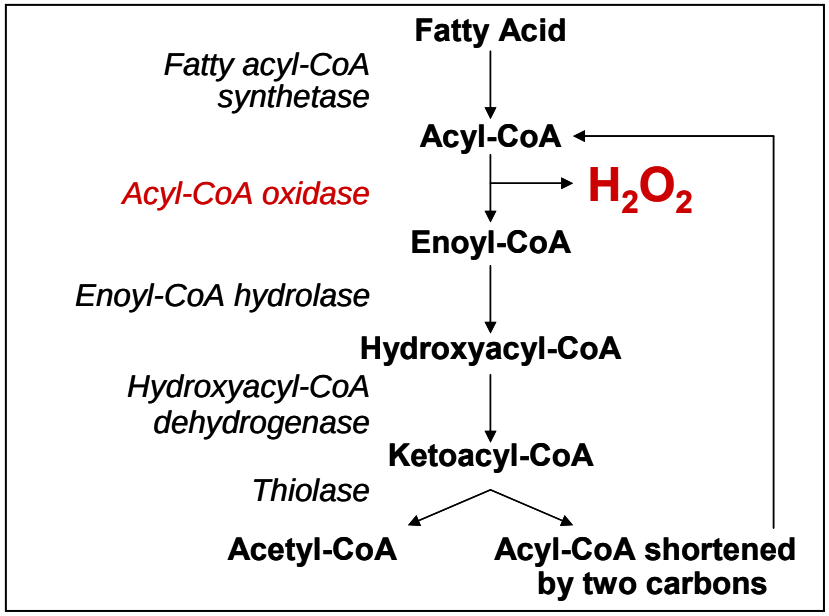
Localization of the main mitochondrial sources of superoxide anion

Component	Localization
Complex I (NADH dehydrogenase)	Inner membrane/ inner side
Complex II (succinate dehydrogenase)	Inner membrane/ inner side
Complex III (ubiquinol–cytochrome *c* reductase)	Inner membrane/ inner side
Complex III (ubiquinol–cytochrome *c* reductase)	Inner membrane/ outer side
External NADH dehydrogenase (yeast)	Inner membrane/ outer side
Glycerolphosphate dehydrogenase	Inner membrane/ outer side
Dehydroorotate dehydrogenase	Matrix
Mono amino oxidase	Outer membrane/ inner side

Turrens, J Physiol, 2003

Peroxisomes as a source of ROS and RNS





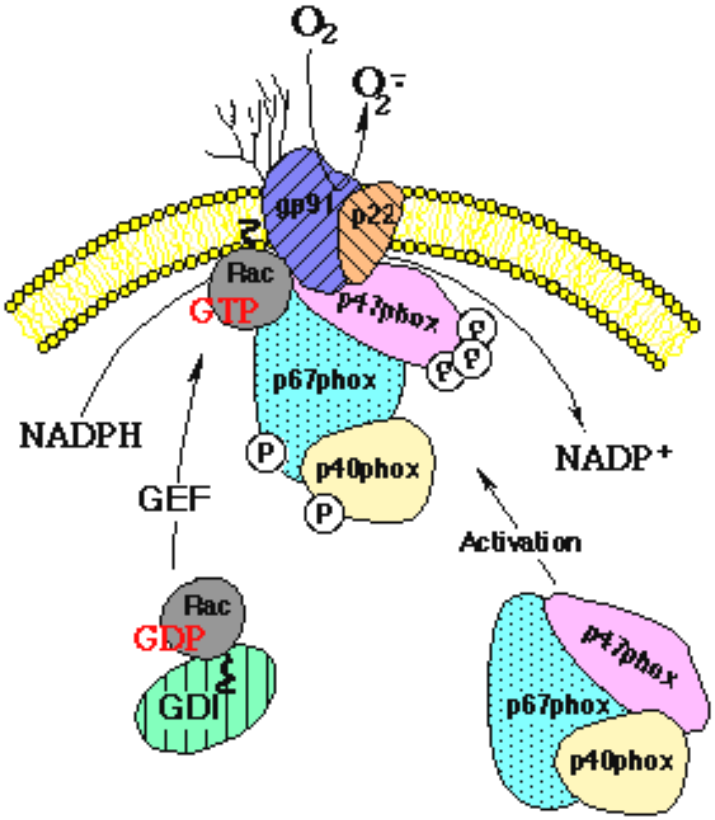
Enzymes in mammalian peroxisomes that generate ROS

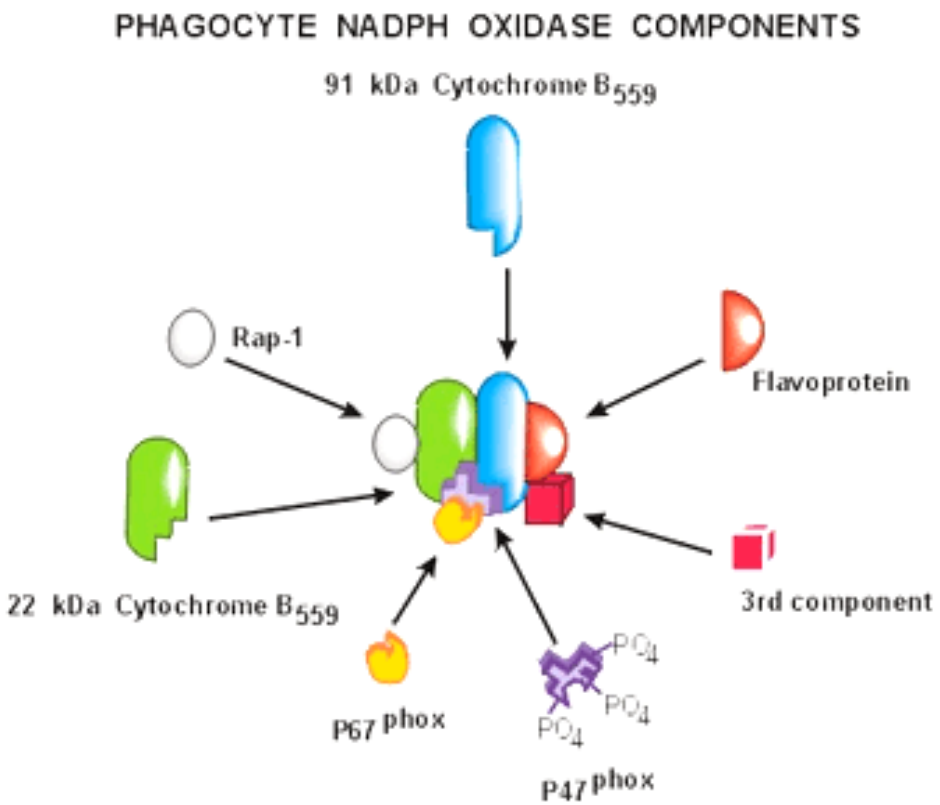
Enzyme	Substrate	ROS
1. Acyl-CoA oxidases		
a) Palmitoyl-CoA oxidase	Long chain fatty acids	H_2O_2
b) Pristanoyl-CoA oxidase	2-Methyl branched-chain fatty acids	H_2O_2
c) Trihydroxycoprostanoyl-CoA oxidase	Bile acid intermediates	H_2O_2
2. D-Amino acid oxidase	D-Proline	H_2O_2
3. D-Aspartate oxidase	D-Aspartate, *N*-methyl-D-aspartate	H_2O_2
4. α-Hydroxyacid oxidase	Glycolate, lactate	H_2O_2
5. Pipecolic acid oxidase	L-Pipecolic acid	H_2O_2
6. Polyamine oxidase	*N*-Acetyl spermine/spermidine	H_2O_2
7. Urate oxidase	Uric acid	H_2O_2
8. Xanthine oxidase	Xanthine	$O_2^{\cdot-}$
9. NO synthase	L-Arginine	$NO^{\cdot}$

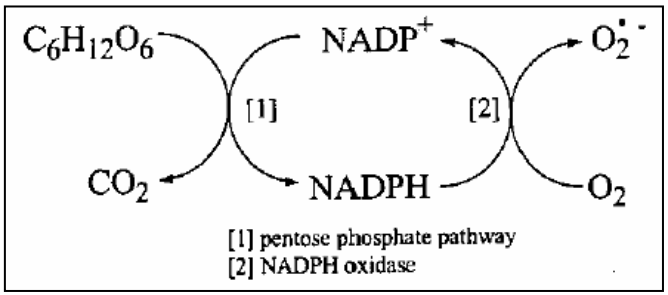
Schader & Fahimi, Histochem Cell Biol, 2004

NADPH oxidase as a source of ROS

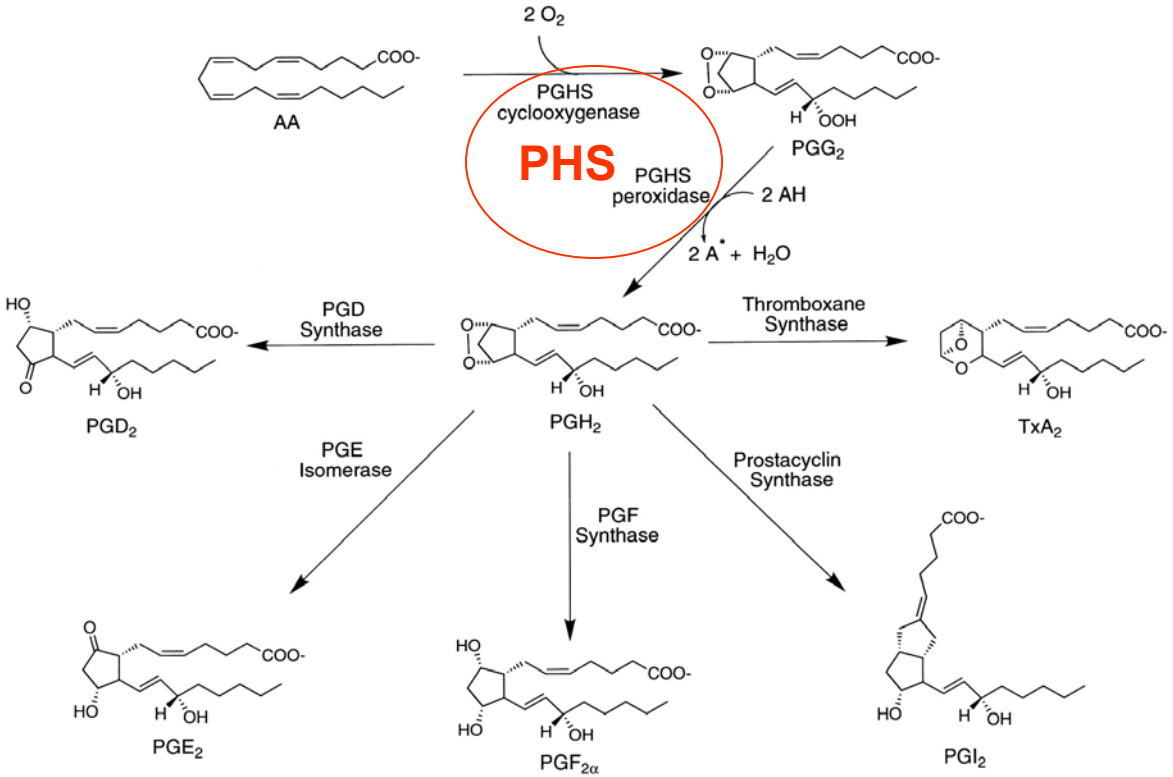
Present mainly in neutrophils (oxidative burst), but also in many other cell types



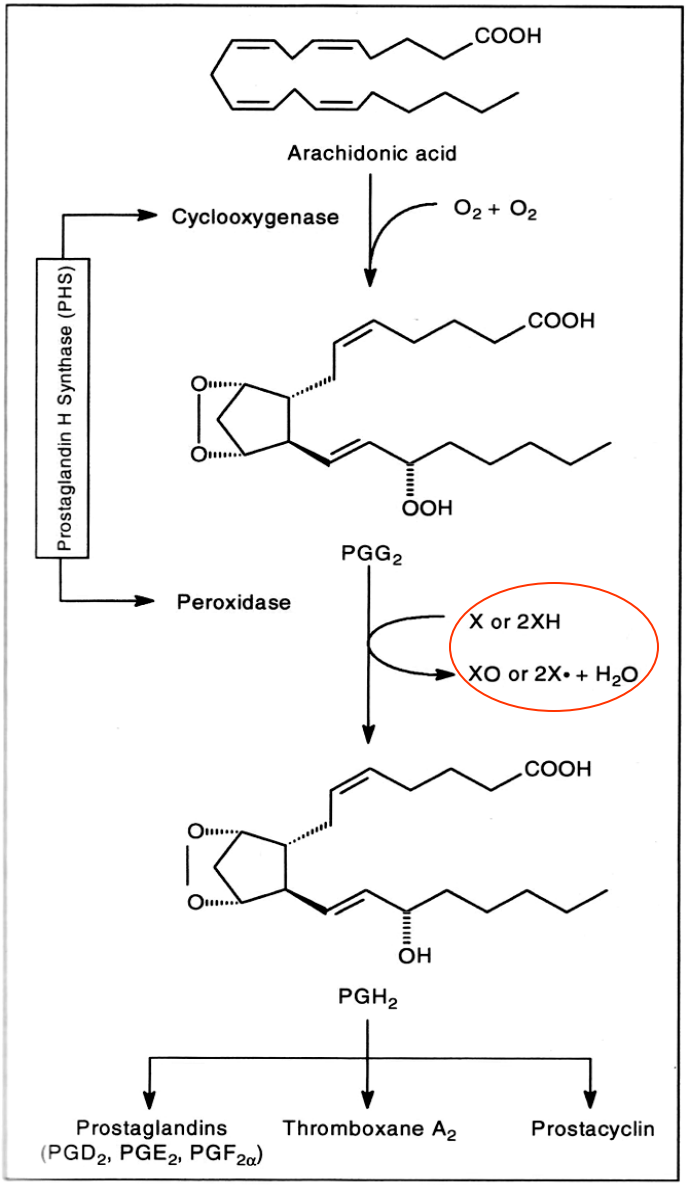




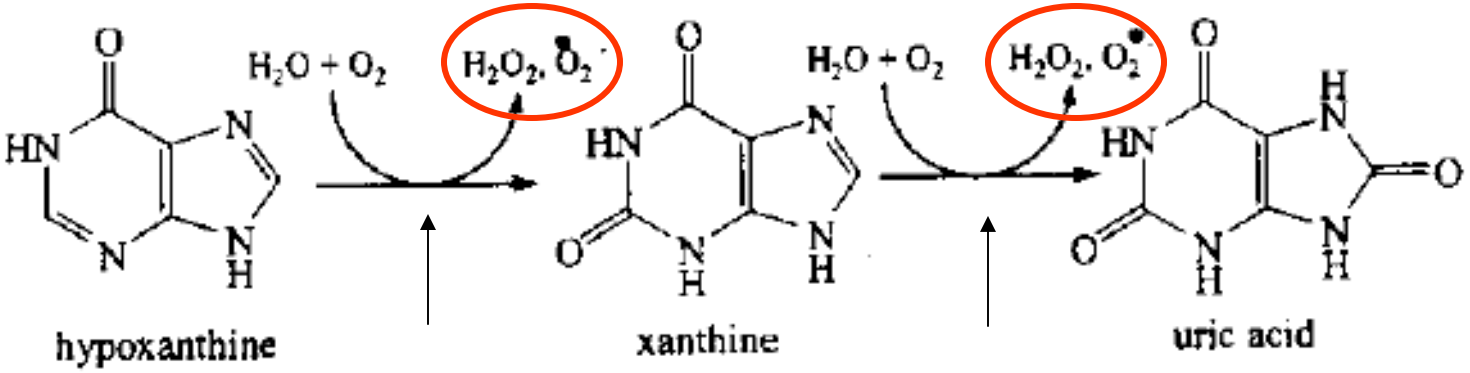
Prostaglandin H Synthase (PHS) as a source of ROS



Co-oxidation of xenobiotics (**X**) during arachidonic acid metabolism to PGH_2

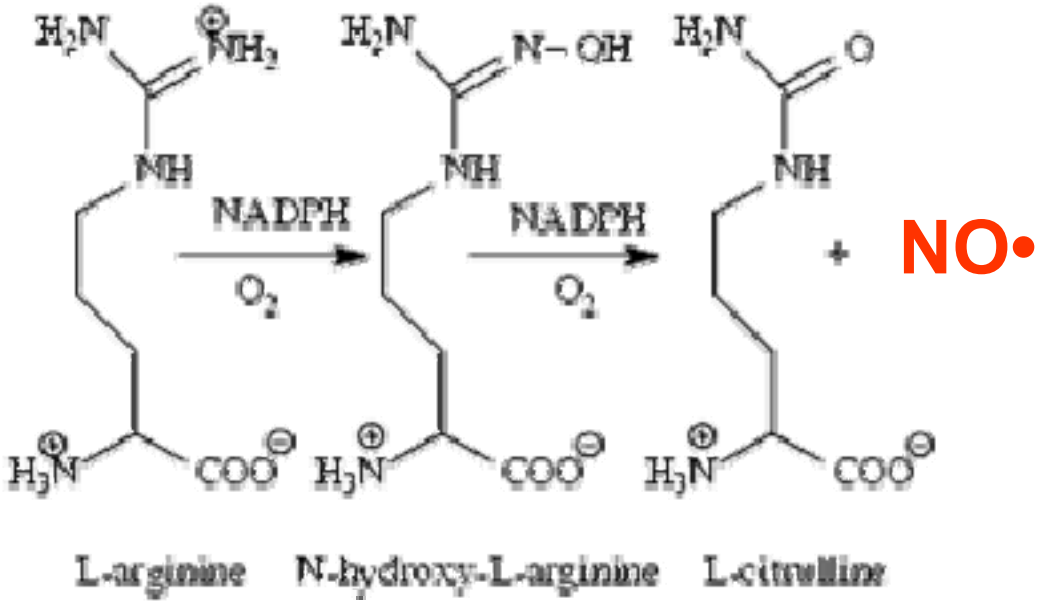


Cytoplasmic sources of ROS and RNS



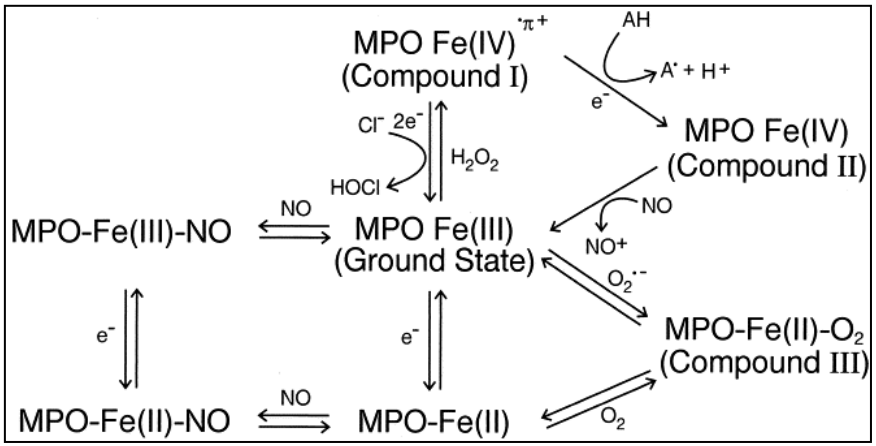
Nitric Oxide Synthases (NOS):

neuronal	nNOS (I)
endothelial	eNOS (III)
inducible	iNOS (II)

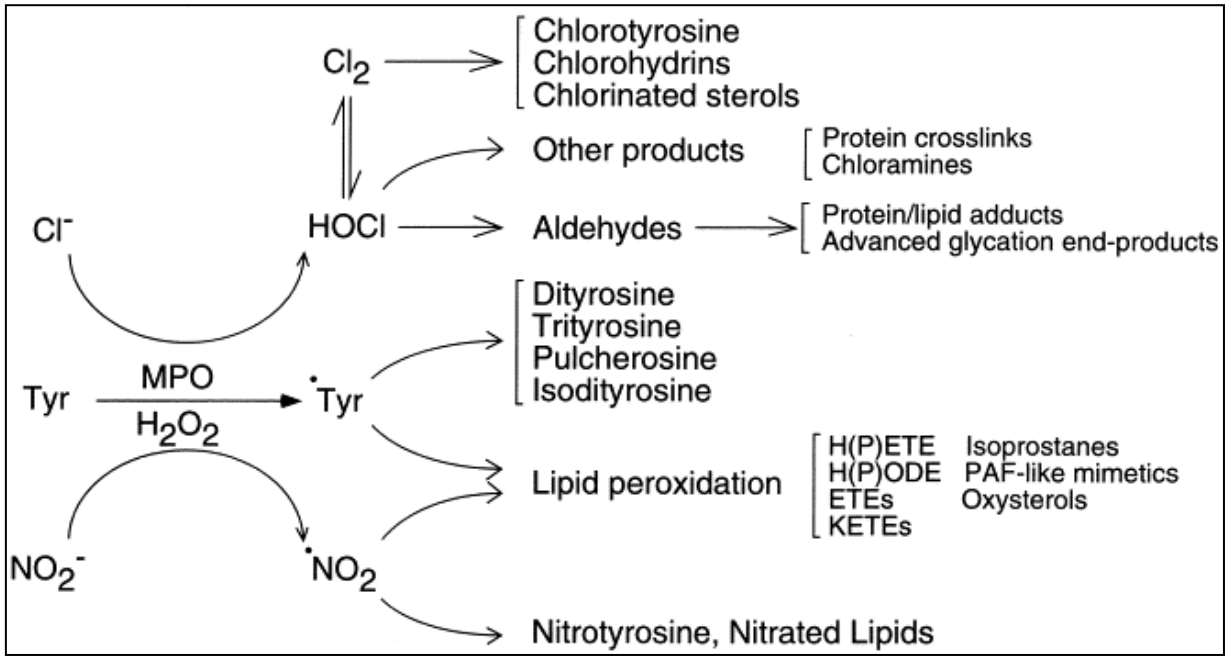


Lysosome as a source of ROS and RNS

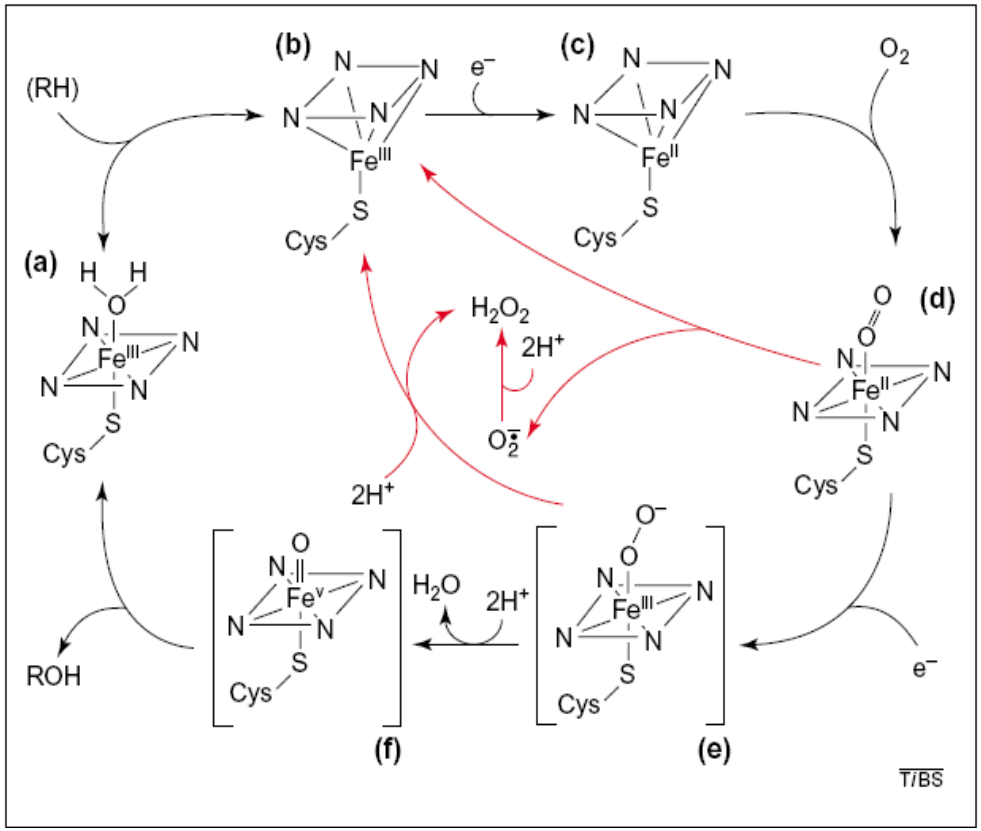
$$Cl^- + H_2O_2 + H^+ \xrightarrow{MPO} HOCl + H_2O$$



Myeloperoxidase undergoes a complex array of redox transformations and produces HOCl, degrades H_2O_2 to oxygen and water, converts tyrosine and other phenols and anilines to free radicals, and hydroxylates aromatic substrates via a cytochrome P450-like activity



Microsomes as a source of ROS (I)



A scheme of the catalytic cycle of cytochrome P450-containing monooxygenases. The binding of the substrate (RH) to ferric P450 (a) results in the formation of the substrate complex (b). The ferric P450 then accepts the first electron from CPR (cytochrome P450 reductase), thereby being reduced to the ferrous intermediate (c). This intermediate then binds an oxygen molecule to form oxycomplex (d), which is further reduced to give peroxycomplex (e). The input of protons to this intermediate can result in the heterolytic cleavage of the O–O bond, producing H_2O and the 'oxenoid' complex (f), the latter of which then inserts the heme-bound activated oxygen atom into the substrate molecule to produce ROH. In eukaryotic monooxygenases, reactive oxygen species (ROS) are produced by 'leaky' branches (red arrows). In one such branch, a superoxide anion radical is released owing to the decay of the one-electron-reduced ternary complex (d). The second ROS-producing branch is the protonation of the peroxycytochrome P450 (e), which forms of H2O2. In addition to these ROS-producing branches, another mechanism of electron leakage appears to be the four-electron reduction of the oxygen molecule with the production of water (Davydov, Trends Biochem Sci, 2001).

Microsomes as a source of ROS (II)

Table 1. Efficiency and coupling in some cytochrome-P450-containing monooxygenases[a]

System	Substrate	NADPH consumption turnover (min^{-1})	Coupling (substrate oxidized per NADPH consumed) (%)	H_2O_2 production per NADPH consumed (%)	O_2^- production per NADPH consumed (%)	Ref.
Liver microsomes of phenobarbital-treated rabbit[b]	None	(8)	–	44.0	36.0	16
	Benzphetamine	(40)	57.6	19.4	23.0	
CYP2B4, micellar reconstituted system	None	22	–	19.0	45.0	17
	Metoxyflurane	66	1.7	0.0	28.0	
	Benzphetamine	86	49.0	0.0	21.0	
CYP3A4, yeast microsomes	None	20	–	20.0	ND	19
	Nefidepine	20	8.0	32.5	ND	
	Quinidine	22	1.1	29.0	ND	
P450cam (CYP101)	Camphor	2411	96.4	2.9	ND	18

[a]Abbreviations: H_2O_2, hydrogen peroxide; ND, not determined; O_2^-, superoxide anion.
[b]The values of NADPH consumption rate given in the original publication are related to the milligram of microsomal protein. The indicative NADPH turnover numbers given here were estimated assuming the average content of cytochrome P450 in the liver microsomes of phenobarbital-treated rabbits to be about 3 nmol mg^{-1} protein[7,14].

Davydov, Trends Biochem Sci, 2001

Exogenous sources of free radicals

- **Radiation**
  UV light, x-rays, gamma rays
- **Chemicals that react to form peroxides**
  Ozone and singlet oxygen
- **Chemicals that promote superoxide formation**
  Quinones, nitroaromatics, bipyrimidiulium herbicides
- **Chemicals that are metabolized to radicals**

  e.g., polyhalogenated alkanes, phenols, aminophenols
- **Chemicals that release iron**
  ferritin

UV radiation

$$H_2O_2 \xrightarrow{UV_B} OH\cdot + OH\cdot$$

UV_A = 320-400 nm
UV_B = 290-320 nm
UV_C = 100-290 nm

- Primarily a concern in skin and eye
- Can also cause DNA damage
- Can form singlet oxygen in presence of a sensitizer

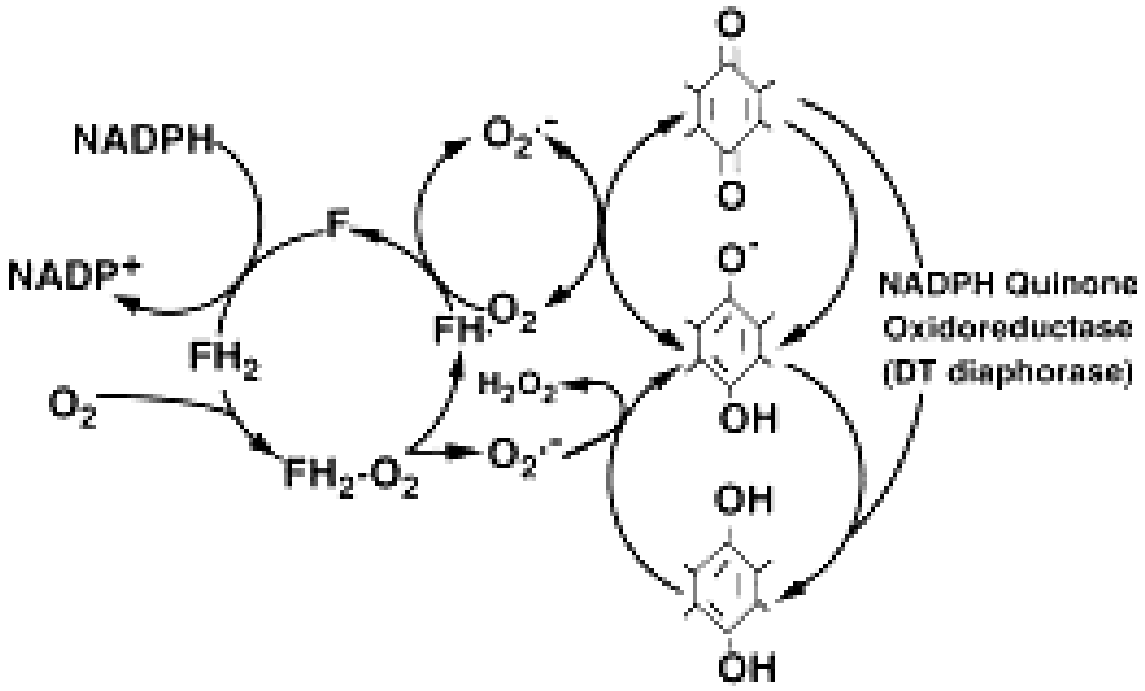
Ionizing radiation

$$2H_2O \xrightarrow{\gamma\text{-rays}} H_2O + e^- + H_2O^*$$

$$H_2O^* \longrightarrow H\cdot + \cdot OH$$

- **High energy radiation will result in ·OH**

Quinone redox cycling as a mechanism to generate ROS



quinone

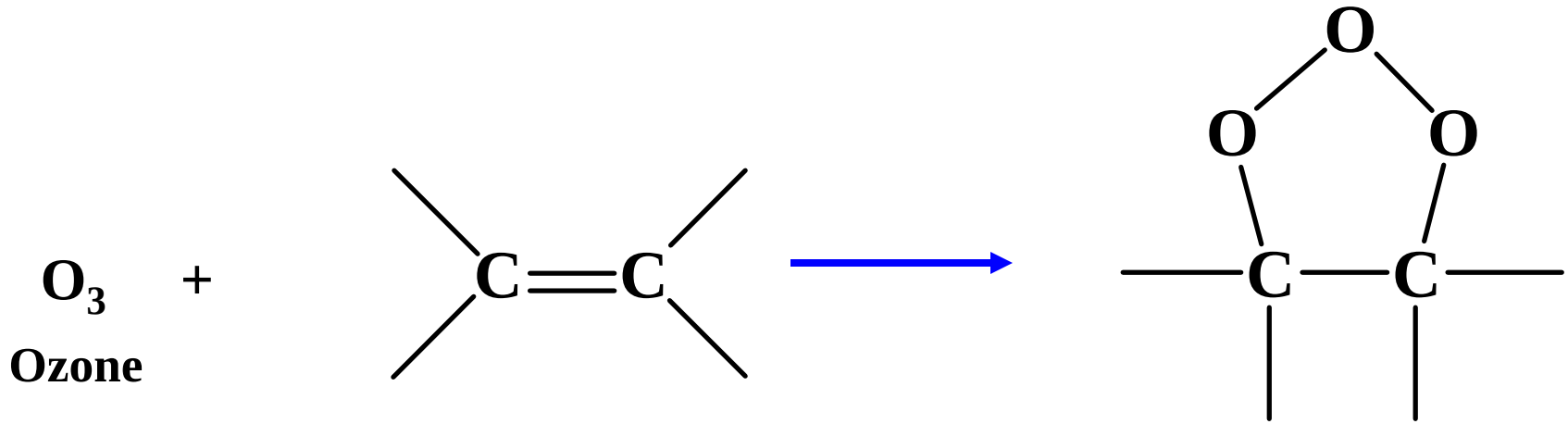
semi-quinone

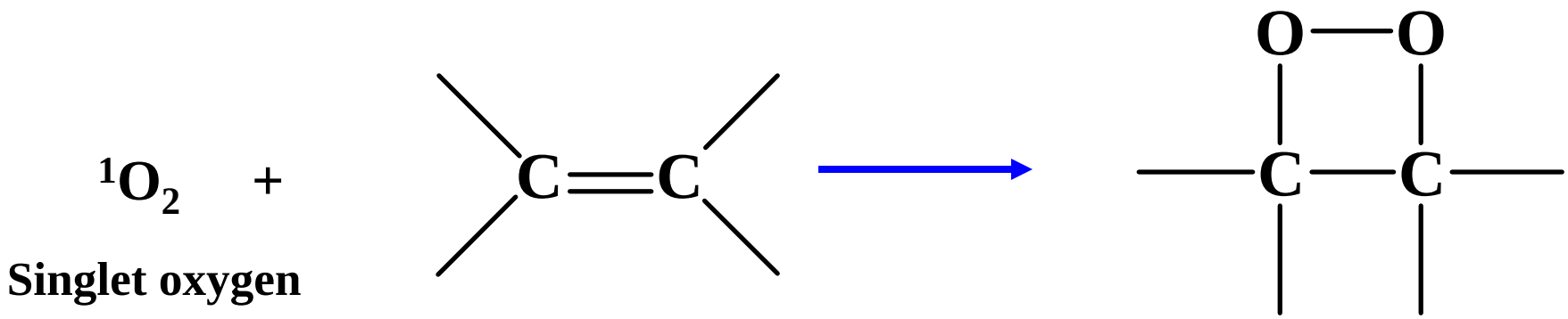
hydro-quinone

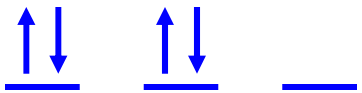
Quinones can be reduced by a flavin-containing enzyme (F) that obtains electrons from NADPH. The one-electron reduction product, a semiquinone, can give up its electron to oxygen forming the superoxide anion, or can receive a second electron yielding a hydroquinone product. A single two-electron reduction step catalyzed by NADPH quinone oxidoreductase, without a semiquinone intermediate, can generate the hydroquinone, which is relatively stable.

From Kehrer JP (2000) Toxicology 149: 43-50

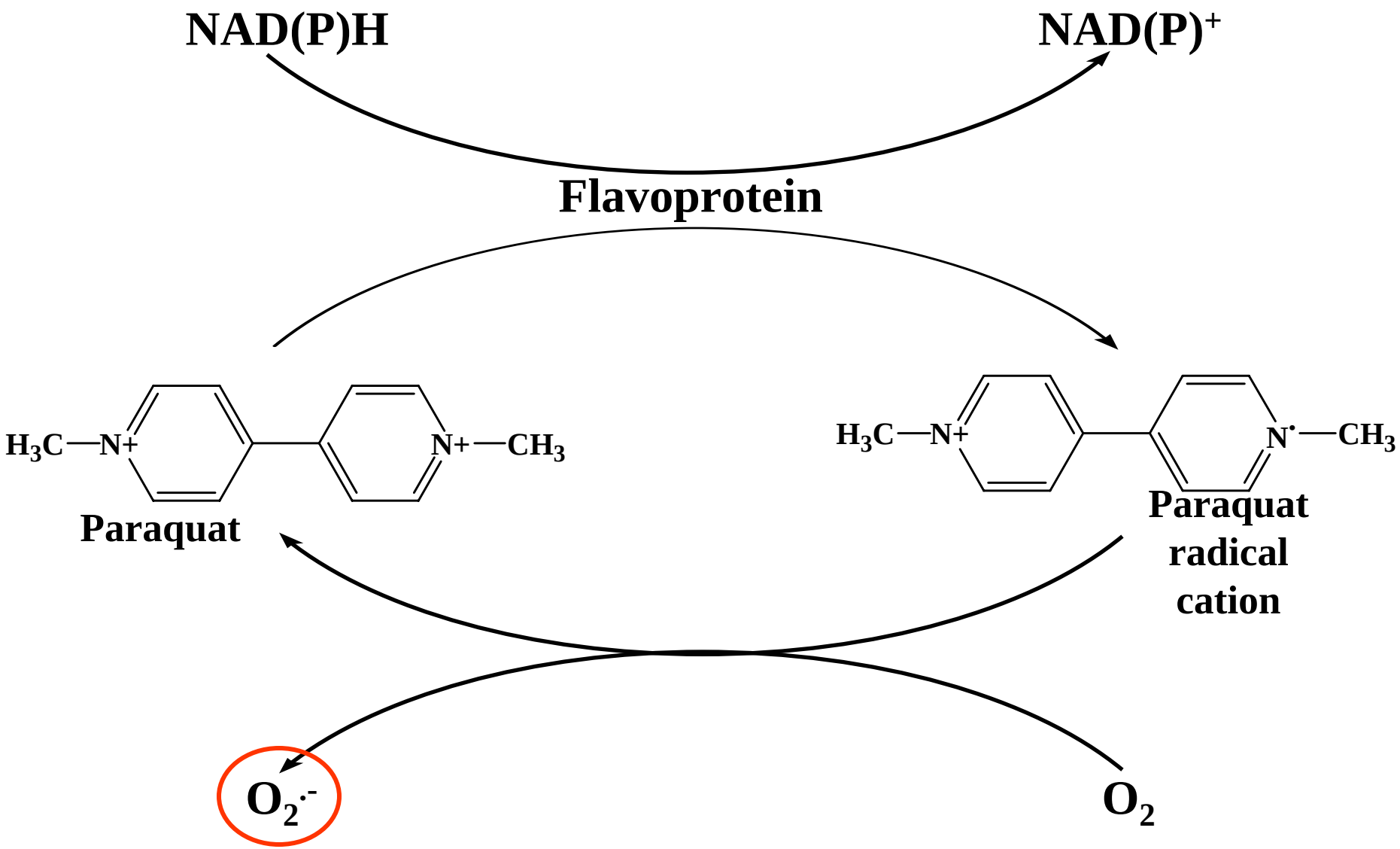
Chemicals that form peroxides







Chemicals that promote $O_2^{\cdot -}$ formation



Chemicals that are metabolized to radicals

Polyhalogenated alkanes

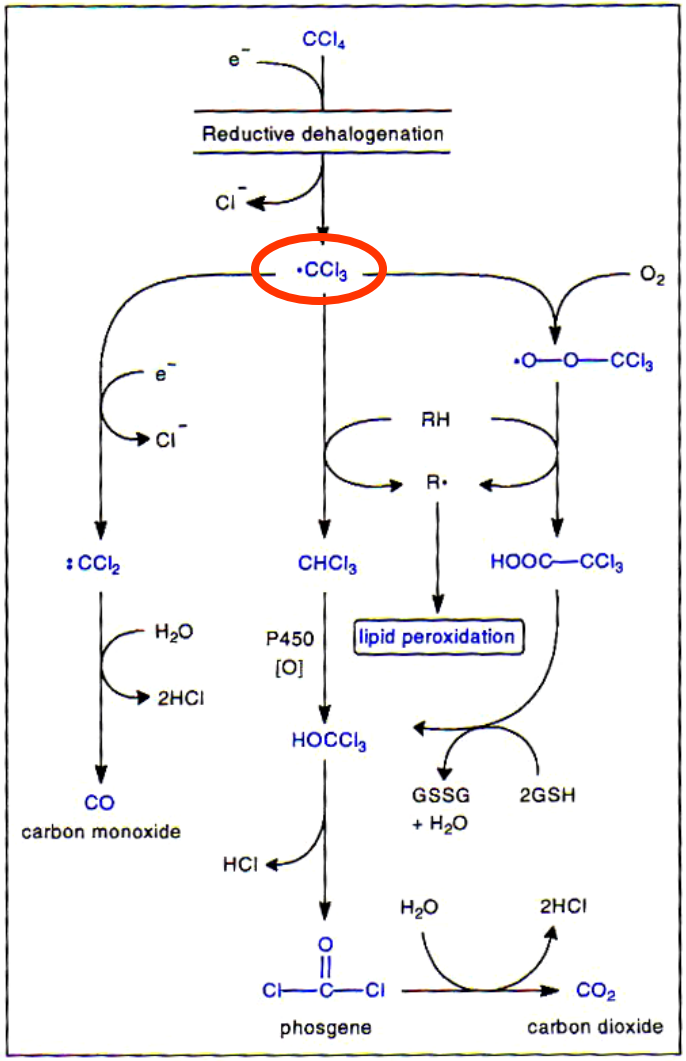


Figure 6-15. Reductive dehalogenation of carbon tetrachloride to a trichloromethyl free radical that initiates lipid peroxidation.

ABBREVIATIONS: RH, unsaturated lipid; R•, lipid dienyl radical; GSH, reduced glutathione; GSSG, oxidized glutathione.

Phenols, aminophenols

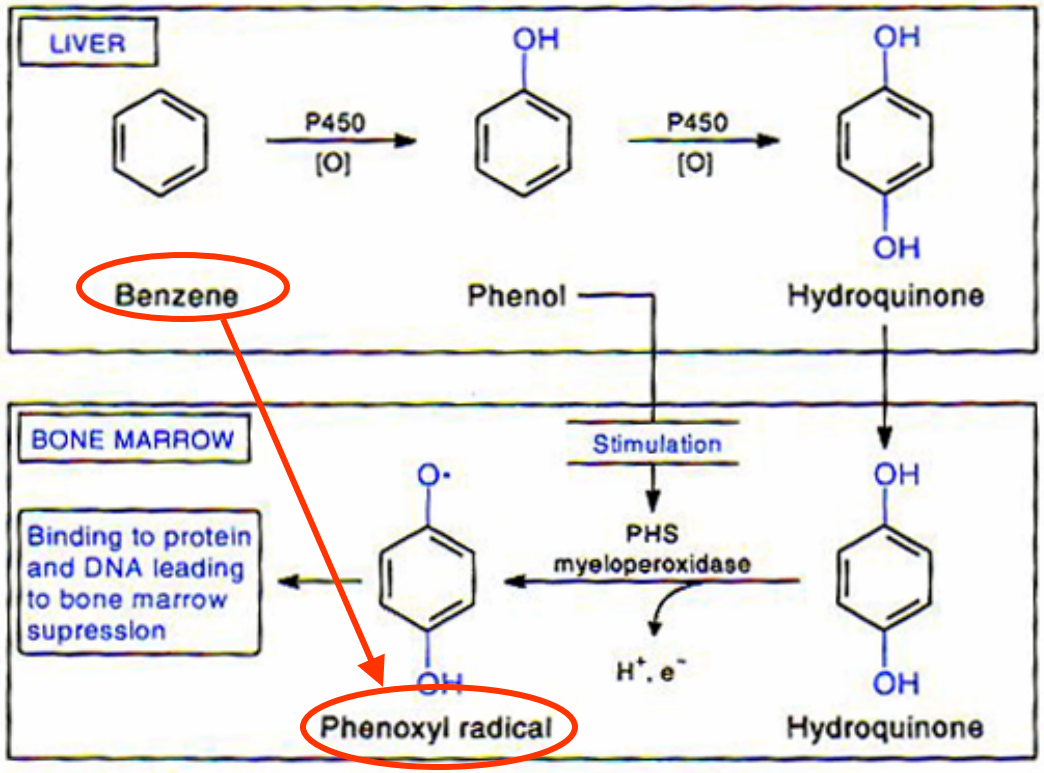


Figure 6-29. Role of cytochrome P450 and peroxidases in the activation of benzene to myelotoxic metabolites.

ABBREVIATION: PHS, prostaglandin H synthase.

Chemicals that are metabolized to radicals

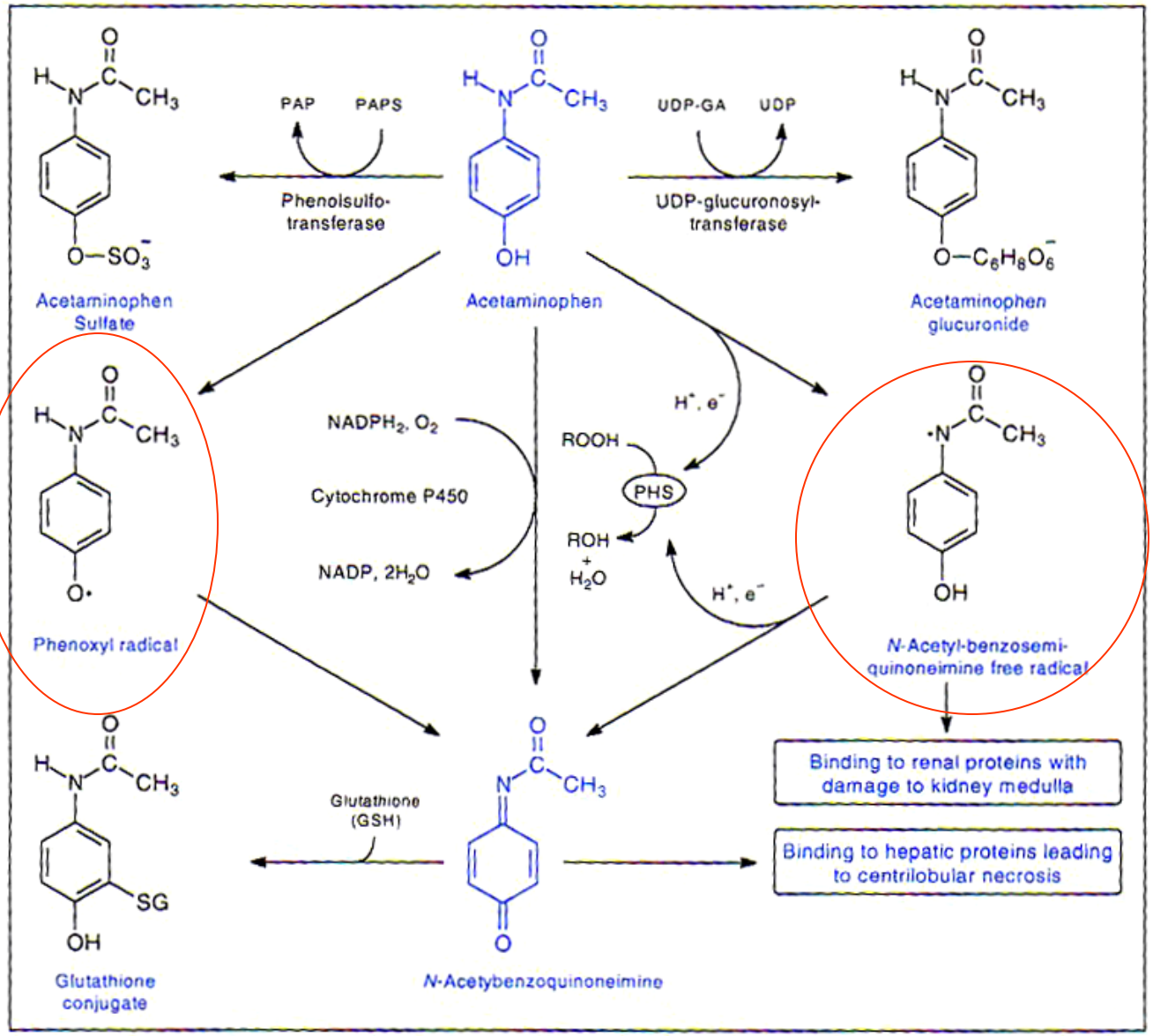
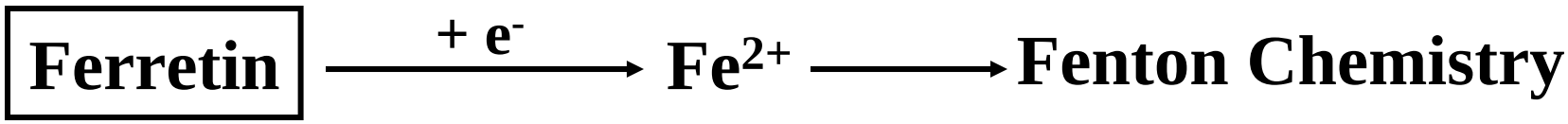


Figure 6-28. Activation of acetaminophen by cytochrome P450, leading to hepatotoxicity, and by prostaglandin H synthase (PHS), leading to nephrotoxicity.

Chemicals that release iron



- **Requires reductant**
- **Promotes ·OH formation**
- **Promotes lipid peroxidation *in vitro***

Oxidative stress and cell damage

- **High doses:**

  directly damage/kill cells

- **Low doses/chronic overproduction of oxidants:**

  activation of cellular pathways

  stimulation of cell proliferation

  damage to cellular proteins, DNA and lipids

Classic lipid peroxidation

1. **Initiation**

   $LH + X\bullet \longrightarrow L\bullet + XH$

2. **Propagation**

   $L\bullet + O_2 \longrightarrow LOO\bullet$

   $LOO\bullet + LH \longrightarrow L\bullet + LOOH$

3. **Termination**

   $2\ LOO\bullet \longrightarrow$ non-radical products

   $L\bullet + LOO\bullet \longrightarrow$ non-radical products

   $L\bullet + L\bullet \longrightarrow$ non-radical products

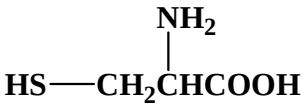
Catalyzed by metals

$$LOOH + Fe^{2+} \longrightarrow OH^{-} + LO^{\cdot} + Fe^{3+}$$

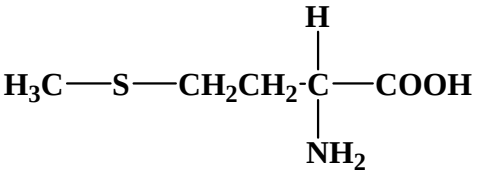
Consequences of lipid peroxidation

- **Structural changes in membranes**
  - alter fluidity and channels
  - alter membrane-bound signaling proteins
  - increases ion permeability
- **Lipid peroxidation products form adducts/crosslinks with non lipids**
  - e.g., proteins and DNA
- **Cause direct toxicity of lipid peroxidation products**
  - e.g., 4-hydroxynonenal toxicity
- **Disruptions in membrane-dependent signaling**
- **DNA damage and mutagenesis**

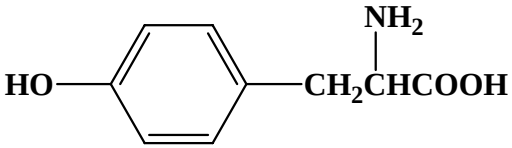
Protein targets for ROS



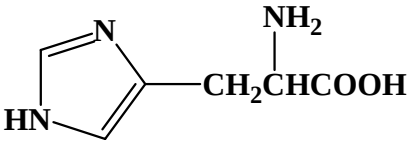
Cysteine



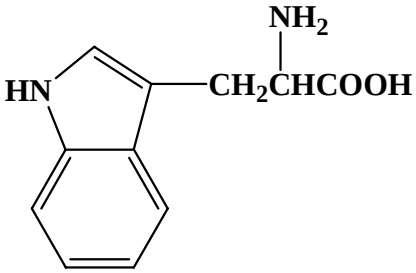
Methionine



Tyrosine



Histidine



Tryptophan

Oxidized proteins and amino acids found in biological systems

2-Oxohistidine
3-Chlorotyrosine
3-Nitrotyrosine
5-Hydroxy-2-aminovaleric acid
Aminomalonic acid
Dimers of hydroxylated aromatic amino acids
Dopa
Hydro(pero)xyleucine
Hydro(pero)xyvalines
N-Formylkynurenine; kynurenine
o- and *m*-tyrosine
p-Hydroxyphenylacetaldehyde
Protein carbonyls

Consequences of protein thiol oxidation

Oxidation of catalytic sites on proteins

loss of function/abnormal function

BUT(!): sometimes it is gain in function!

Formation of mixed sulfide bonds

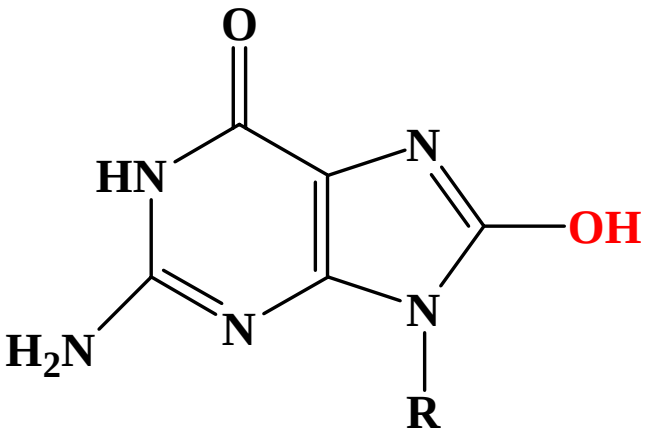
Protein-protein linkages (RS-SR)

Protein-GSH linkages (RS-SG)

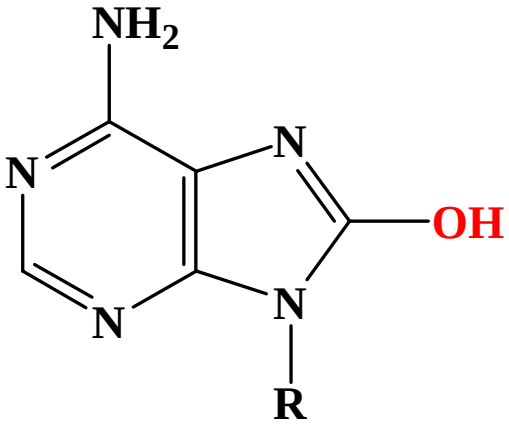
Alteration in 2^o and 3^o structure

Increased susceptibility to proteolysis

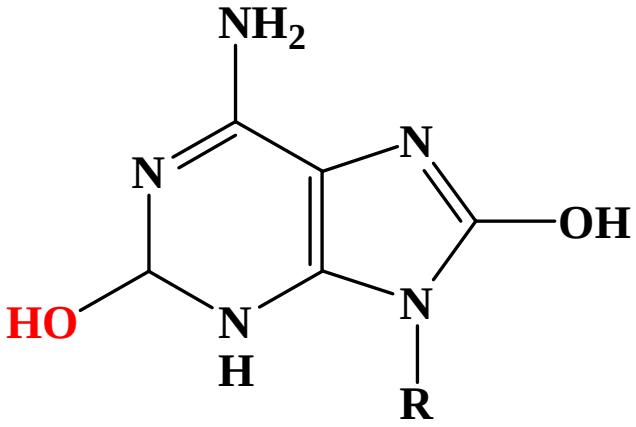
DNA oxidation products



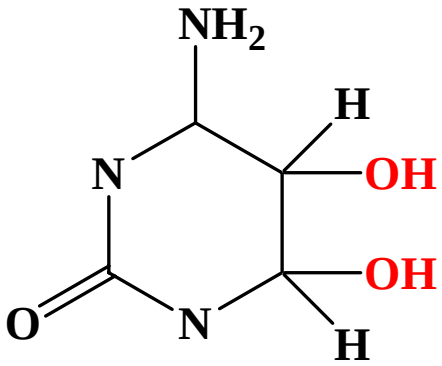
8-hydroxyguanine



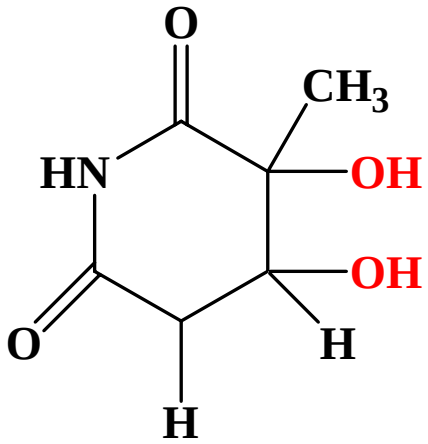
8-hydroxyadenine



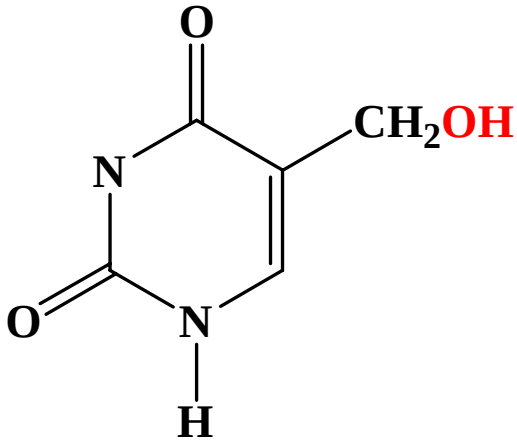
2-hydroxyadenine



5,8-dihydroxycytosine

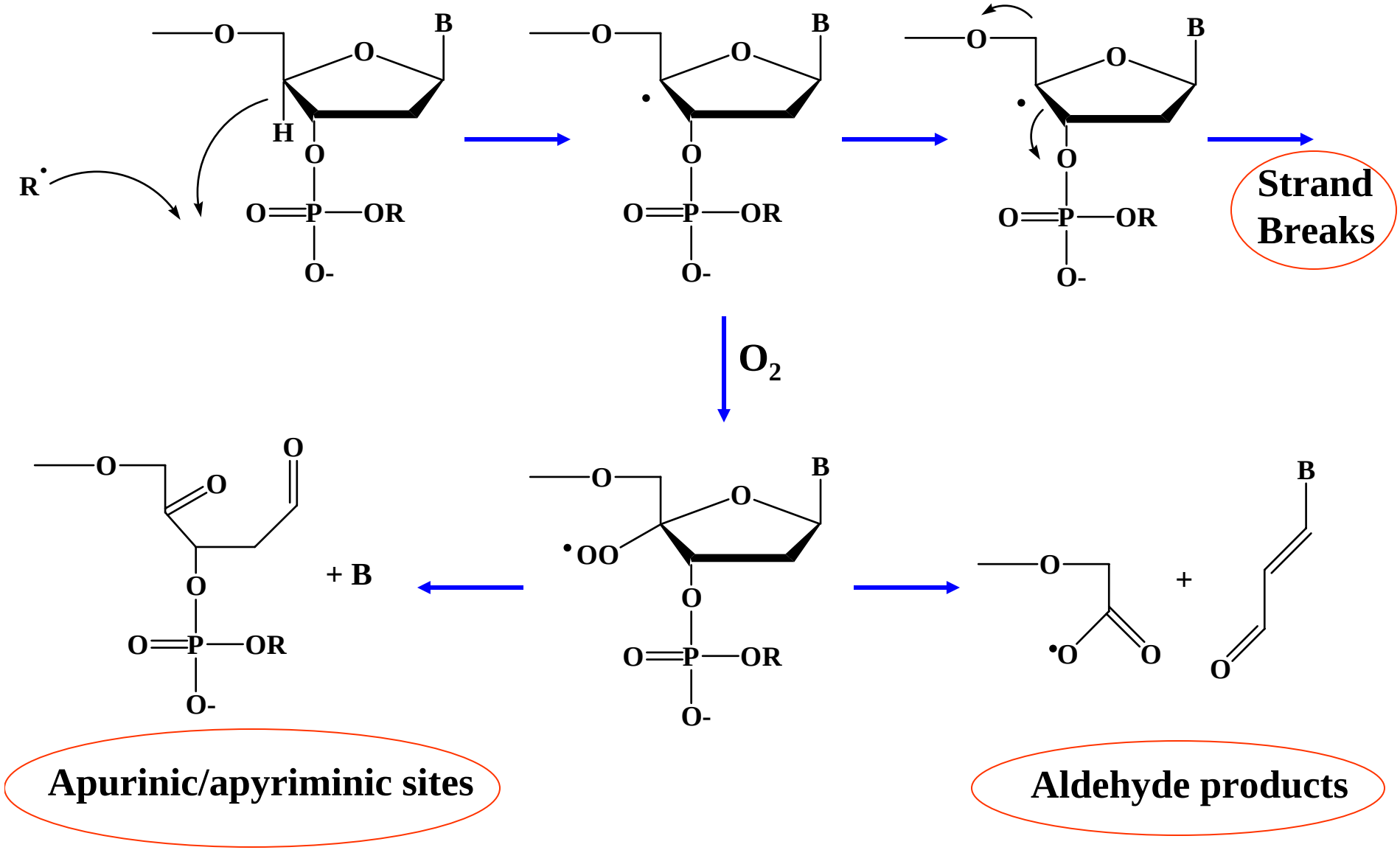


thymidine glycol



5-hydroxymethyluracil

Oxidation of deoxyribose (DNA backbone)



Consequences of DNA oxidation

- **DNA adducts/AP sites/Strand breaks**

  mutations
  initiation of cancer

- **Stimulation of DNA repair**

  can deplete energy reserves (PARP)
  imbalanced induction of DNA repair enzymes
  induction of error prone polymerases
  activation of other signaling pathways

Defenses against Prooxidants

1. **Prevention of prooxidant formation**
2. **Interception of prooxidants**
3. **Breaking the chain of radical reactions**
4. **Repair of damage caused by prooxidants**

ANTIOXIDANT: a substance that is able, at relatively low concentrations, to compete with other oxidizable substrates and, thus, to significantly delay or inhibit the oxidation of other substrates

Prevention of prooxidant formation

Physical prevention:

Behavioral: - avoidance

Barriers: - organismal level

- organ level

- cellular level

Biochemical prevention:

Control of prooxidant molecules:

- transition metal chelators

- catalytic control of O_2 reduction

Control of prooxidant enzymes:

- blockade of stimuli

- inhibition of enzymes

Examples of preventative 'antioxidants'

Anti-inflammatory agents

Nitric oxide synthase inhibitors

Metal chelators:

- Metallothionein
- Transferrin
- Lactoferrin

NADPH oxidase inhibitors

Xanthine oxidase inhibitors

Interception of prooxidants

‘Classical’ antioxidant:

Intercepts species, once formed

Excludes from further damaging activity

Transfers species from critical parts of cell

Important considerations for interception reactions:

Speed of reaction (rate constant)

Concentration of intercepting species *in vivo*

Is reaction truly a detoxication pathway?

Is reaction catalytically recyclable?

Chain breaking antioxidants

Example of radical chain-reaction: lipid peroxidation

ROO• (peroxyl radicals) are often the chain-carrying radicals

Chain-breaking oxidants act by reacting with peroxyl radicals:

"Donor" antioxidants (tocopherol, ascorbate, uric acid,…)

$$LOO\bullet + TOH \longrightarrow LOOH + TO\bullet$$

"Sacrificial" antioxidants (Nitric oxide):

$$LOO\bullet + NO\bullet \longrightarrow LOONO$$

Good chain-breaking antioxidant:

- both **ANT** and **ANT•** should be relatively UNreactive
- **ANT•** decays to harmless products
- does not add O_2 to make a new peroxyl radical
- is renewed (recycled)

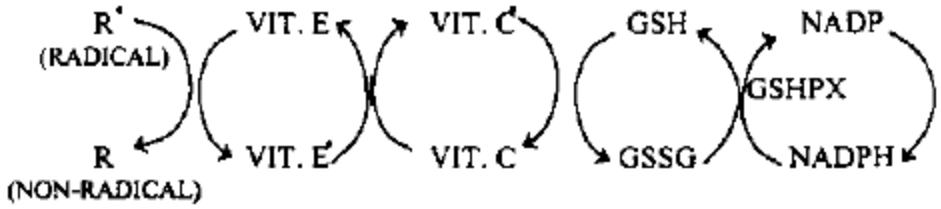
a) Initiation of the peroxidation by an oxidizing radical, X', by abstraction of a bis-allylic hydrogen, forming a pentadienyl radical.

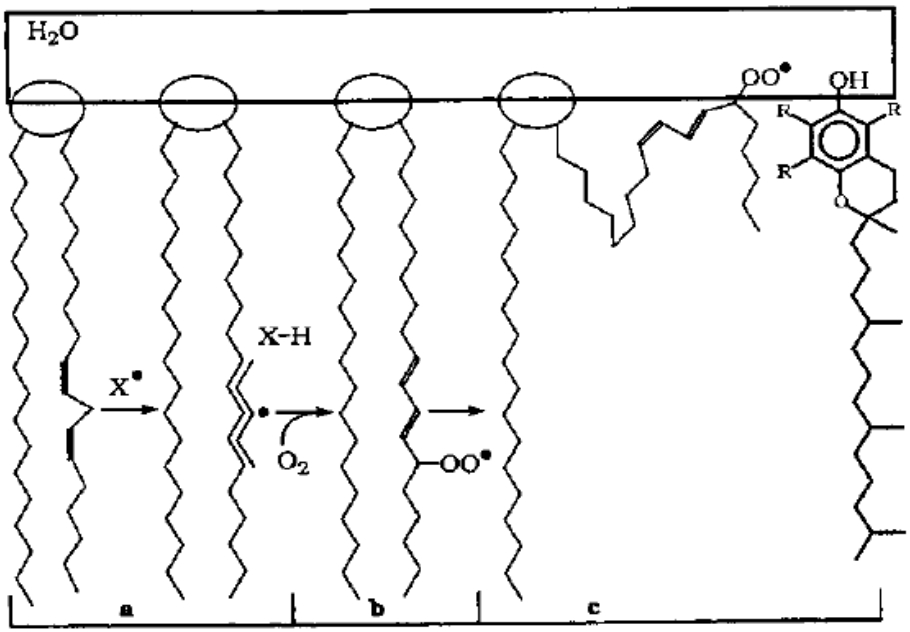
b) Oxygenation to form a peroxyl radical and a conjugated diene.

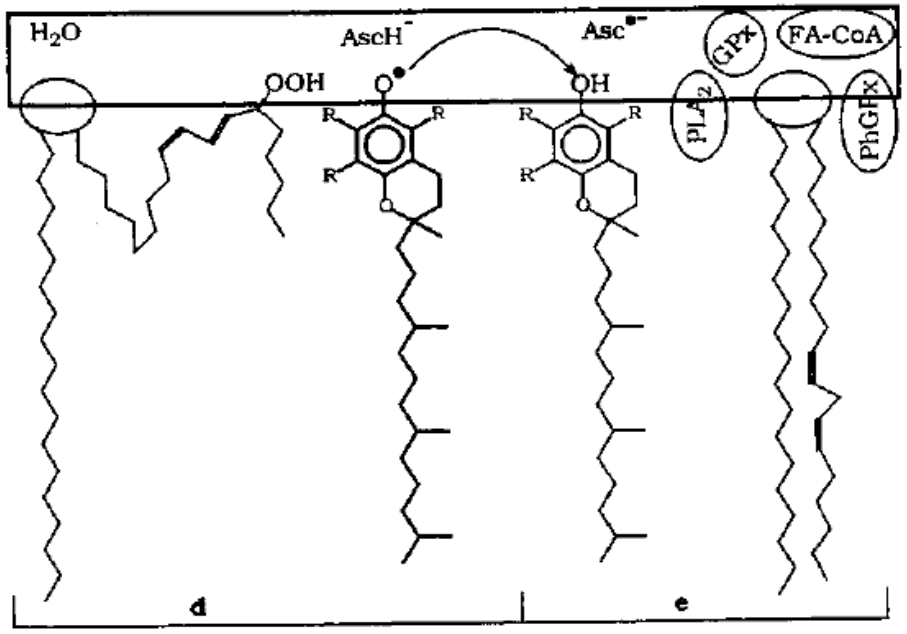
c) The perolcyl radical moiety partitions to the water-membrane interface where it is poised for repair by tocopherol.

d) The tocopheroxyl radical can be repaired by ascorbate.

e) Tocopherol has been recycled by ascorbate; the resulting ascorbate radical can be recycled by enzyme systems. The enzymes phospholipase AZ (PLAZ), phospholipid hydroperoxide glutathione peroxidase (PhGPx), glutathione peroxldase (GPx), and fatty acyl-coenzyme A (FA-CoA), cooperate to detoxify and repair the oxidized fatty acid chain of the phospholipid.







Cellular antioxidants

Small Molecules

-Water soluble: glutathione, uric acid, ascorbate (Vit. C)

-Lipid soluble: α-tocopherol (Vit. E), β-carotene, coenzyme Q

Proteins

-Intracellular: SOD (I and II), glutathione peroxidase, catalase

-Cell membrane: SOD (III), ecGPx, plasma proteins (e.g. albumin)

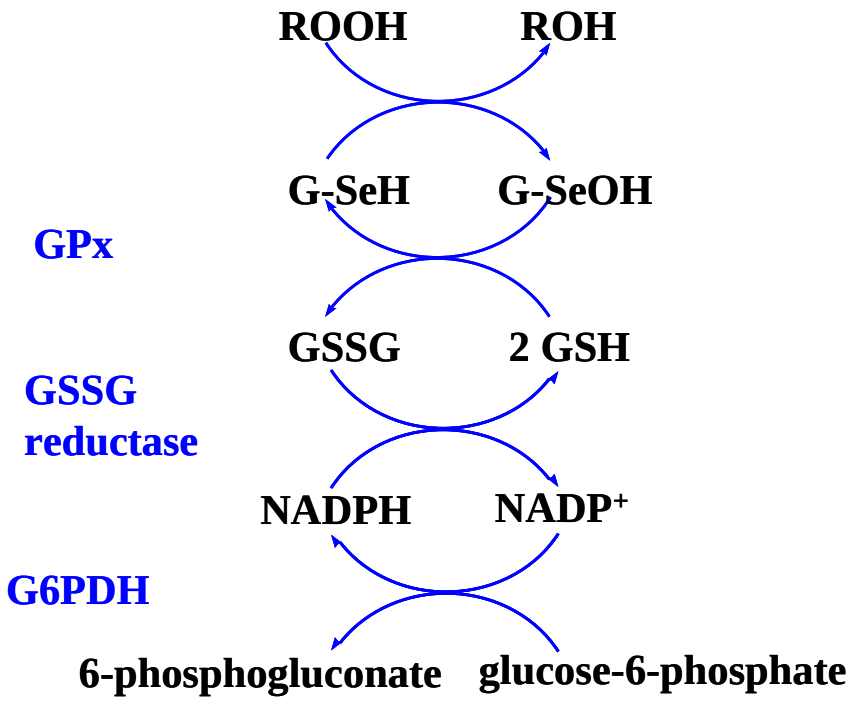
-Extracellular: phospholipid hydroperoxide GPx (PHGPx)

See additional information on antioxidant enzymes in handout material

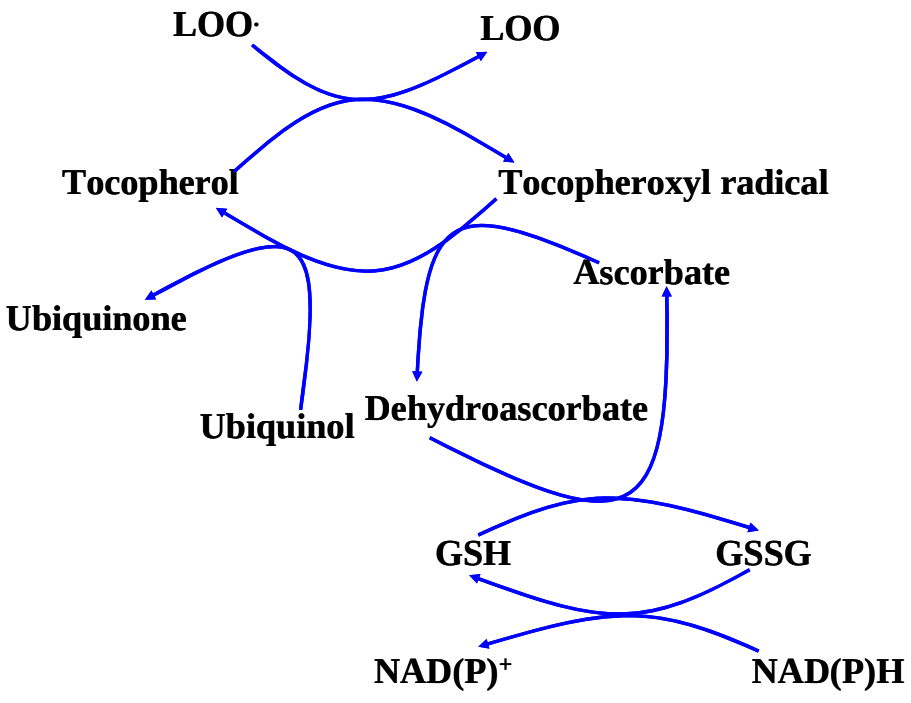
'Antioxidant Network'

Catalytic maintenance of antioxidant defense
Non-scavenging enzymes (re-reduce antioxidants)
Dependence on energy status of cell
Glucose most important 'antioxidant'

Catalytic reduction of peroxides



Catalytic reduction of lipid radicals



Repair of damage caused by prooxidants

Protection not perfect

Repair of damaged products

proteins and lipids

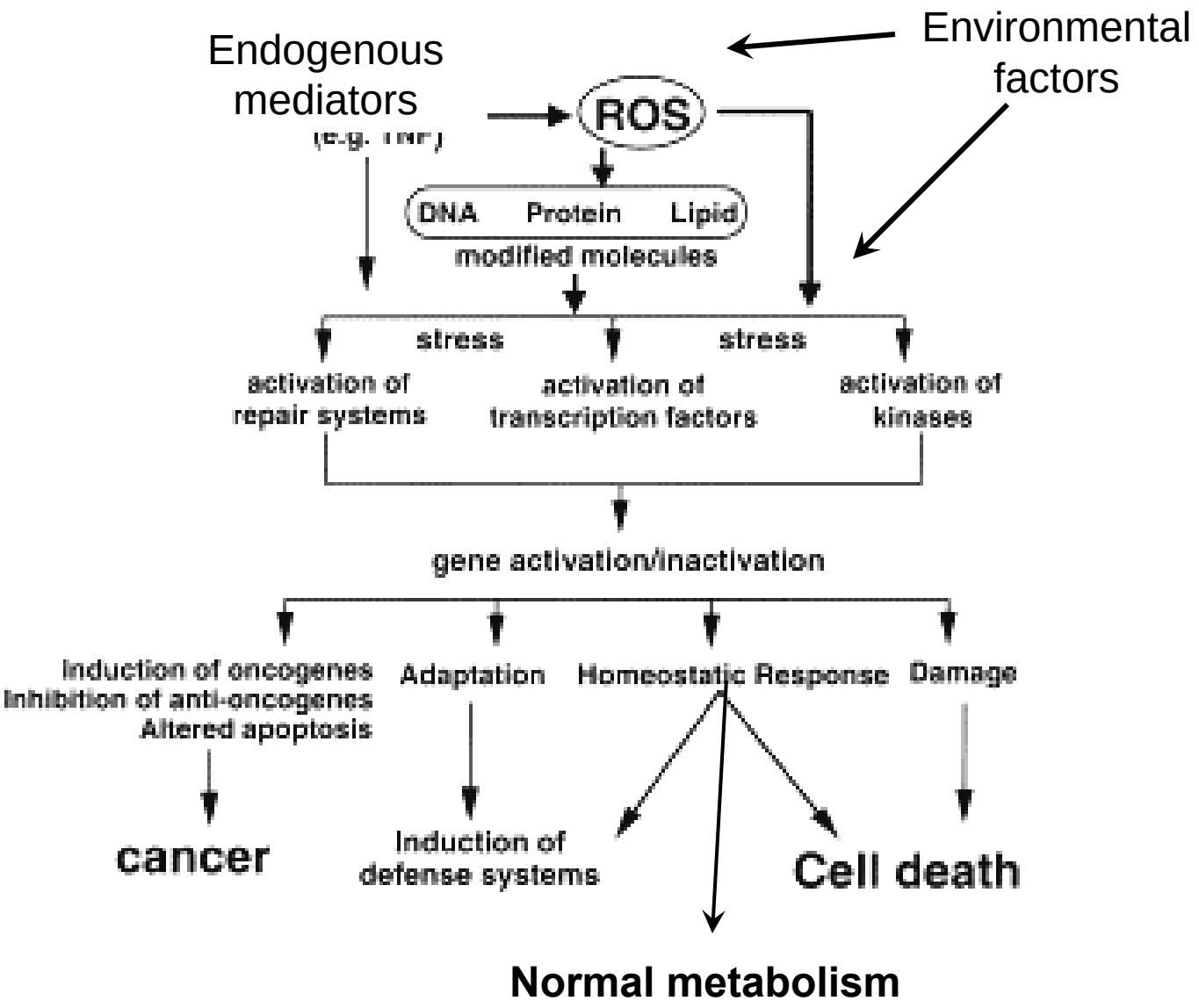
-rereduction and degradation

DNA

-repair enzymes

Cell death (apoptosis/necrosis)

Free Radicals and cell function



Signal transduction:

Regulated sequence of biochemical steps through which a stimulant conveys a message, resulting in a physiologic response

Agonist

↓

Receptor

↓

Primary effectors (enzymes or channels)

↓

Second messenger(s)

↓

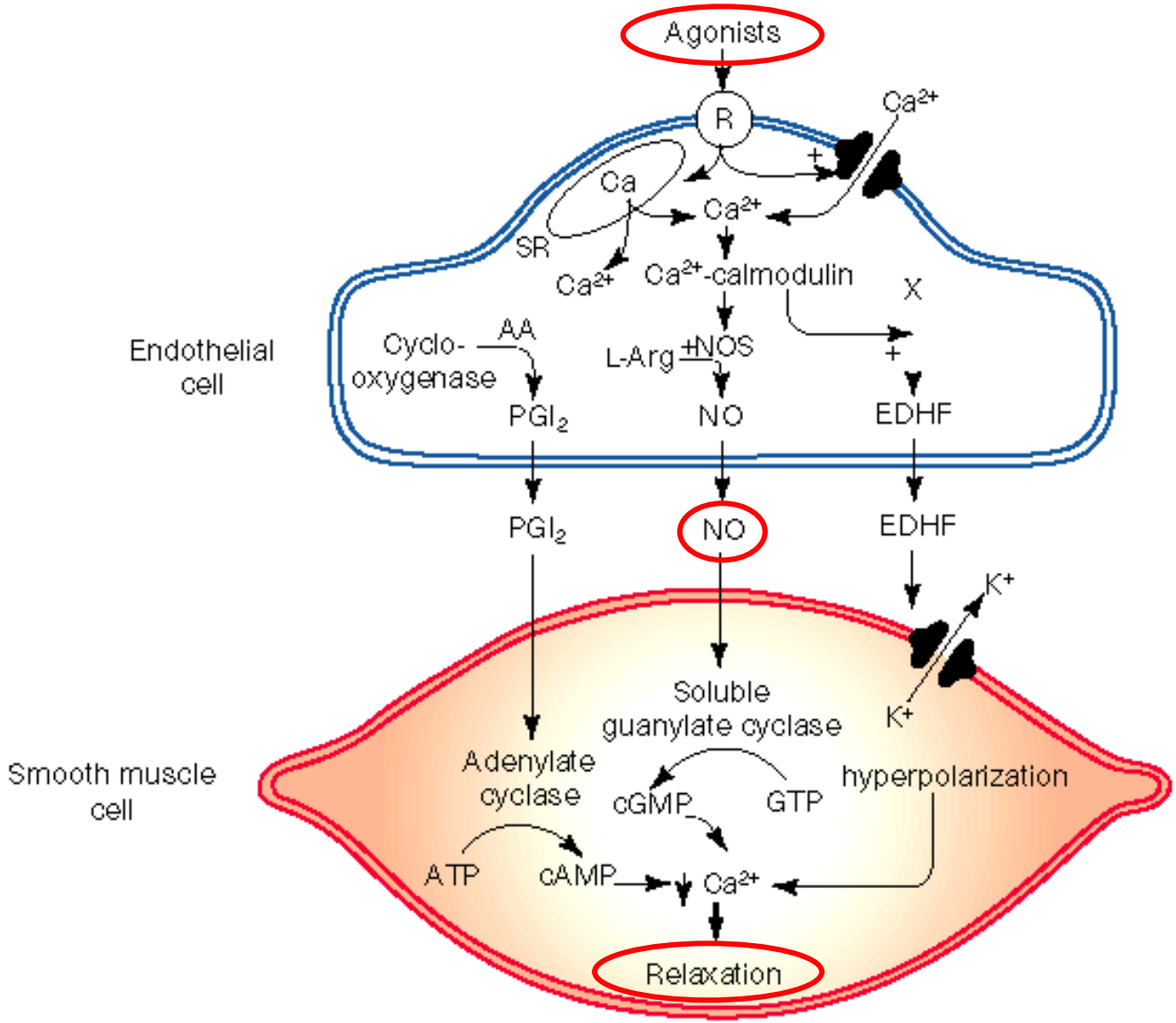
Secondary effectors (enzymes, other molecules)

Oxidants can act to modify signal transduction

How free radicals can be involved in signaling?

- Heme oxidation
- Oxidation of iron-sulfur centers in proteins
- Changes in thiol/disulfide redox state of the cell
- Change in conformation → change in activity
- Oxidative modification of proteins: degradation, loss of function, or gain of function
- Oxidative modification of DNA: activation of repair, and/or apoptosis
- Oxidative modification of lipids: disruption of membrane-associated signaling, DNA damage, and formation of protein adducts

NO· signaling in physiology



Nitric **O**xide **S**ynthase

$O_2^{-\bullet}$ → NO• → ONOO-

Binds to heme moiety of guanylate cyclase

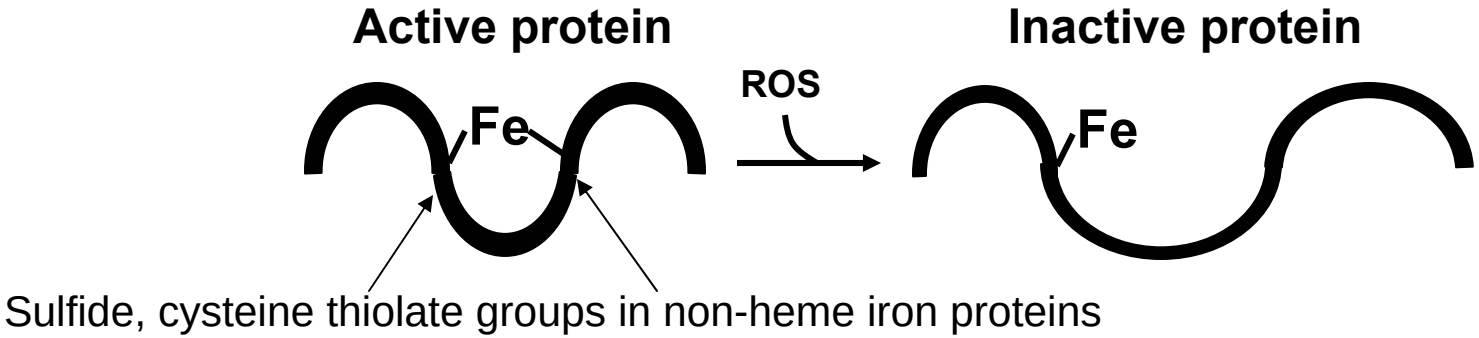
Conformational change of the enzyme

Increased activity (production of cGMP)

Modulation of activity of other proteins (protein kinases, phospho-diesterases, ion channels)

Physiological response (relaxation of smooth muscles, inhibition of platelet aggregation, etc.)

Iron-sulfur proteins and free radical signaling



Mammalian (4Fe-4S) aconitase (citric acid cycle, citrate →isocitrate):

- Contains non-heme iron complex Fe-S-Cys
- ROS and RNS disrupt Fe-S clusters and inhibit aconitase activity
- Binding site is exposed: binding to ferritin mRNA and transferrin receptor
- Participates in regulation of iron homeostasis (iron-regulatory protein-1)

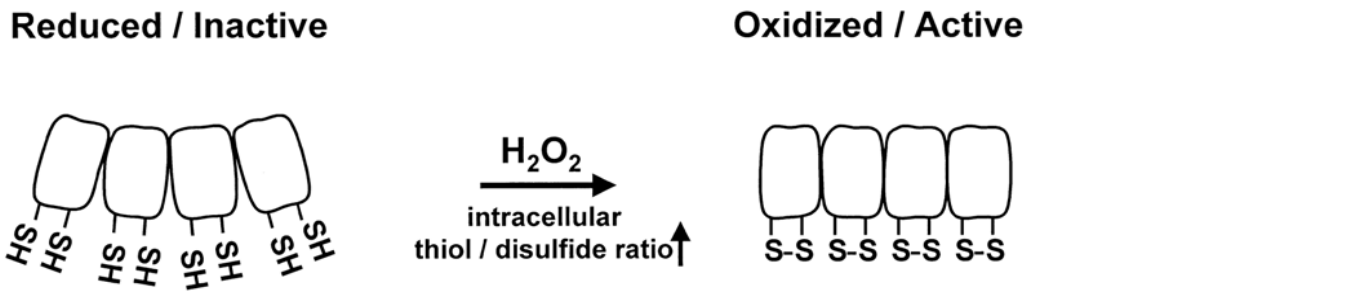
Bacterial SoxR protein:

- Contains two stable (2Fe-2S) centers that are anchored to four cysteine residues near carboxy-terminus of the protein
- Under normal conditions these iron-sulfur centers are reduced
- When oxidized, SoxR changes configuration and is able to activate transcription of oxidative stress-related proteins (MnSOD, G6PDH, etc.)

Thiol/disulfide redox state and signaling by ROS

Bacterial OxyR is a model case of a redox-sensitive signaling protein that may be activated by either H_2O_2 or changes in intracellular GSH content

Schematic Model of OxyR activation

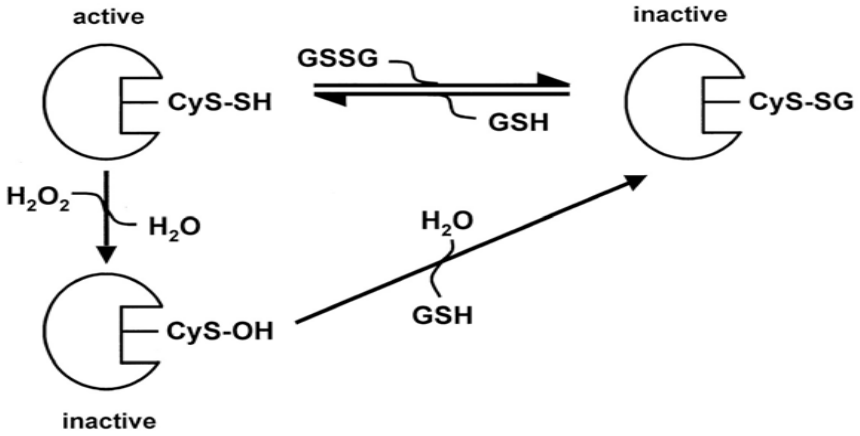


From Dröge W. (2002) *Physiol Rev* 82:47-95

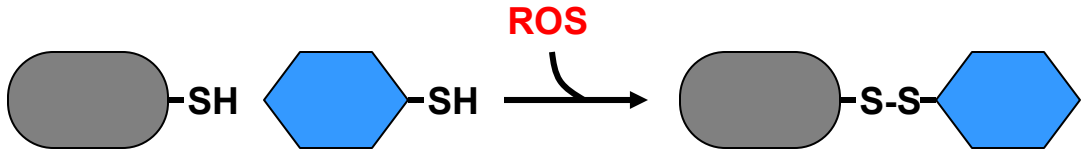
- No new protein synthesis occurs to activate OxyR, but it acts by ↑transcription (MnSOD, catalase, GSH reductase, glutaredoxin 1, thioredoxin, etc.)
- Both reduced and oxidized OxyR can bind promoter regions of these genes but they exhibit different binding characteristics: oxidized much more active
- OxyR response is autoregulated since glutaredoxin and thioredoxin can reverse formation of disulfide bonds thus inactivating OxyR

Thiol/disulfide redox state and signaling by ROS

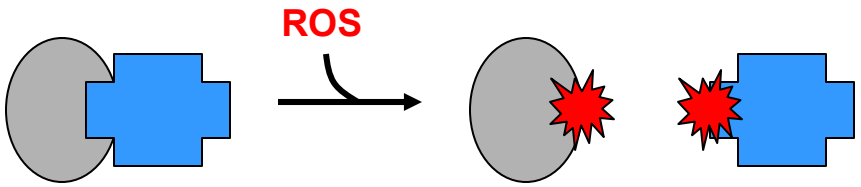
Mixed disulfide formation



Disulfide formation

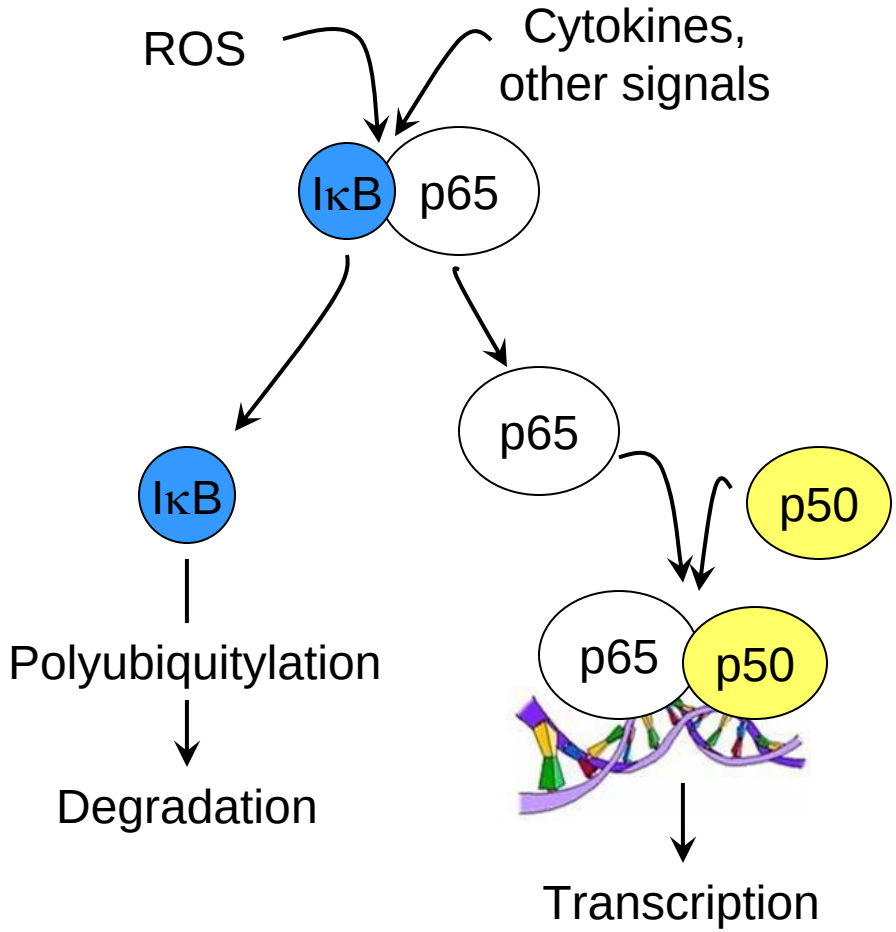


Alteration of protein-protein interactions

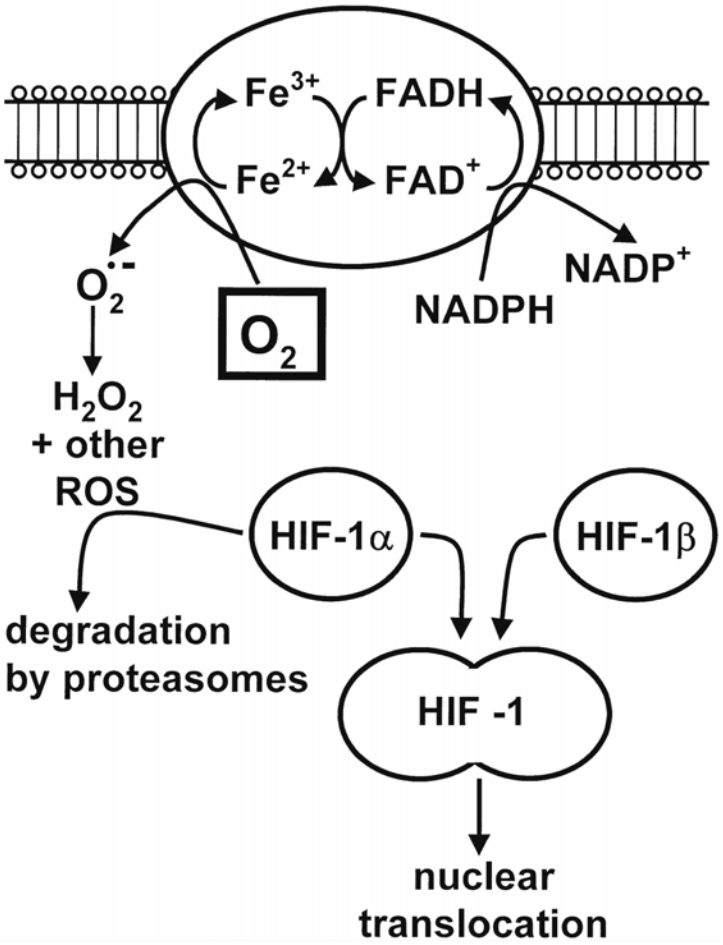


Redox signaling by protein degradation

Nuclear Factor κB

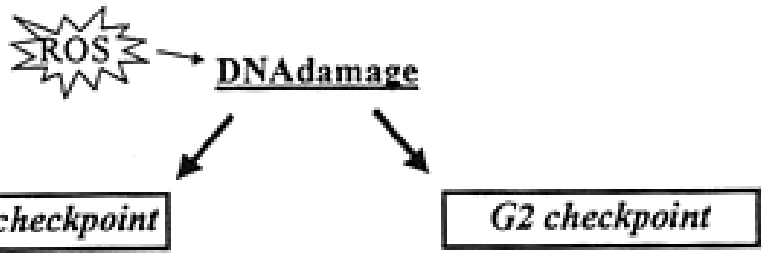


Hypoxia-Inducible Factor 1 (HIF-1)



From Dröge W. (2002) *Physiol Rev* 82:47-95

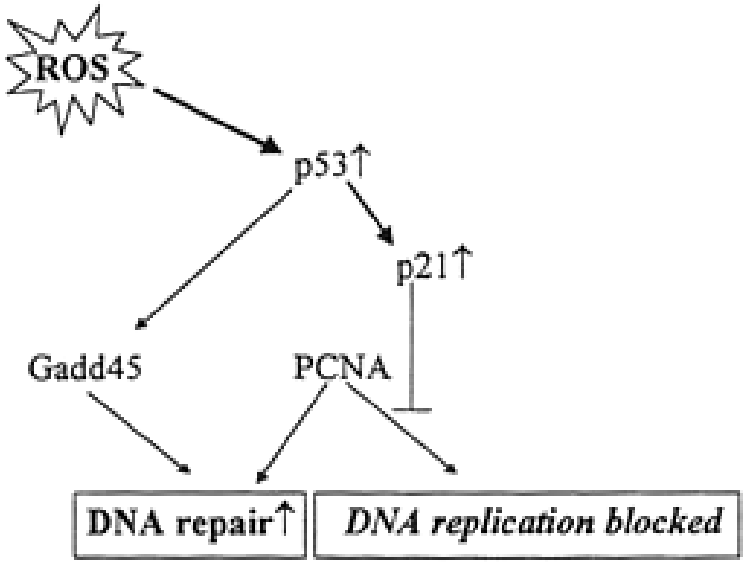
Redox signaling by DNA damage



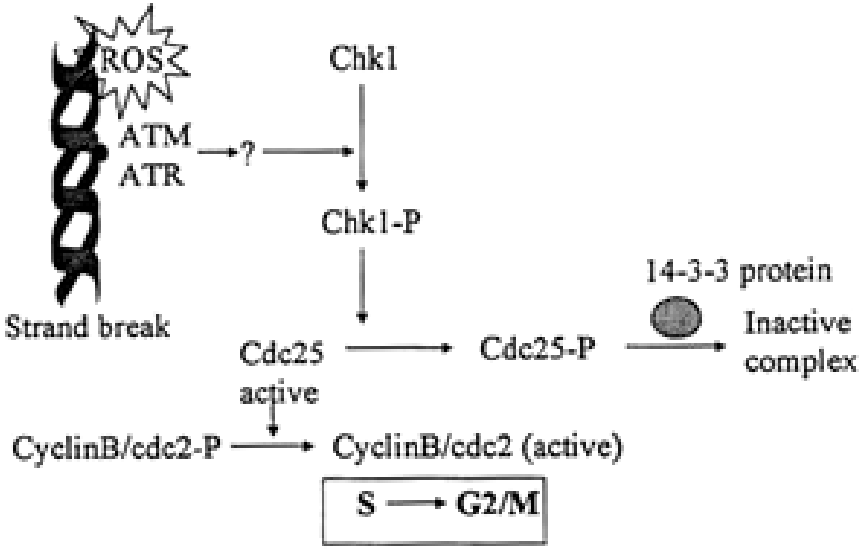
Arrests DNA replication.
Allows *repair* before replication.
Protects against replication errors: mutations

Prevents segregation of unreplicated/damaged chromosomes.
Protects against chromosomal aberrations.

DNA Replication arrest

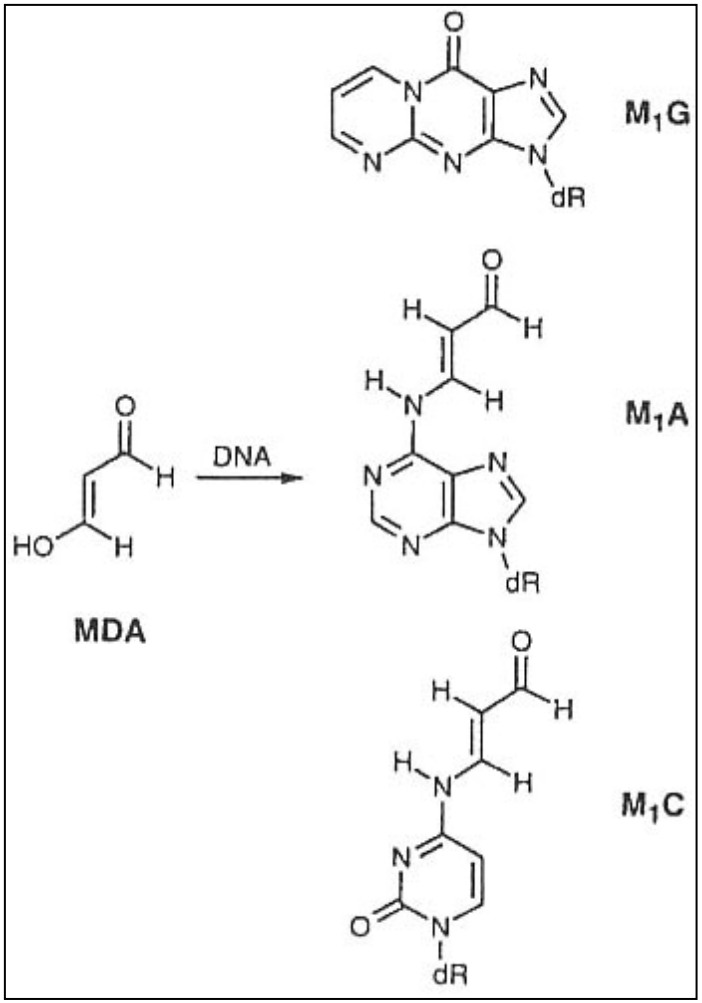


G2 arrest

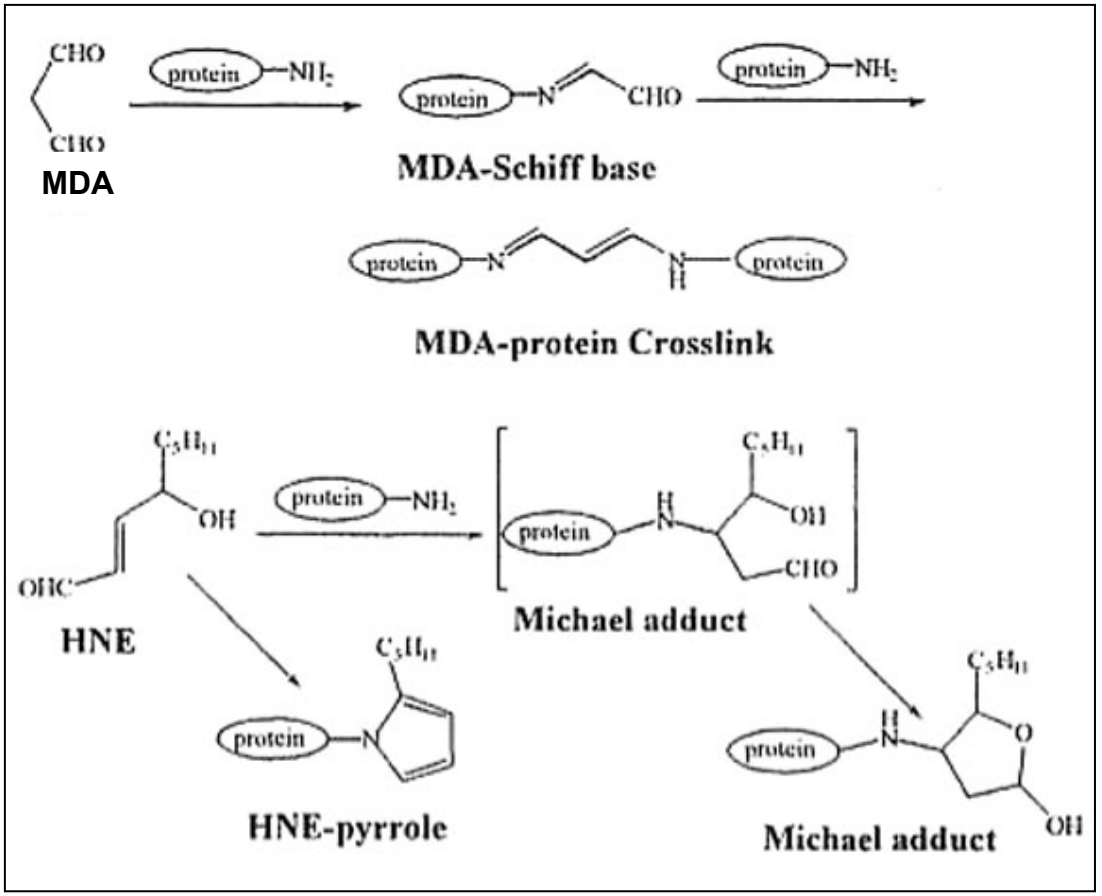


Redox signaling by lipid peroxidation

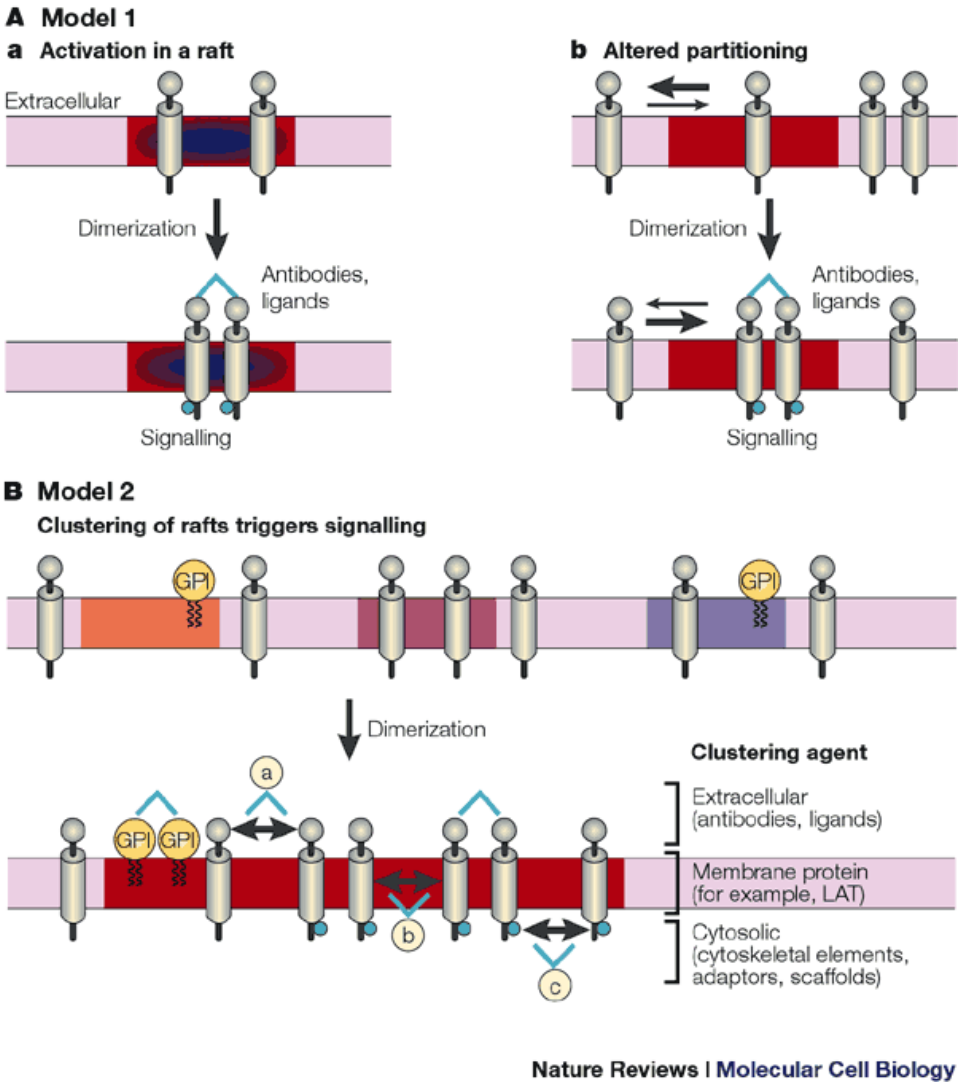
DNA adducts



Protein adducts



Membrane lipids and their role in signaling



Nature Reviews Molecular Cell Biology **1**; 31-39 (2000)

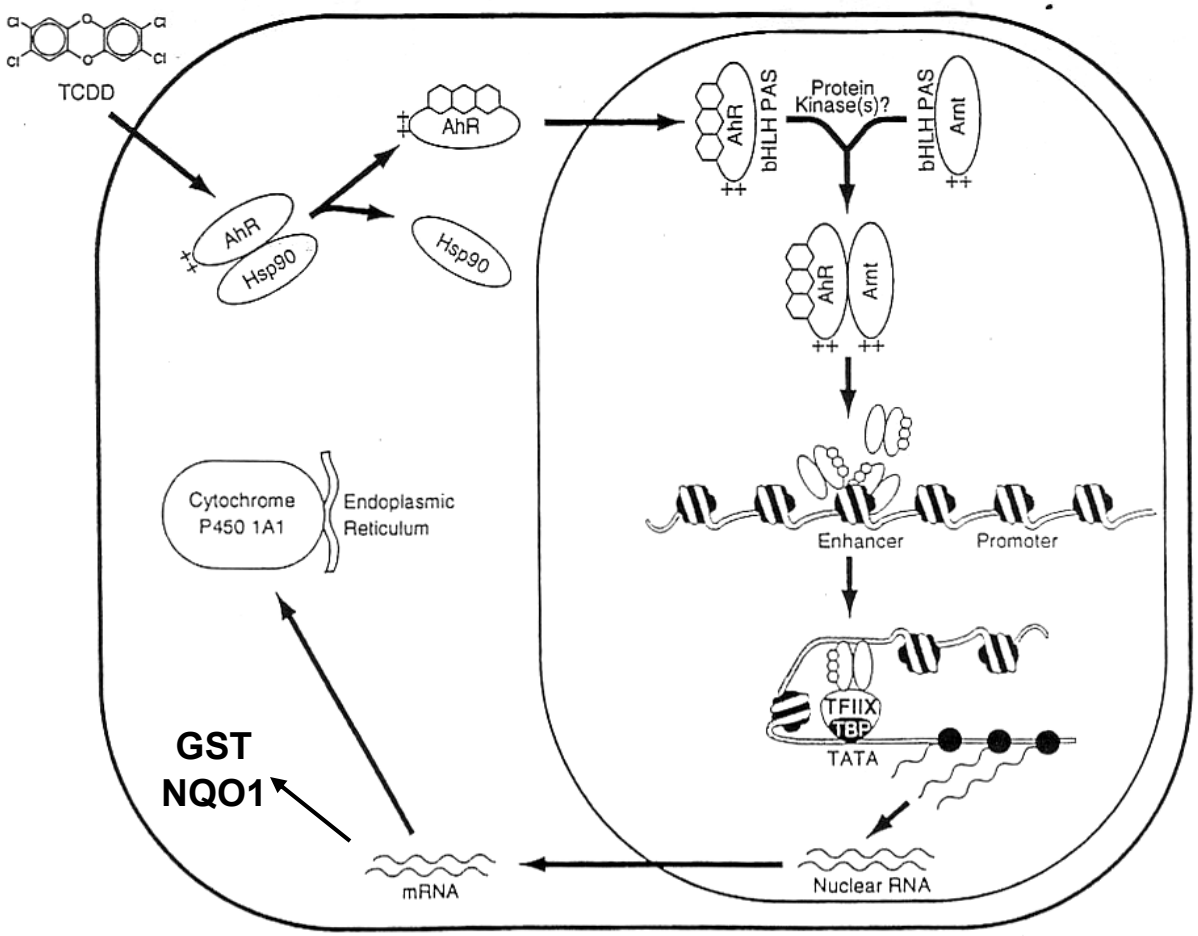
Models of how signalling could be initiated through raft(s). **A** | In these models, signalling occurs in either single rafts (Model 1) or clustered rafts (Model 2). Following dimerization the protein becomes phosphorylated (blue circle) in rafts. In single rafts this can occur by activation **a** | within the raft, or **b** | by altering the partitioning dynamics of the protein. **B** | In the second model we assume that there are several rafts in the membrane, which differ in protein composition (shown in orange, purple or blue). Clustering would coalesce rafts (red), so that they would now contain a new mixture of molecules, such as crosslinkers and enzymes. Clustering could occur either extracellularly, within the membrane, or in the cytosol (a–c, respectively). Raft clustering could also occur through GPI-anchored proteins (yellow), either as a primary or co-stimulatory response. Notably, models 1 and 2 are not mutually exclusive. For instance, extracellular signals could increase a protein's raft affinity (for example, similar to the effect of single versus dual acylation) therefore drawing more of the protein into the raft where it can be activated and recruit other proteins, such as LAT, which would crosslink several rafts.

Lipid peroxidation products and receptor signaling

There are dramatic differences in the mechanism of action of the different bioactive lipids. There are differences in the receptors to which each lipid binds.

Lipid	Receptor
22-OH Chol	LXR
12 HETE	PPAR
IsoPGE2	EP-1 receptor
Iso PGF2alpha	TP receptor
POVPC	unknown G protein coupled receptor
POVPC	CD36
LG/apoB	Scavenger Receptor
MDA ApoB	SRA

Bi-functional and mono-functional inducers



Modified from Whitlock et al. (1996) FASEB J. 10:809-818

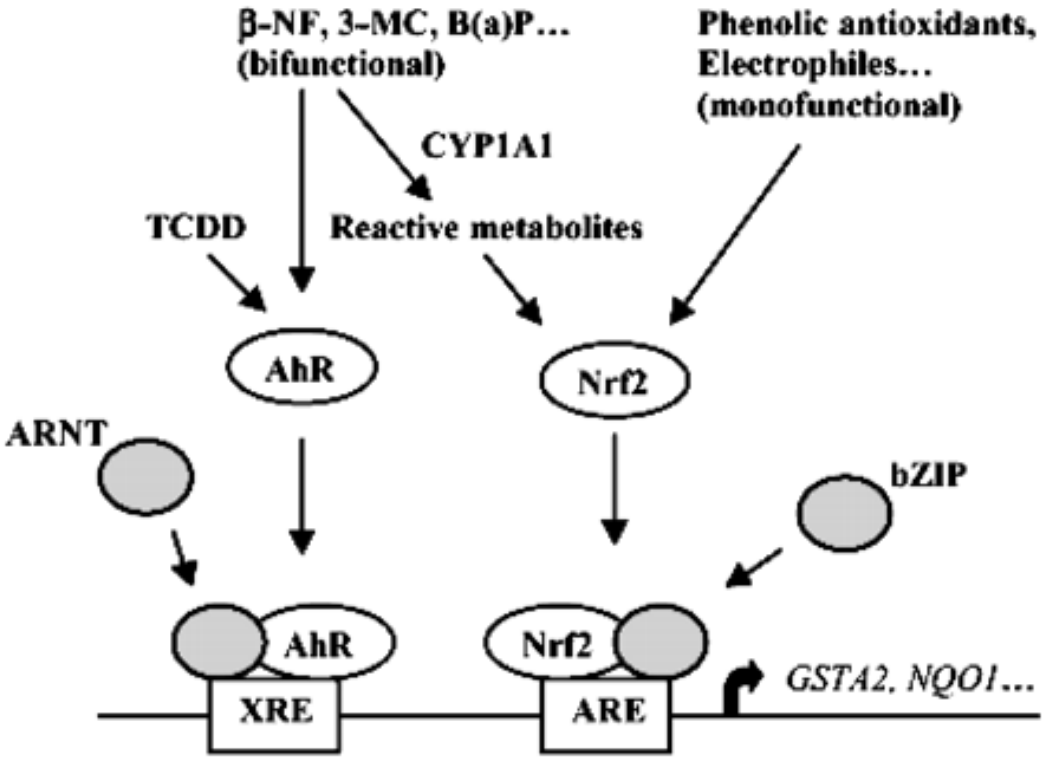
Bi-functional inducers:

Compounds that can induce both Phase I (mono-oxygenases) and Phase II (GST, NQO1) enzymes

Mono-functional inducers:

Compounds that can only regulate Phase II enzymes

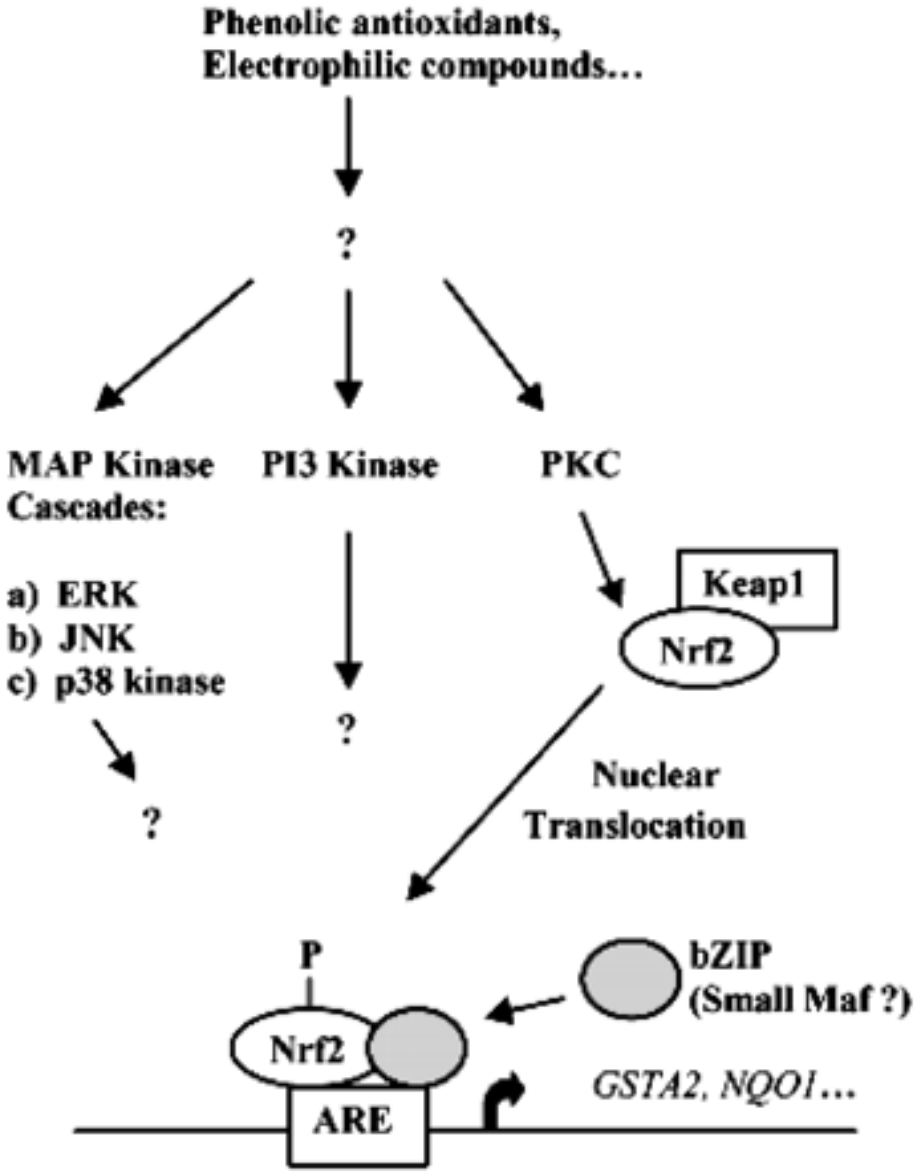
Antioxidant Response Element (ARE)



Transcriptional regulation of the rat *GSTA2* and *NQO1* genes by bifunctional and monofunctional inducers. The bifunctional inducers and the dioxin TCDD bind to and activate the AhR, which then translocates into the nucleus and associates with ARNT to activate transcription through the XRE. The bifunctional inducers can also activate transcription through the ARE via a separate pathway following their biotransformation into reactive metabolites that have characteristics of the monofunctional inducers. The monofunctional inducers can only act through the ARE-mediated pathway. 3-MC, 3-methylcholanthrene; B(a)P, benzo(a)pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

From Nguyen et al. (2003) Annu Rev Pharmacol Toxicol. 43:233-260

Nrf2 and activation of ARE



Signaling events involved in the transcriptional regulation of gene expression mediated by the ARE. Three major signaling pathways have been implicated in the regulation of the ARE-mediated transcriptional response to chemical stress. In vitro data suggest that direct phosphorylation by PKC may promote Nrf2 nuclear translocation as a mechanism leading to transcriptional activation of the ARE. Nrf2 has been proposed to be retained in the cytoplasm through an interaction with Keap1 and it is possible that phosphorylation of Nrf2 may also cause the disruption of this interaction. Nrf2 binds to the ARE as a dimer with bZIP (small Mafs?) complex.

From Nguyen et al. (2003) Annu Rev Pharmacol Toxicol. 43:233-260

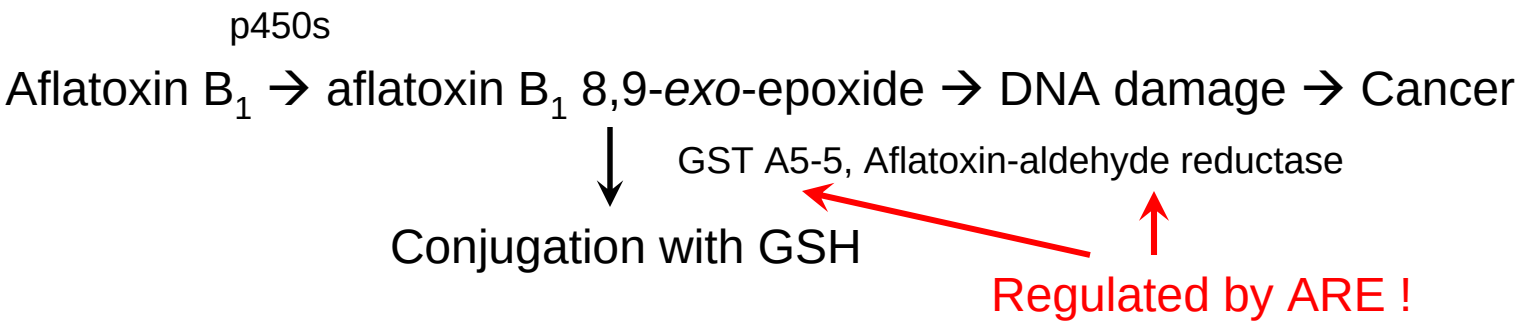
ARE/NRF2 as a link between metabolism of xenobiotics and antioxidants

Traditional paradigm:

"Antioxidants are good because they protect by eliminating ROS"

However,

some synthetic and naturally occurring antioxidants (e.g., polyphenols such as ethoxyquin, coumarin, quercetin) can induce genes under control of ARE that provides protection against chemical carcinogens.



Pathological conditions that involve oxidative stress

- Inflammation
- Atherosclerosis
- Ischemia/reperfusion injury
- Cancer
- Aging

Inflammation

“Vicious cycle” of inflammation and damage:

Immune cells migrate to the site of inflammation

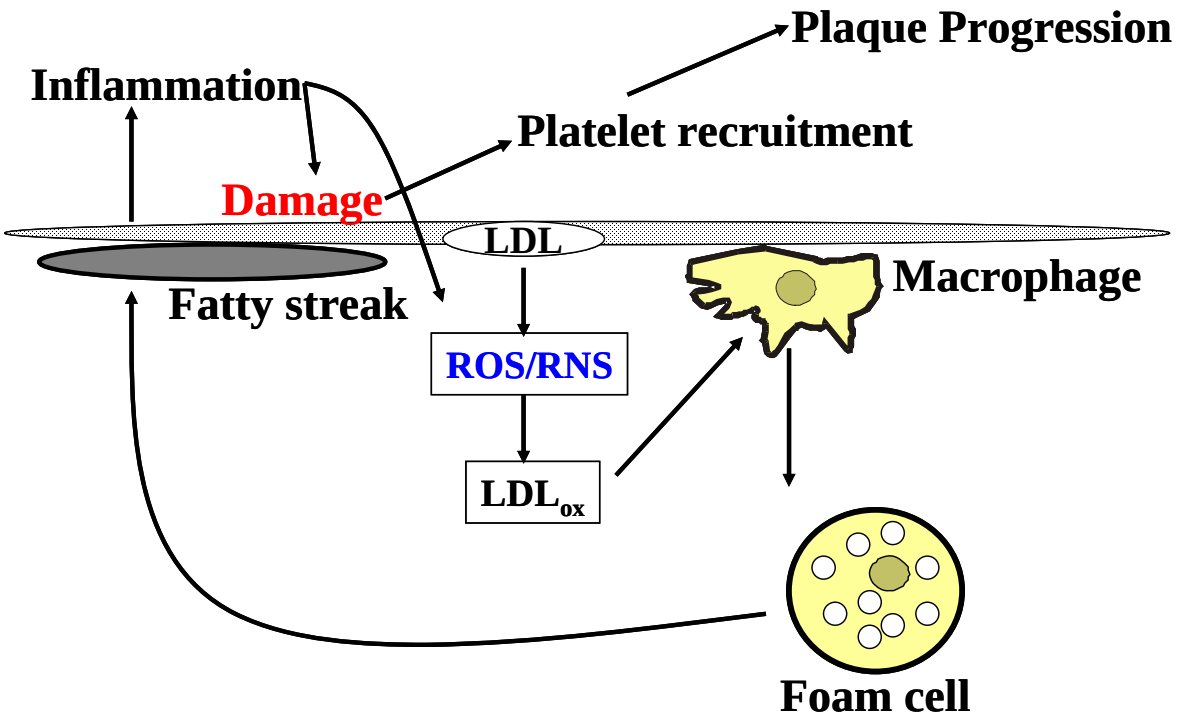
↓

Produce oxidants to kill bacteria

↓

Oxidants directly damage tissue → (back to: Immune cells migrate to the site of inflammation)

Atherosclerosis



ROS appear to be critical in progression:

Oxidation of LDL key early step

Macrophages accumulate products become foam cells

Immune and platelet response

Progression of atherosclerotic plaque

Ischemia/Reperfusion Injury

Ischemia

Cessation of blood and/or oxygen supply

ATP degraded to hypoxanthine

Proteolysis increases and pH decreases

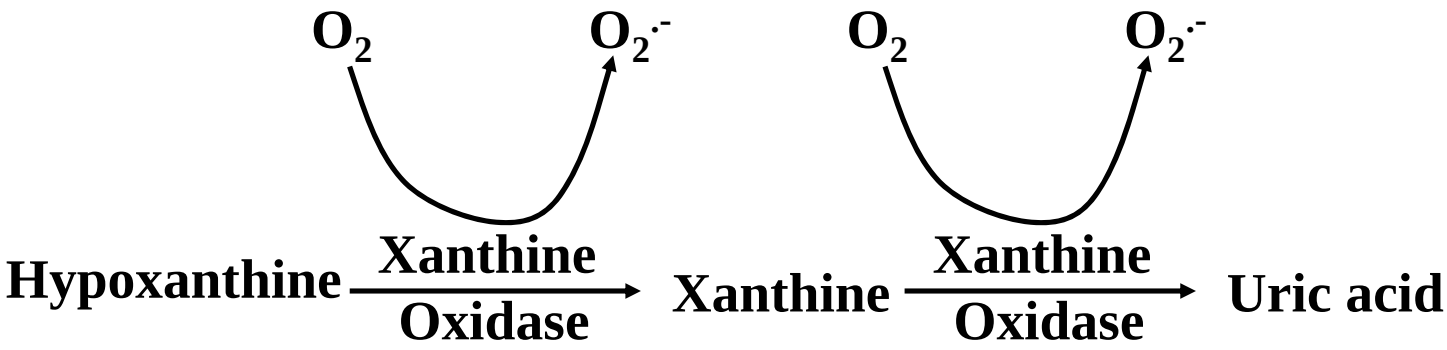
xanthine dehydrogenase → xanthine oxidase

Tissue damage mild (when not prolonged)

Reperfusion

'Oxygen paradox': generation of free radicals from XO

'pH paradox': protective in ischemia, damaging in reperfusion



Cancer and ROS

Initiation

DNA oxidation may lead to mutation

Promotion

Promoters can be oxidants

- **e.g., benzoyl peroxide**

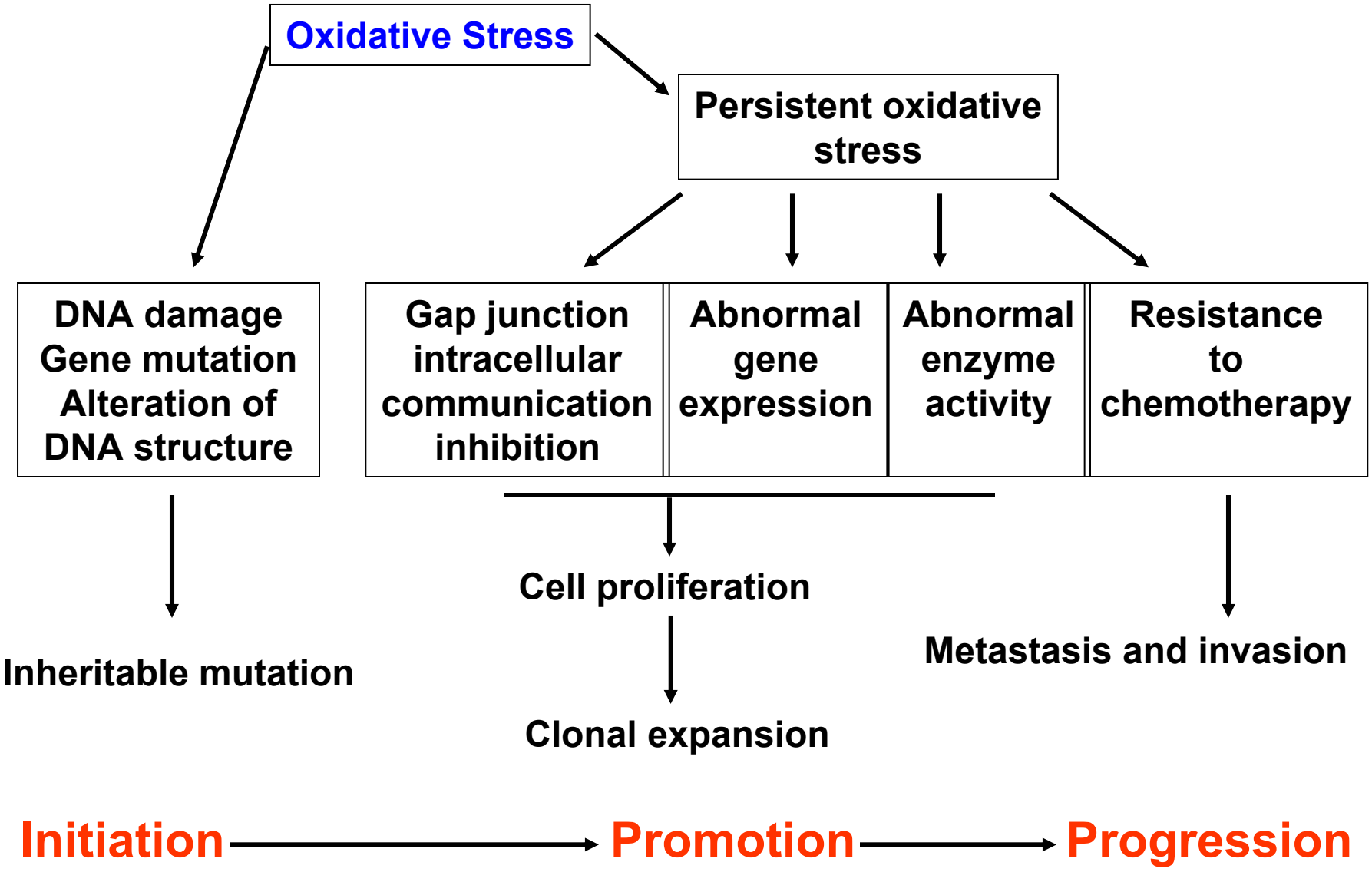
Promoters can stimulate oxidant production

- **e.g., phorbol esters**

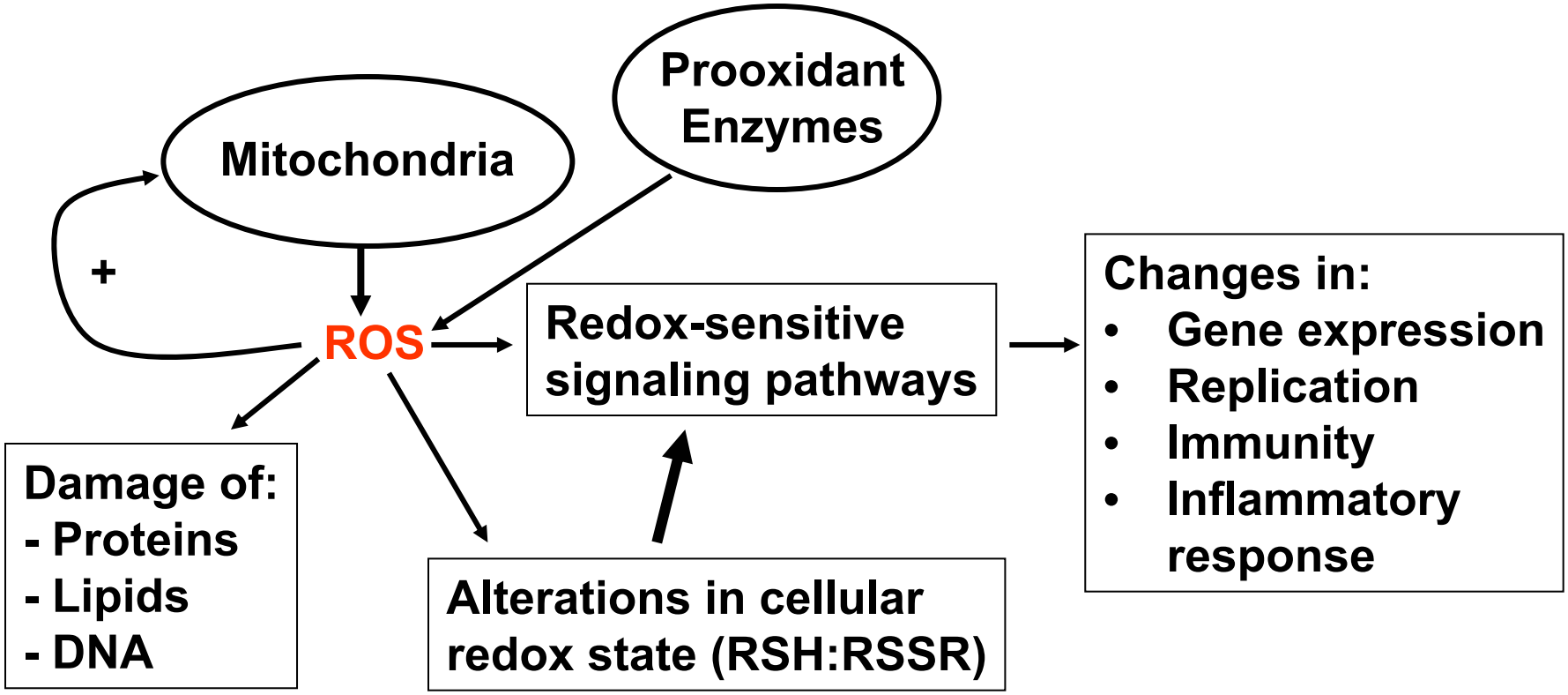
ROS may cause changes that lead to promotion

- **e.g., promitogenic signaling molecules**

Cancer and ROS



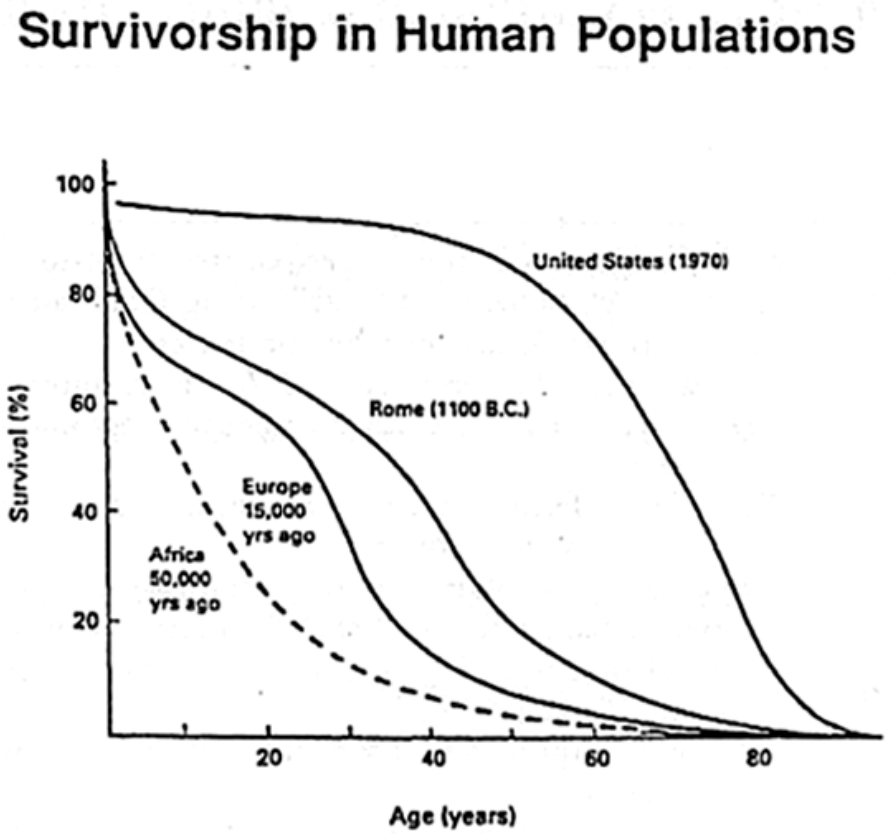
Oxidative stress hypothesis of aging
"Wear and Tear Model"

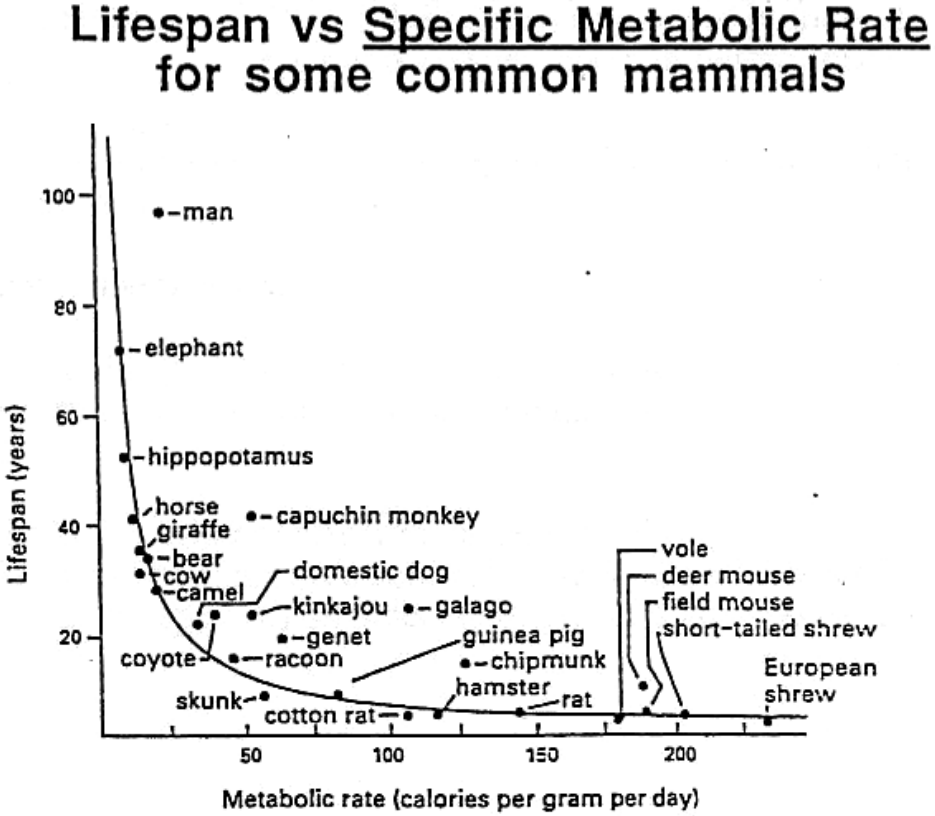


Questions remaining:

- Are ROS the primary effectors of senescence?
- Is the course of senescence controlled by ROS metabolism?
- Is species lifespan per se determined by ROS metabolism?

Oxidative stress hypothesis of aging

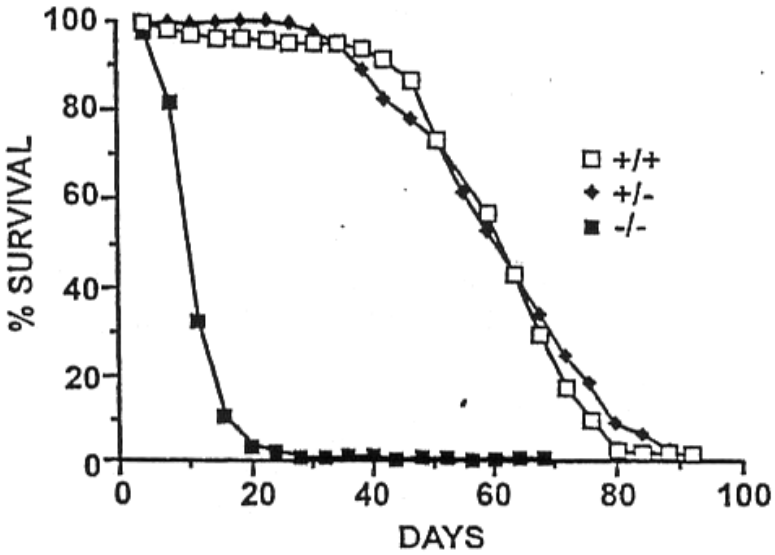




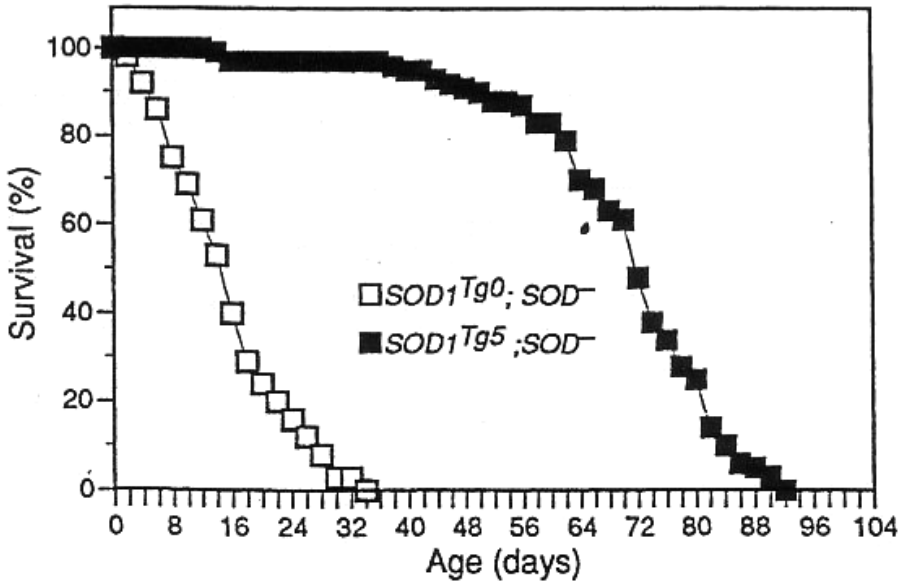
Adapted from Cutler, R. (1884), in A.K. Roy and B. Chatterjee Eds., *Molecular Basis of Aging*. Academic Press, Orlando Fl.

Oxidative stress hypothesis of aging

Drosophila Longevity (SOD1 mutants)



Rescue of SOD-null Lifespan with a Genomic SOD1 Transgene



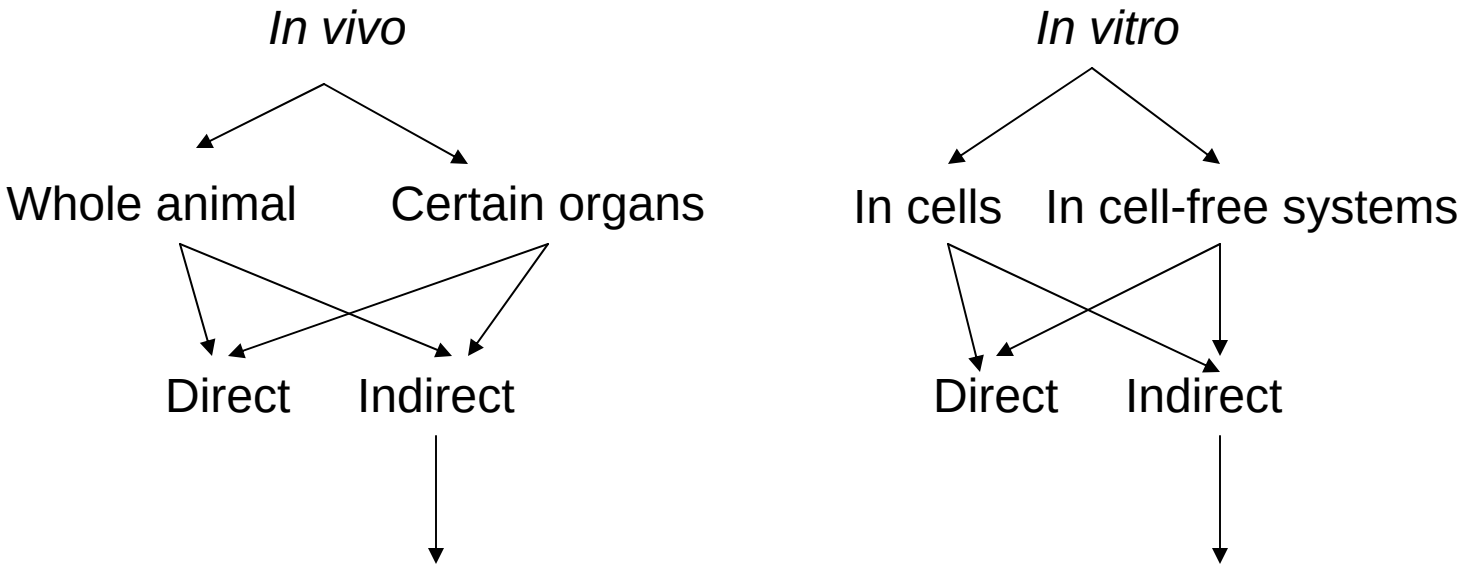
Parkes et al. (1998). Transgenic analysis of the cSOD-null phenotypic syndrome in Drosophila. Genome *41*, 642-651.

Expression of SOD1 and/orCAT: Effects on Drosophila Lifespan

Transgene	Mean Lifespan (%)
SOD1	up to 48% increase
CAT	variable negative to no increase
SOD1 + CAT	variable negative to weak positive increase

Sun, J., and Tower, J. (1999). FLP recombinase-mediated induction of Cu/Zn-superoxide dismutase transgene expression can extend the life span of adult *Drosophila melanogaster* flies. Mol Cell Biol *19*, 216-28.

Measurement of Free Radicals



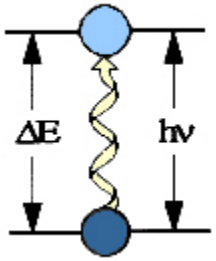
Detection of radical-modified molecules:

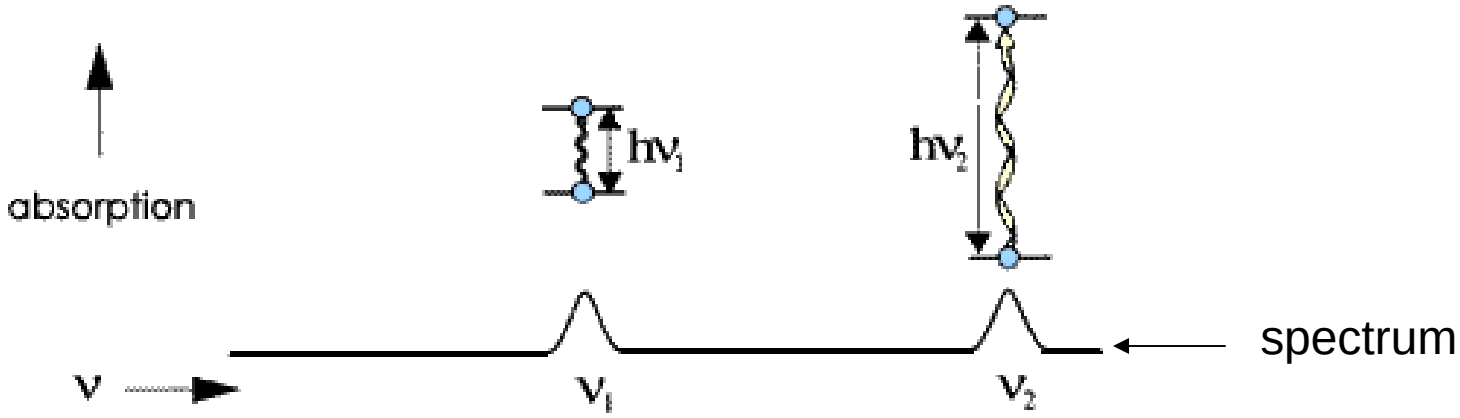
- Exogenous probes
- Proteins
- Lipids
- DNA

Direct detection of Free Radicals: EPR

EPR (Electron Paramagnetic Resonance) is the resonant absorption of microwave radiation by paramagnetic systems in the presence of an applied magnetic field.

Spectroscopy is the measurement and interpretation of the energy differences between the atomic or molecular states. With knowledge of these energy differences, you gain insight into the identity, structure, and dynamics of the sample under study. We can measure these energy differences, $\Delta\mathbf{E}$, because of an important relationship between $\Delta\mathbf{E}$ and the absorption of electro-magnetic radiation. According to Planck's law, electromagnetic radiation will be absorbed if: $\Delta\mathbf{E} = \mathbf{h}\nu$ where **h** is Planck's constant and ν is the frequency of the radiation.





The energy differences we study in EPR spectroscopy are predominately due to the interaction of unpaired electrons in the sample with a magnetic field produced by a magnet in the laboratory.

From www.bruker-biospin.com

Detection of Free Radicals: EPR

EPR can detect and measure free radicals and paramagnetic species (e.g., oxygen):

- high sensitivity
- no background
- definitive and quantitative

Direct detection: e.g., semiquinones, nitroxides

Indirect detection: spin trapping (superoxide, NO, hydroxyl, alkyl radicals)

Intact tissues, organs or whole body can be measured,

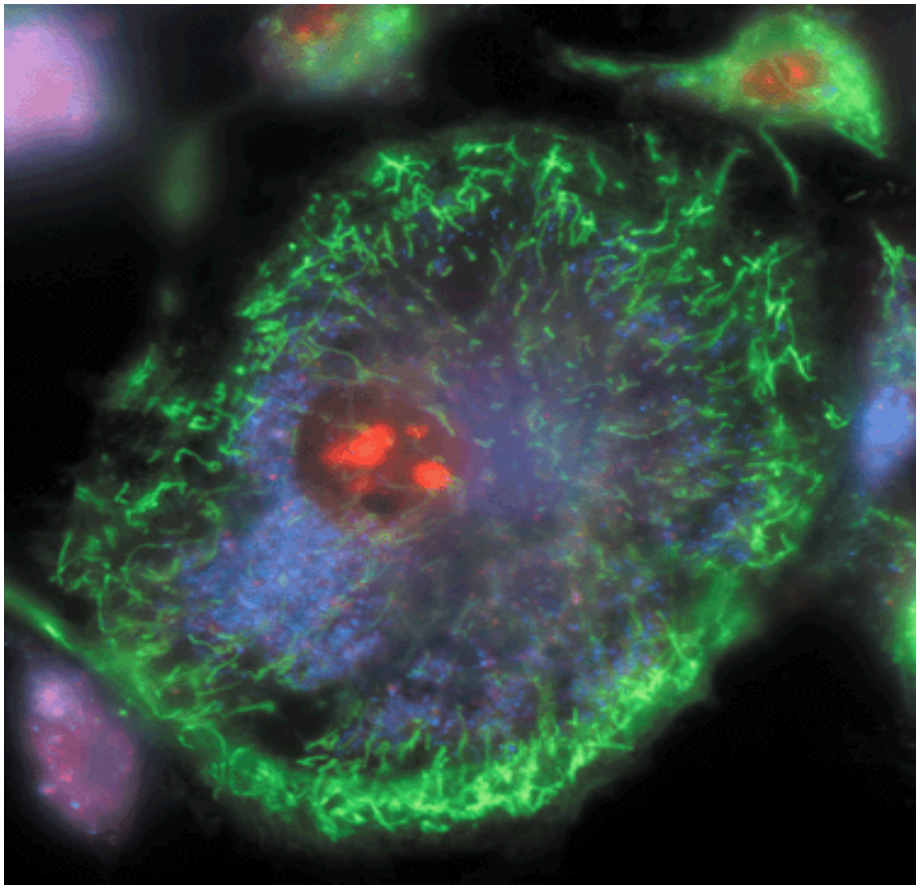
but biological samples contain water in large proportions that makes them dielectric. Thus frequencies of the EPR spectrometers should be adjusted (reduced) to deal with “non-resonant” absorption of energy (heating) and poor penetration

Detection of Free Radicals: Fluorescent Probes

ROS/RNS

↓

Chemical compound → Fluorescent chemical compound



Live bovine pulmonary artery endothelial cells (BPAEC) were incubated with the cell-permeant, weakly blue-fluorescent dihydroethidium (D-1168, D-11347, D-23107) and the green-fluorescent mitochondrial stain, MitoTracker Green FM (M-7514). Upon oxidation, red-fluorescent ethidium accumulated in the nucleus. The image was acquired using a CCD camera controlled by MetaMorph software (Universal Imaging Corporation). From Molecular Probes, Inc.

Detection of Free Radicals: Modified Proteins

TABLE 1

Oxidative Modifications of Proteins

Modification	Amino acids involved	Oxidizing source[a]
Disulfides, glutathiolation	Cys	All, $ONOO^-$
Methionine sulfoxide	Met	All, $ONOO^-$
Carbonyls (aldehydes, ketones)	All (Lys, Arg, Pro, Thr)	All
Oxo-histidine	His	γ-Ray, MCO, $^1O_2$
Dityrosine	Tyr	γ-Ray, MCO, $^1O_2$
Chlorotyrosine	Tyr	HOCl
Nitrotyrosine	Tyr	$ONOO^-$
Tryptophanyl modifications (*N*-formyl)kynurenine	Trp	γ-Ray
Hydro(pero)xy derivatives	Val, Leu, Tyr, Trp	γ-Ray
Chloramines, deamination	Lys	HOCl
Lipid peroxidation adducts (MDA, HNE, acrolein)	Lys, Cys, His	γ-Ray, MCO (not HOCl)
Amino acid oxidation adducts	Lys, Cys, His	HOCl
Glycoxidation adducts	Lys	Glucose
Cross-links, aggregates, fragments	Several	All

[a] MCO-metal catalyzed oxidation; All = γ-ray, MCO, HOCl, ozone, $^1O_2$.

From Shacter E (2000) Drug Metab Rev 32:307-326

TABLE 3

Forms of Protein Oxidative Modification That Have Been Found in Biological Tissues

Modification	Disease/tissue
Carbonyls	
Glutamine synthetase	Aging, Alzheimer's, ischemia/reperfusion
IgG	Rheumatoid arthritis
Kidney proteins	Chronic estrogen exposure
Lung proteins	Hyperoxia
Brain proteins	Ischemia/reperfusion
Eye lens proteins	Cataracts
Muscle proteins	Muscular dystrophy (chicken)
Unidentified proteins	Aging
Unidentified proteins	Parkinson's disease
Unidentified proteins	Neonatal lung fluids/ventilators
Methionine sulfoxide	
α-1-proteinase inhibitor	Smoker, bronchitis lung fluids, synovial fluid
Lipid peroxidation adducts	
LDL	Atherosclerosis
Glutathiolation (SH oxidation)	
Carbonic anhydrase III	Aging
Muscle proteins	Muscular dystrophy (chicken)
Unidentified proteins	Activated monocytes
3-Chlorotyrosine, dityrosine	
LDL	Atherosclerosis
Hydro(pero)xyleucine	
Unidentified proteins	Cataracts
Nitrotyrosine	
Unidentified proteins	Acute inflammatory lung tissue Atherosclerosis Rheumatoid arthritis
Aggregates	
IgG	Rheumatoid arthritis

TABLE 4

Methods for Detection of Oxidative Protein Modifications

Modification	Methods of detection	Ref.[a]
Disulfides	SDS–gel electrophoresis (±β-ME); DTNB	30
Thiyl radicals	Electron spin resonance spectroscopy	34
Glutathiolation	RP-HPLC/mass spectroscopy; IEF; ^{35}S–Cys/Chx/SDS-PAGE	31, 57, 122, 123
Methionine sulfoxide	CNBr cleavage/amino acid analysis	105, 106
Carbonyls	DNPH-coupled assays: Western blot/ELISA/immunocyto-chemistry/HPLC/A_{370}	89–91, 93, 94, 97
	Reduction with NaB^3H_3	100, 101
2-Oxo-His	Amino acid analysis	39
Dityrosine	Fluorescence; proteolysis or hydrolysis/HPLC	51, 74
Chlorotyrosine	Hydrolysis/nitroso-napthol/HPLC; HBr hydrolysis-GC/MS	44, 45
Nitrotyrosine	Immunoassay; hydrolysis/HLPC; HPLC/electrochemical detection	124–128
Tryptophanyl	Fluorescence; amino acid analysis (alk. hydrolysis); proteolysis/ MS	129
Hydroperoxides	KI/I_3^-/spectroscopy; $NaBH_4$/ hydrolysis/OPA–HPLC	19
Lipid peroxidation adducts	$NaBH_4$/hydrolysis/OPA–HPLC; hydrolysis–GC/MS; DNPH; immunoassays	28, 29, 54, 55
Amino acid oxidation adducts	$NaCNBH_3$ reduction/hydrolysis/ H^1–NMR/MS	47
Glycoxidation adducts	Derivitization—GC/MS	58
Cross-links, aggregates, fragments	SDS–gel electrophoresis; HPLC	43, 130, 131

Note: DTNB: dithiobisnitrobenzoate; IEF: isoelectric focusing; DNPH: dinitrophenylhydrazine; CNBr: cyanogen bromide; Chx: cyclohexamide; OPA: *o*-phthaldialdehyde.

[a] References for some methods currently in use. Other methods are also available.

Detection of Free Radicals: Modified Lipids

Methods to quantify lipid peroxidation:

- measurement of substrate loss
- quantification of lipid peroxidation products (primary and secondary end products)

The IDEAL assay for lipid peroxidation:

- accurate, specific, sensitive
- compounds to be quantified are stable
- assay applicable for studies both *in vivo* and *in vitro*
- easy to perform with high throughput
- assay is economical and does not require extensive instrumentation

BUT: no assay is ideal

most assays are more accurate *in vitro* than *in vivo*

little data exist comparing various methods *in vivo*

Detection of Free Radicals: Modified Lipids

Assays of potential use to quantify lipid peroxidation *in vivo* and *in vitro*:

Fatty Acid analysis:

commonly used to assess fatty acid content of biol. fluids or tissues

Method: Lipid extraction→transmethylation of fatty acids→separation by GC (HPLC)→quantification

Advantages: easy, equipment readily available, data complement other assays

Disadvantages: impractical for a number of in vivo situations, measures disappearance of substrate only, may not be sensitive enough

Conjugated Dienes:

- peroxidation of unsaturated fatty acids results in the formation of conjugated diene structures that absorb light at 230-235nm → spectrophotometry
- useful for *in vitro* studies
- inaccurate measure in complex biological fluids (other compounds absorb at 234 nm – purines, pyrimidines, heme proteins, primary diene conjugate from human body fluids is an non-oxygen containing isomer of linoleic acid –octadeca-9-cis-11-trans-dienoic acid)

Advantages: easy, equipment readily available, provides info on oxidation of pure lipids *in vitro*

Disadvantages: virtually useless for analysis of lipid peroxidation products in complex biological fluids

Detection of Free Radicals: Modified Lipids

Assays of potential use to quantify lipid peroxidation *in vivo* and *in vitro*:

Lipid Hydroperoxides:

primary products of lipid peroxidation

Method: several exist, chemiluminescent based are most accurate: LO• +isoluminol → light (430 nm)

Advantages: more specific and sensitive than other techniques

Disadvantages: probes are unstable, ex vivo oxidation is a major concern, equipment $$

Thiobarbituric Acid-Reactive Substances (TBARS)/MDA:

most commonly used method for lipid peroxidation

measures MDA – a breakdown product of lipid peroxidation

Method: sample is heated with TBA at low pH and pink chromogen → TBA-MDA adduct is formed
absorbance is quantified at 532 nm or fluorescence at 553 nm

Advantages: easy, equipment readily available, provides info on oxidation of pure lipids *in vitro*

Disadvantages: not reliable for analysis of lipid peroxidation products in complex biological fluids or *in vivo*

Detection of Free Radicals: Modified Lipids

Assays of potential use to quantify lipid peroxidation *in vivo* and *in vitro*:

Alkanes:

volatile hydrocarbons generated from scission of oxidized lipids:

N-6 fatty acids → pentane, N-3 fatty acids → ethane

Method: collection of gas phase from an in vitro incubation or exhaled air (animals or humans) → GC

Advantages: integrated assessment of lipid peroxidation in vivo

Disadvantages: cumbersome technique, takes several hours. atmospheric contamination, 1000-fold differences in normal exhaled pentane in humans were reported

F_2-Isoprostanes:

arachidonic acid-containing lipids are peroxidized to PGF_2-like compounds (F_2-Iso)

formed independent of cyclooxygenase

generated in large amounts *in vivo*

have biological activity

Method: lipid extraction, TLC and derivatization → GC-MS

Advantages: stable molecules, equipment available, assay precise and accurate, can be detected in all fluids and tissues, normal ranges in agreement between labs

Disadvantages: samples must be stored @-70C, only a small fraction of possible arachidonic acid oxidation products, analysis is labor intensive and low throughput

Detection of Free Radicals: Modified DNA

1. **GC/MS**
   Advantage: detects the majority of products of oxidative damage
   Disadvantage: requires derivatization before analysis which can artifactually inflate the amount of oxidative damage observed; requires expensive instrumentation
2. **HPLC-EC**
   Advantage: very sensitive and accurate technique; requires less expensive equipment than GC/MS
   Disadvantage: can only detect electrochemically active compounds (8-oxodG, 5-OHdCyd, 5-OHdUrd, 8-oxodA)
3. **Immunoaffinity Isolation with Detection by ELISA or HPLC-EC**
   Advantage: can be used for concentration of oxidized bases from dilute solutions (urine, culture medium) for quantitation
   Disadvantage: confines analysis to only one modified base at a time
4. **Postlabelling:** [3H]acetic anhydride, enzymatic 32P, dansyl chloride
   Advantage: requires very little DNA and has at least an order of magnitude more sensitivity than some of the other techniques
   Disadvantage: presence of significant background from cellular processes and analytical work-up
5. **DNA Strand Breaks:** Nick translation, alkaline elution, alkaline unwinding, supercoiled gel mobility shift, comet assay
   Advantage: can be very sensitive
   Disadvantage: when viewing in whole cells, not always specific for oxidative lesions; may indicate DNA repair
6. **DNA Repair Assays:** Formamidopyrimidine glycosylase, endonuclease followed by strand break assay
   Advantage: sensitive measure of specific types of damage
   Disadvantage: limited to the types of damage for which repair enzymes have been identified

Pitfalls of DNA Damage Assay Techniques

1. **Isolation of DNA**

a. Phenol extraction: breakdown products of phenol can introduce oxidative damage to DNA as it is isolated
b. Contamination by metals (Fe)
c. Photochemical reduction leads to DNA oxidation

2. **Derivatization of DNA for GC/MS**

a. Isolation of DNA
b. Acid hydrolysis
c. Silylation

TABLE 1. Relative Concentrations of 8-oxo-dG (freqeuncy per 10^5 dG) in Lymphocyte DNA*

Tissue	Method	8-oxo-dG (8-oxo-gua) per 10^5 dG	Reference
Lymphocytes	HPLC-EC	2.9	(52)
Leukocytes (MN, PMN)	HPLC-EC	1.1	(53)
Leukocytes	HPLC-EC	4	(54)
Leukocytes	HPLC-EC	0.43	(55)
Leukocytes (MN)	HPLC-EC	3.7	(38)
Leukocytes (MN, PMN)	HPLC-EC (with anaerobic preparation)	0.24, 0.31	(17)
Breast cancer	GC-MS	165	(56)
Lymphocytes	GC-MS	33	(57)
Lymphocytes	GC-MS	30	(58)

GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography. MN, mononucleocytes; PMN, polymorphonucleocytes.

From Collins AR (1999) Bioessays 21:238-246