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METHODICAL POINTING
from the course
"BIOLOGICAL MEMBRANES AND REGULATION OF METABOLISM"
(part 2. Regulation of metabolism: cell signaling)

for the master's degrees of a 1 course of department of biochemistry with English of educating

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It is ratified to printing by meeting of scientific advice of The Institute of Biology (10.04.2013, protocol №11)

Kyiv-2013

ЗМІСТ

ADAR - Double-stranded RNA-specific adenosine deaminase
BH4 - tetrahydrobiopterin
cADP-ribose - cyclic ADP-ribose
CaMK - calmodulin-dependent serine/threonine protein kinases
cAMP – cyclic adenosine monophosphate
CAPK - ceramide-activated protein kinase
CAPP - ceramide activated protein phosphatases
CAT - catalase
CDase - ceramidase
CerK - ceramide kinase
cGMP - cyclic guanosine monophosphate
COX - cyclooxygenase
C1P - Ceramide 1-phosphate
CYP - cytochrome P-450
DAG - diacylglycerol
dsRNA - double-stranded RNA
EDRF - endothelium derived relaxation factor
EF-hand
EGFR - epidermal growth factor receptor;
eNOS - endothelial (macrofagal) NOS
ER – endoplasmatic reticulum
ERKs - extracellular signal-related kinases
FAD - flavin adenine dinucleotide
FLAP - 5-lipoxygenase activating protein
FMN - flavin mononucleotide
GAPs - GTPase Activating Proteins
GAS – IFN-γ-activated site
GEFs - Guanine Nucleotide Exchange Factors
GPCRs - G-Protein-Coupled Receptors
GPx - glutathione peroxidase
Grb2 - growth factor receptor binding protein
Grxs - glutaredoxins
GSH - glutathione
GSH-reductase - glutathione reductase
GSSG - glutathione disulfide
HIF-1 - hypoxia-inducible factor-1
IFN - interferon
IFNAR - IFN-α receptor
IFNGR – IFN-β -receptor
IkB - inhibitor of NF-kB
IKK - IκB kinase
IL-1Rs - interleukin-1 receptors
iNOS - induced NOS
IP3 - inositol-1,4,5- triphosphate
IP4 (inositol-1,3,4,5-tetraphosphate)
IRFs - Interferon regulatory factors
IRS-1 - insulin-receptor substrate
ISGF3 - IFN-stimulated gene factor 3
ISGs - IFN stimulated genes
ISREs - IFN-stimulated response elements
JAKs - Janus Kinases
JNKs - c-Jun NH2-terminal kinases

LPS -lipopolysaccharide
LRR - leucine-rich repeat
MAPKs - Mitogen Activated Protein Kinases
MAPKK - kinase MAPK
MAPKKK - kinase kinase MAPK
MDA5 - Melanoma Differentiation-Associated protein 5
MHC - major histocompatibility complex molecules
MPO - Lysosomal myeloperoxidase
Mx proteins - myxovirus proteins
MyD88 - Myeloid differentiation factor 88
NF-kB - Nuclear factor kappa B
nNOS - neuronal NOS
NO - nitric oxide
NOS - nitric oxide synthetase
NOX - NADPH oxidase
NRPTPs - nonreceptor protein tyrosine phosphatases
NRTKs - nonreceptor tyrosine protein kinases
NSAIDs - non-steroidal anti-inflammatory drugs
PAMPs - pathogen associated molecular patterns
PDGFR - platelet-derived growth factor receptor
PDK1 – PI-3,4,5-P3-dependent kinase
PGH2 -prostaglandin H2
PH domains - Plekstrin homology domains
PHGPx - phospholipid hydroperoxide glutathione peroxidase
Phox – phagocyte NADPH oxidase
PI - Phosphatidylinositol
PI-4,5-P2 - phosphatidyl-inositol-bis-phosphate
PI-3,4,5-P3 - phosphatidyl-inositol-triphosphate
PI3K - phosphatidyl inositide 3-kinase
PkA - Protein Kinase A (=cAMP Dependent Protein Kinase, CADPK)
PkB - Protein kinase B
PkC - Protein kinase C
PkG - cGMP dependent protein kinase (=CGDPK)
PkR - Protein kinase R
PLC - Phospholipase C
PRR - pattern recognition receptors
Prxs - peroxiredoxins
PTB - phosphotyrosine-binding domains
PTK - Protein tyrosine kinases
PTPs - Protein tyrosine Phosphatases
Ras – from “rat sarcoma”
RIG-I - retinoic acid-inducible gene 1
ROS – reactive oxygene species
RPTPs - receptor protein tyrosine phosphatases
RR - ryanodin receptor
RTKs - receptor tyrosine protein kinases
SH2 domain - Src Homology 2
SK - sphingosine kinase
SOD - superoxide dismutase
S1P - sphingosine-1-phosphate
S1P-lyase - sphingosine-1-phosphate lyase
SPP1 - spingosine-1-phosphate phosphatase,

S1PR – receptors for sphingosine-1-phosphate
SR - sarcoplasmic reticulum
STATs - Signal Transduction Activators of Transcription
TIR domain - Toll-IL1-R domain
TLR - Toll-like Receptor
TNFα - tumor necrosis factor α
TRAM - TRIF-related adaptor molecule
TRIF - Toll/IL-1 receptor domain-containing adaptor inducing IFNβ
Trxs - thioredoxins
XO - xanthine oxidase
2’-5’-OA - 2'-5' oligoadenylate
2’-5’-OAS - 2'-5' oligoadenylate synthetase
5-HPETE - 5-hydroperoxyeicosatetraenic acid
5’-LO - 5’-lipoxygenase

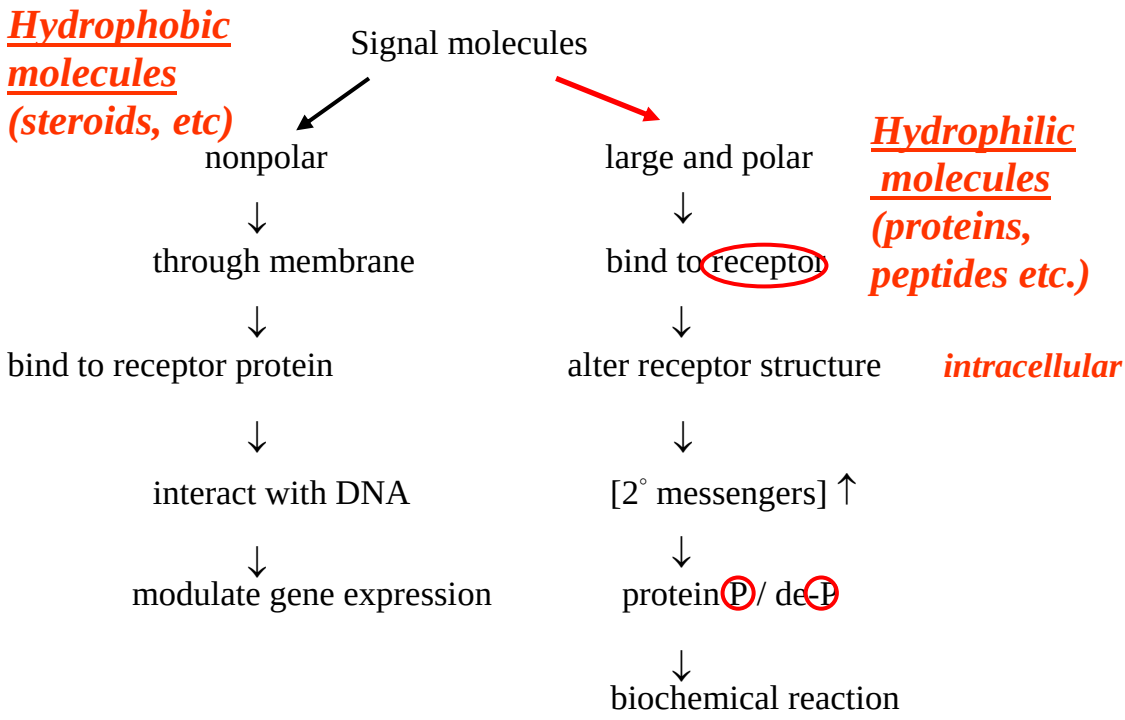
1. The main signaling cascades of cell: principle and function

1.1. Cell signaling by polar and nonpolar molecules.

Extracellular signal molecules bind to either cell-surface receptors or intracellular receptors.

Most signal molecules are **hydrophilic (primary messengers)** and are therefore unable to cross the plasma membrane directly; instead, they bind to cell-surface receptors, which in turn generate one or more signals inside the target cell.

Some small signal molecules, by contrast, diffuse across the plasma membrane and bind to receptors inside the target cell either in the cytosol or in the nucleus (as shown here). Many of these small signal molecules are **hydrophobic** and nearly insoluble in aqueous solutions; they are therefore transported in the bloodstream and other extracellular fluids after binding to carrier proteins, from which they dissociate before entering the target cell.



Hydrophilic ligands bind to receptors located on the external surface of the plasma membrane with conformational change in receptors. Ligand·receptor complex couples to an enzyme, located on the internal surface of the cell membrane, activates it and stimulates the conversion of some metabolite into a second messenger. This second messenger then is responsible for the manifestations of all of the effects of the primary messenger inside the cell (changes in enzyme activities and alterations in gene expression). In a large number of cases, the second messenger acts by stimulating or inhibiting protein phosphorylation/ dephosphorylation cascades.

Cascades have certain advantages to include signal divergence, convergence and amplification.

The three main enzymes which are second messenger producers are:

1. adenylate cyclase (adenylate cyclase cascade; second messenger is cAMP);
2. guanylate cyclase (guanylate cyclase cascade; second messenger is cGMP);
3. phospholipase C (phosphoinosytol cascade; second messengers are inositol-1,4,5- triphosphate – IP3; diacylglycerol – DAG; Ca2+).

1.2. Receptor Classes

Cell surface receptors bind signaling molecules include peptide hormones, catecholamines, insulin, growth factors, cytokines, etc. There are:

1. Ion channel-linked cell surface receptors;
2. G-protein-linked or **G**-**P**rotein-**C**oupled **R**eceptors (GPCRs);
3. Receptor-enzyme (tyrosine protein kinases et al).

There are next subsequent events after ligand binding to these receptor groups:

- triggering an ↑ or ↓ in the cytosolic concentration of a second messengers (1,2);

- activation of cell-surface receptor that triggers a phosphorylation cascade and acts as a scaffold to recruit and activate other intracellular proteins (3)

G-**P**rotein-**C**oupled **R**eceptors or **serpentine receptors** belong to the largest family of cell surface receptors. Different ones respond to a wide variety of mediators including hormones, neurotransmitters and local mediators. They are integral membrane proteins with a common structural motif, **7 transmembrane α-helices**, so **seven-helix receptors** are thus called. Approximately 800 different GPCRs are encoded in the human genome (receptors to epinephrine, acetylcholine, serotonin etc). Different G-protein coupled receptors for same compound activate different trimeric G-proteins, so the same signal can have different effects in different tissues.

Intracellular receptors bind signaling molecules include steroid hormones, retinoids, thyroxine, etc. Receptor-hormone complex acts a transcription factor to alter transcription of certain genes or has effects on cellular biological membranes.

1.3. G-Proteins and Small GTP-binding proteins

G-Proteins are members of a family of the guanyl nucleotide regulatory proteins. They are trimeric proteins which have three chains: α, β and γ. They bind guanine nucleotides and function to couple integral membrane receptors to target membrane-bound enzymes.

Initially Gα has bound GDP, and α, β and γ subunits are complexed together. The complex βγ subunits inhibits Gα. β and γ chains form a tight complex βγ that anchors G-protein to the plasma membrane - γ subunits have covalently attached lipid anchors that bind a G-protein to the plasma membrane cytosolic surface.

Inactive G-protein so has a bound GDP. After GDP dissociates from Gα new GTP is bound. This causes Gα to dissociate from Gβγ. Gα binds to membrane-bound enzyme (adenylate cyclase, phospholipase C for different G-proteins), altering its activity. The complex of Gβγ that is released when Gα binds GTP is itself an effector that binds to and activates or inhibits several other proteins.

The family of heterotrimeric G proteins includes:

- Gs and Gi coupled to *adenylate cyclase*;
- Gq coupled to *phospholipase C*;
- transducin, involved in sensing of light in the retina;
- G-proteins involved in odorant sensing in olfactory neurons.

Behind of trimeric G proteins there is a larger family of small GTP-binding switch proteins, related to Gα.

Small GTP-binding proteins include (roles indicated):

- initiation & elongation factors (protein synthesis).
- Ras (growth factor signal cascades).
- Rab (vesicle targeting and fusion).
- ARF (forming vesicle coatomer coats).
- Ran (transport of proteins into & out of the nucleus).
- Rho (regulation of actin cytoskeleton)

All GTP-binding proteins differ in conformation depending on whether GDP or GTP is present at their nucleotide binding site.

Generally, GTP binding induces the active state.

Most of small GTP-binding proteins depend on ***helper proteins*** - **GAPs (G**TPase **A**ctivating **P**roteins, which promote GTP hydrolysis) and **GEFs (G**uanine **N**ucleotide **E**xchange Factors, which promote GDP/GTP exchange). Activation of G proteins involves GTP displacement of GDP bound to the α subunit and dissociation of the complex from the γβ subunits, and this exchange facilitated by a GEF protein, specific for Gs. The GTPase activity associated with the α subunit slowly hydrolyzes the bound GTP to GDP which is facilitated by a GAP, specific for Gs.

	G proteins vs. small G proteins **(divergent evolution)**	
	G proteins	**small G proteins**
M	30-35 kD	20-25 kD
Structure	heterotrimer	monomer (similar to G_α)
Receptors which activate/inhibit G protein	7TM	Dimerization of receptor tyrosine protein kinases
GTPase activity	GTPase act.	GTPase act. (low) GTPase-activating proteins (GAPs): facilitate GTP hydrolysis

An example of small GTP-binding proteins is protein Ras, which localized to the inner surface of plasma membrane. There are three 21-kD Ras proteins in mammalian cells:

- H-Ras (from Harvey rat sarcoma: a loss of the ability to hydrolyze GTP through to this pathology);
- K-Ras (from Kirsten rat sarcoma);
- N-Ras (from Neuroblastoma rat sarcoma)

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1.4. Adenylate cyclase cascade

1. ***Hormone binding, usually to an extracellular domain of 7-helix-receptor***, causes a ***conformational change in the receptor that is transmitted to a G-protein*** on the cytosolic side of the membrane.

2. After that Gα releases GDP and binds GTP (***GDP-GTP exchange***). Substitution of GTP for GDP causes another conformational change in Gα, so ***Gα-GTP dissociates from the inhibitory βγ complex and can now bind to and activate or inhibit Adenylate Cyclase***.

There are two kinds of G proteins that can influence on Adenylate Cyclase - ***Gs protein***, which stimulates adenylate cyclase activity, and ***Gi protein***, which inhibits it. Gs and Gi proteins differ by Gα subunits (***stimulatory Gsa*** and ***inhibitory Gia***). Different effectors and their receptors induce Giα to exchange GDP for GTP than those that activate Gsα. And the same effector after binding with different receptors can activate either Giα or Gsα. For example, epinephrine can increase or decrease Adenylate Cyclase activity, depending upon the receptor to which it binds: β adrenergic receptors couple to Gs, whereas α2 adrenergic receptors couple to Gi.

Cholera toxin catalyzes covalent modification of Gsα in which ADP-ribose is transferred from NAD+ to an arginine residue at the GTPase active site of Gsα. This ADP-ribosylation prevents GTP hydrolysis by Gsα and the stimulatory G-protein is permanently activated.

Pertussis toxin (whooping cough disease) catalyzes ADP-ribosylation at a cysteine residue of the inhibitory Giα, making it incapable of exchanging GDP for GTP and the inhibitory pathway is blocked.

ADP-ribosylation is a general mechanism by which activity of many proteins is regulated, in eukaryotes (including mammals) as well as in prokaryotes.

3. ***Adenylate Cyclase, activated by the stimulatory Gα-GTP, catalyzes synthesis of cAMP***. Adenylate cyclase is subjected to both stimulatory and inhibitory input from ligand·receptor complexes via G-proteins (and other sources). As mentioned above, Gs has stimulatory effects on adenylate cyclase and Gi has inhibitory effects.

Adenylate Cyclase (Adenylyl Cyclase) catalyzes:

$ATP \rightarrow cAMP + PPi$

Adenylate cyclases have 10 mammalian isoforms. They are integral membrane proteins with 60% similarity (50%-90% in cytoplasmic regions), that consist of two bundles of six transmembrane segments (M1, M2). Two catalytic domains extend as loops into the cytoplasm: C1a/C1b cytoplamic domains (360-390 amino acids) and C2a/C2b cytoplasmic domains (255-330 amino acids).

4. ***Protein Kinase A (cAMP Dependent Protein Kinase, CADPK, PkA) activates by cAMP and catalyzes transfer of phosphate from ATP to OH of serine or threonine residues of various cellular proteins, altering their activity***.

PkA in the resting state is a complex of 2 catalytic subunits (C) and 2 regulatory subunits (R): R2C2.

Each regulatory subunit (R) of PkA contains a pseudosubstrate sequence, like the substrate domain of a target protein but with Ala substituting for the Ser/Thr. The pseudosubstrate domain of (R), which lacks a hydroxyl that can be phosphorylated, binds to the active site of (C), blocking its activity. When each (R) binds 2 cAMP, a conformational change causes (R) to release (C):

R2C2 + 4 cAMP → R2cAMP4 + 2C

The catalytic subunits can then catalyze phosphorylation of Ser or Thr on target proteins.

Turning off the signal:

1. ***Receptor desensitization*** varies with the hormone. So, the ligand-activated receptor can be phosphorylated on select Ser/Thr residues by GRK (G-coupled receptor kinase, e.g. BARK - β adrenergic receptor kinase). These phosphorylated residues provide a docking site for arrestin that resulting in *inactivation*/*desensitization.* In some instances, arrestin binding targets the receptor for clathrin-dependent endocytosis. β-Arrestin may also bind a cytosolic phosphodiesterase, bringing this enzyme close to where cAMP is being produced, contributing to signal turnoff.

2. Gα hydrolyzes GTP to GDP + Pi (GTPase activity of Gα). ***The presence of GDP on Gα causes it to rebind to the inhibitory βγ complex and Adenylate Cyclase is no longer activated***.

3. ***Phosphodiesterases catalyze hydrolysis of cAMP to AMP***.

4. ***Protein Phosphatase catalyzes removal by hydrolysis of phosphates that were attached to proteins via PkA.*** There exist several families of protein phosphatases. They remove phosphate from amino acid side chains. Members of each subfamily of phosphatases have similar structures but often carry out diverse functions depending upon their association with an array of accessary subunits that regulate enzyme activity and also target catalytic subunits to particular substrates or parts of cells.

Signal amplification is an important feature of signal cascades:

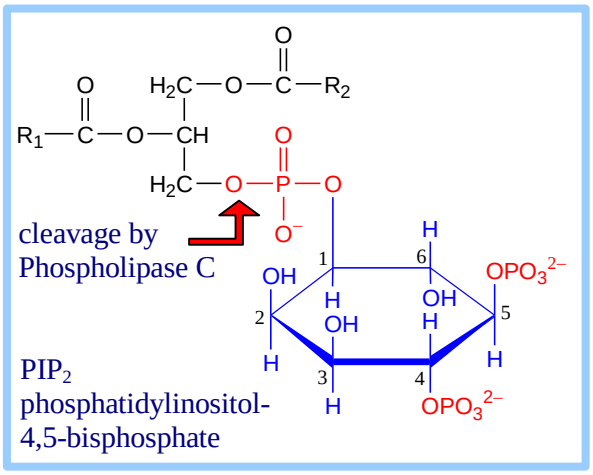
- One hormone molecule can lead to formation of many cAMP molecules.
- Each catalytic subunit of PkA catalyzes phosphorylation of many proteins during the life-time of the cAMP.

1.5. Phosphatidylinositol Signal Cascade

The first stage of this signal cascade is the same of one of Adenylate cyclase cascade. As mentioned above***, hormone binding to GPCR causes a conformational change in the receptor that is transmitted to a G-protein*** (one **Gq-protein in Phosphatidylinositol Signal Cascade).**

Gα subunit of Gq-protein then activates enzyme phospholipase C. It is cytosolic enzyme and act on membrane-inserted phosphoinositide substrates. ***Phospholipase C hydrolyzes phosphatidyl-inositol-bis-phosphate (PI-4,5-P2) to produce second messengers inositide triphosphate (IP3) and diacyl glycerol (DAG).***

There are two forms of Phospholipase C (PLC): β and γ. *PLC- β is activated by G-protein-coupled receptors and Gq-protein, PLC- γ is activated by receptor tyrosine kinases*. Different isoforms of PLC have different regulatory domains, and thus respond to different signals.



PLC-γ binds to activated receptor protein-tyrosine kinases via its SH2 domains (pg. 16). Tyrosine phosphorylation increases PLC-γ activity, stimulating the hydrolysis of PI-4,5-P2.

So, cleavage of PI-4,5-P2, catalyzed by PLC, yields 2 second messengers:

- inositol-1,4,5-trisphosphate (IP3) - hydrophilic compound
- diacylglycerol (DG) - hydrophobic compound

The effect of IP3 is to stimulate Ca^{2+} release from intracellular stores in the ER by activation of Ca^{2+}-released channels in ER membranes. *The IP3 receptor* is a transmembrane protein, probably with two transmembrane domains in the vicinity of the C terminus.

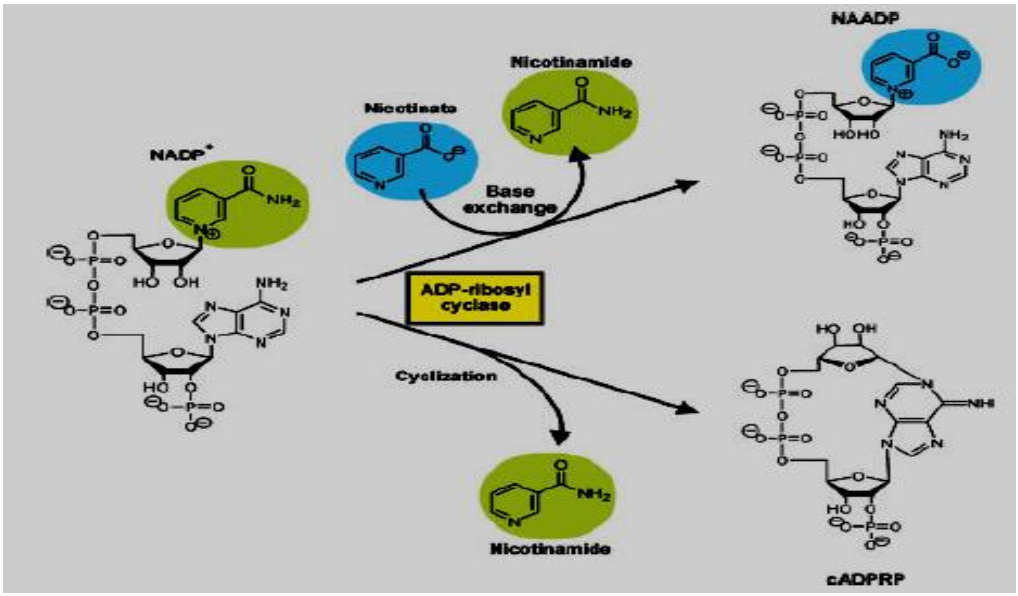
The active receptor is composed of four identical subunits. It is assumed that the Ca^{2+}-channel is formed by the C-terminal transmembrane element and that the binding site for InsP3 is localized in the large cytoplasmic region of the receptor. Opening of the InsP3 receptor is subject to complex regulation involving Ca^{2+}, Mg^{2+} and ATP, in addition to IP3. So, the IP3 gated calcium release channels are regulated by a positive feed back mechanism - calcium binds back to the channels to increase the calcium release.

Signal turn-off includes removal of Ca^{2+} from the cytosol via Ca^{2+}-ATPase pumps and degradation of IP3.

SR has another Ca^{2+}-channels to mobilization of Ca^{2+} from itself - *ryanodin receptors (RR),* which take their name from the plant alkaloid ryanodin – compound, that can stimulate RR. These receptors are ligand-gated Ca^{2+} channels too, have high structural homology with IP3 receptor and are involved in Ca^{2+} signal conduction in many excitatory cells.

In some cell types (including cardiac muscle cells and pancreatic cells), another "second messenger", the **cyclic ADP-ribose** (cADP-ribose), is involved in opening of the ryanodin receptors. The cADP-ribose is formed from NADP by an enzymatic pathway with the help of an *ADP-ribosyl cyclase*. ADP-ribosyl cyclase, in base exchange mode, can catalyze replacement of

the nicotinamide group of NADP (yellow) with nicotinic acid to generate NAADP. ADP-ribosyl cyclase can also catalyze cyclization of NADP to cADPRP.



Ca^{2+} stored in the ER is released to the cytosol, where it may bind calmodulin, or help activate Protein Kinase C.

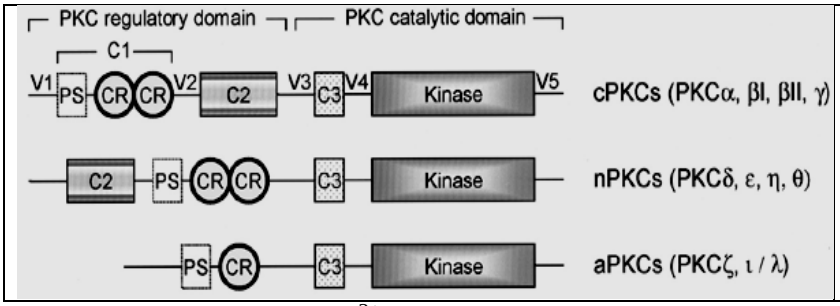
Calmodulin **(**17-kD, 150 a/a) is a member of *EF-hand protein family* (not an enzyme) which serve as a calcium sensor and allosteric activator of target proteins. It has 4 calcium-binding sites (a *helix-loop-helix unit, = EF hand)* and is activated when $[Ca^{2+}]_{cytosol} > 500$ nm.

Calmodulin consists of two globular lobes joined by a long alpha helix. Each lobe contains two EF hands, so there are 4 calcium binding sites in whole molecule. The calcium binding domain - EF-hand - is formed by two helices separated by a loop. Calcium binding loop is composed of residues containing side chain oxygen groups. The EF hand motif (helix-loop-helix structure) is present in more than 100 known proteins, for example, many enzymes (multifunctional calmodulin-dependent protein kinases (CaM Kinase), plasma membrane Ca^{2+}-ATPase pump, adenylyl cyclase, phosphodiesterase, phosphoprotein phosphatases, phospholipase A2, troponin C, etc).

Most Ca^{2+} effects are mediated by the ***calmodulin-dependent serine/threonine protein kinases (CaMKs)***. Examples of CaMKs are myosin light-chain kinase (activates smooth muscle contraction), phosphorylase kinase (activates glycogen breakdown), CaM kinase II (found in all cells but enriched in nervous system - Ca^{2+} influx in neurons activates CaM kinase II to phosphorylate tyrosine hydroxylase, the rate limiting enzyme in catecholamine synthesis, and may play a role in memory and learning).

Another second messenger of Phosphatidylinositol Signal Cascade, DAG, remains in membrane. It has two potential signaling roles: activates protein kinase C (major function) and can be cleaved to release arachidonic acid, which then used to synthesize eicosanoids – compounds, which are messengers of pain and inflammation.

Protein kinase C (PkC) is a family of serine/threonine protein kinases which includes molecules whose activities require stimulation by DAG, phosphatidylserine and Ca^{2+} together. The first type of PkC is the classical PkC, but the other three types have been found by predominantly molecular biological techniques, isolated and characterized.



V1-V5 – variable domains
C1 – phorbol ester and DAG-binding domain
C2 – Ca^{2+}-binding domain
C3 – ATP-binding domain
C4 – substrate binding and kinase domain
C1-C4–conservative domains

Rise in cytosolic Ca^{2+} resulting in PkC translocation from the cytosol to the cytoplasmic face of the plasma membrane where PkC binds to DAG and is activated, after that it activates transcription factors that regulate genes involved in proliferation.

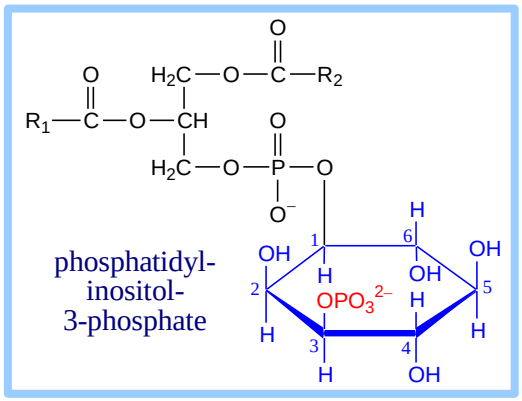
The role and importance of PkC in controlling cell division and proliferation is revealed by the action of *phorbol esters*. Phorbol esters are polycyclic alcohol derivatives that can activate PkC (mimic DAG effects) and induce carcinogenesis.

Turn off of the signal.

1. The lifetime of IP3 in a cell is very short: IP3 is rapidly dephosphorylated and inactivated. ***Sequential dephosphorylation of IP3 by enzyme-catalyzed hydrolysis produces inositol,*** a substrate for synthesis of PI (phosphatidylinositol). After synthesis of PI specific **kinases** convert PI to PI-4,5-P2 (PI-4,5-P2) by transferring Pi from ATP to OH at positions 4 and 5 of the inositol ring.

IP3 also may be phosphorylated via specific kinases to IP4, IP5 or IP6 which have signaling roles. E.g., the IP4 (inositol-1,3,4,5-tetraphosphate) in some cells stimulates Ca^{2+} entry, perhaps by activating plasma membrane Ca^{2+} channels.

Several metabolic pathways lead from phosphatidyl inositol to compounds with "second messenger" character. **One main pathway**, the formation of DAG and IP3 from PI-4,5-P2, has already been described. **Other major compounds *of regulatory importance can be formed by phosphorylation at the 3' position of the inositol part of PI. This reaction is catalyzed by a class of enzymes known as phosphatidyl inositide 3-kinases (PI3K).***



The PI3K phosphorylate various phosphatidyl inositol compounds at the 3' position. The products of this reaction are ***different phosphoinositide derivatives phosphorylated at the 3 position, of which PI-3,4,5-P3 has the greatest regulatory importance***. A substrate for synthesis of PI-3,4,5-P3 is PI-4,5-P2. PI-3,4,5-P3, like cAMP, has the function of a messenger substance that activates effector molecules in the sequence for further signal conduction. So, various signaling proteins which have *PH domains* (Plekstrin homology domains) bind to PI-3,4,5-P3 in cell membrane to promoting their membrane association.

Many observations indicate that PI3K functions as a signal protein that receives signals on the cytoplasmic side of the cell membrane and transmits them further, although its primary role is to produce membrane-localized messenger substances.

PI3K is activated via three pathways: by growth factor receptors (which are receptor tyrosine protein kinases); by direct interaction or via the Ras protein; with uses the βγ-subunits of heterotrimeric G proteins liberated upon activation of G protein-coupled receptors, GPCR.

The SH2 domain of the p85 subunit of PI3K mediates an interaction with tyrosine residues of growth factor receptors. Thus, binding of the PI3K to tyrosine phosphate residues of the activated PDGF receptor is observed. Another binding partner is the insulin receptor substrate (IRS).

In both cases, it is assumed that the binding of the SH2 domain of p85 to the tyrosine residue of the signal protein serves to target the PI3K to its membrane-localized substrate.

The PI3K has been identified as a part of the Ras signaling pathway. Signals originating from transmembrane receptors can be transmitted from the Ras protein to PI3K. In this case, the PI3K acts as the effector molecule of the Ras protein.

Gβγ dimers directly activate the PI3K β and γ subtypes. In this way, a variety of extracellular signals can be transmitted via G protein-coupled receptors and G proteins to PI3-kinase and its effectors.

The product of the PI3K reaction is PI-3,4,5-P3 which binds to PH domains of various signaling proteins promoting their membrane association and activation. So, PI-3,4,5-P3 regulates the activity of a series of protein kinases, including the serine/threonine-specific Akt kinases, protein kinase C enzymes, and the tyrosine-specific Tec kinases.

The first target protein of PI-3,4,5-P3 to be characterized was ***Protein Kinase B (PkB, also called Akt kinase)***. Akt kinase is a serine/threonine-specfic protein kinase which regulates multiple biological processes including glucose metabolism, apoptosis, gene expression, and cellular proliferation. The signaling pathway for Akt kinase illustrates the central role of PI3K and PI-3,4,5-P3 in growth factor controlled signal paths that lead from the cell membrane into the cytosol and the nucleus.

Akt becomes activated when it is recruited from the cytosol to the plasma membrane surface by binding via its PH domain to products of PI3K, e.g., PI-3,4,5-P3. Other kinases at the cytosolic surface of the plasma membrane then catalyze phosphorylation of PkB, activating it. For example, PDK1 (PI-3,4,5-P3-dependent kinase) also has PH domain and binding to PI-3,4,5-P3 too. Membrane translocation of PDK1 causes activation of its kinase activity. Active PDK1 then phosphorylates PkB for its full activation. Activated PkB phosphorylates a variety of target proteins that prevent apoptotic death (Bad) and regulate transcription and other metabolic processes. ***Downstream metabolic effects of PkB include stimulation of glycogen synthesis, stimulation of glycolysis, and inhibition of gluconeogenesis***.

Overall, activation of PI3K stimulates cell growth and proliferation and inhibits apoptosis. A suppressing effect is exerted by the tumor suppressor PTEN which hydrolyzes and thus inactivates PI-3,4,5-P3.

The concentration of PI-3,4,5-P3 in the cell depends both on the rates of synthesis by PI3K and the rates of hydrolysis of its phosphate residues. Several inositol polyphosphate phosphatases have been identified that remove the phosphates at position 3 or 5 of the inositol moiety.

2. ***DAG is rapidly hydrolyzed. This reaction produces free fat acids and glycerol***. ***Arachidonic acid, which released in this process, then used to synthesize eicosanoids – messengers of pain and inflammation. Another pathway of DAG metabolism is its converting to phosphatidic acid by diacylglycerol kinase***.

3***. The calcium that enters the cytosol is rapidly pumped out of the cell by Ca^{2+}-ATPases in ER and plasma membrane.***

4. Ser/Thr phosphatases dephosphorylate PkC and CaM kinase targets.

1.6. Lipid-soluble hormones action

Lipid-soluble hormones (small, hydrophobic steroid and thyroid hormones and the hormonal form of vitamin D) are secreted by glandular cells and released into bloodstream (usually transported via shuttle proteins). After that hormone molecules enter the cytoplasm of cells. In the cytoplasm of target cells, the hormone molecules bind to steroid hormone receptor proteins. The hormone-receptor complexes are translocates into the nucleus, where they activate the transcription of the primary response genes. Transcription of the primary response genes followed by translation, results in the production of primary response proteins. The primary response proteins usually inhibit further transcription of their own genes, and they may activate transcription of secondary response genes.

All nuclear hormone receptors have the short DNA-binding domain, which has 9 cysteine residues. A receptor protein in its inactive state is bound to inhibitory proteins. The binding of ligand to the receptor causes the ligand-binding domain of the receptor to clamp shut around the ligand, the inhibitory proteins to dissociate, and coactivator proteins to bind to the receptor's transcription-activating domain, thereby increasing gene transcription.

1.7. Guanylyl Cyclase Systems

There are three types of guanyl cyclases:

- *membrane bound with one transmembrane segments*, those mechanism analogous to that of the cAMP system;
- *membrane bound with two or more transmembrane segments*, its ligands or mechanism of activation is not yet known;
- *soluble guanylyl cyclases*

The first type is easy. ***Hormone binds to receptor and stimulates the activity of guanylyl cyclase, resulting in an increase in the intracellular concentration of cyclic GMP (cGMP). The cGMP then binds to a cGMP dependent protein kinase (CGDPK, PkG) and activates the catalytic activity, which in turn phosphorylates protein substrates utilizing ATP as the phosphate donor.*** *Turn off signal*: Phosphodiesterase destroys the generate cGMP and there are phosphoprotein phosphatases to dephosphorylate the CGDPK-phosphorylated proteins.

The thirty system is a little bit more complicated since there has to be a way for the hormone· receptor complex to pass the signal to the soluble guanylyl cyclase. This implies the presence of another second messenger, and it turns out that ***this messenger is nitric oxide (NO).***

Guanylyl Cyclases with a Single Transmembrane Segment function as receptors that contain an extracellular ligand-binding domain and various intracellular domains that are required for the ligand-regulated activation of the enzyme. ***As ligands for the guanylyl cyclase receptors, peptides with vasodilatory properties like the atrial natriuretic peptide have been identified. The receptor-type guanylyl cyclases are therefore also termed natriuretic peptide receptors, NPR.***

The receptors exist in a homodimeric transmembrane form, and its ***intracellular guanylyl cyclase domain is activated by peptide binding to the extracellular domains***. A complicated series of reactions follow activation, which include phosphorylation of an intracellular kinase-homology domain, ATP binding and finally activation of cGMP synthesis.

Like cAMP, 3’-5’-cGMP is an intracellular messenger substance. Analogous to cAMP, cGMP is formed by catalysis via guanylyl cyclase from GTP. Although the guanylyl cyclases catalyze a similar reaction as the adenylyl cyclases, the two enzyme classes differ considerably in structure and mechanism of activation.

Soluble Guanylyl Cyclases exist as heterodimers and are regulated by the second messenger NO. A heme group that confers NO-sensitivity is bound at the N-terminus of these

enzymes. NO binding to the heme group results in activation of the guanylyl cyclase activity. The second messenger function of cGMP is directed towards three targets:

* cGMP-dependent protein kinases
* Cation channels
* cAMP-specific phosphodiesterases

Stimulation of the activity of nitric oxide synthetase (NOS) resulting in the production of NO from arginine. NO diffuses to soluble guanylyl cyclase and stimulates its activity resulting in the production of cGMP.

1.8. Test questions

1. ***DAG and IP3 are two important second messengers. How do they interfere with the signaling of Ca^{2+}?***

A) IP3 opens IP3-gated Ca^{2+}-channels in the plasma membrane and DAG activates Ca^{2+}-dependent protein kinase C

B) IP3 opens IP3-gated Ca^{2+}-channels in the ER membrane and DAG activates Ca^{2+}-dependent protein kinase A

C) IP3 opens IP3-gated Ca^{2+}-channels in the ER membrane and DAG activates Ca^{2+}-dependent protein kinase C

D) IP3 blocks the Ca^{2+}-ATPase in the plasma membrane and DAG activates Ca^{2+}-dependent protein kinase C

2. Cholera toxin _____, while pertussis toxin _____:

A) binds to adenylate cyclase-coupled receptors; binds to Ras

B) displaces GTP from α-subunit of Gs protein; displaces GTP from α-subunit of Gi protein

C) ADP ribosylates α-subunit of Gs protein at the GTPase catalytic site; ADP ribosylates α-subunit of Gi protein.

D) covalently modifies α-subunit of Gs protein so it remains with βγ-subunits; covalently modifies α-subunit of Gi protein so it remains with βγ-subunits

E) covalently modifies α-subunit of Gs protein at the βγ-subunit-binding site; alters the ability of Ras to cleave GTP

3. Phorbol esters are:

a) phosphodiesterase activators; b) NOS inhibitors; c) phospholipase C activators; e) protein phosphatase calcineurin inhibitors; f) NADPH-oxidase activators; g) protein kinase A activators; h) protein kinase C activators; i) some forms of adenylate cyclase activators

4. The pathway of PkB activation requires:

a) protein kinase C; b) phospholipase C; c) protein kinase A; d) phosphatidylinositol derivatives phosphorylated at the 3 position, for example, phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P3); e) PI3K; f) phosphodiesterase; j) kinase PDK1

5. What compound is ryanodin receptor activator?

a) cyclic ADP-ribose; b) inositol-1,4,5-trisphosphate; c) ryanodin; d) diacylglycerol; e) cAMP; f) ADP

2. Tyrosine phosphorylation and its regulatory role

There are known near 500 protein kinases in human. Approximately 400 from them are cytosolic serine/threonine kinases (protein kinase A (PkA); protein kinase C (PkC); protein kinase G (PkG); mitogen activated protein kinases (MAPKs); protein kinase B (PkB/Akt); Ca^{2+}, calmoduline-dependent protein kinase (CaMK); phosphatydylinositol-3-kinase (PI3K); phosphatydylinositol dependent kinase-1 (PDK-1)) and approximately from them are tyrosine protein kinases: transmembrane (insuline receptor, receptor of epidermal grows factor (EGFR) etc) and cytoplasmic (Src, Janus kinases etc).

Protein kinases regulate signalling pathways and cellular processes that mediate metabolism, transcription, cell-cycle progression, differentiation, cytoskeleton arrangement and cell movement, apoptosis, intercellular communication, and neuronal and immunological functions.

Regulation of proteins by phosphorylation is one of the most common modes of regulation of protein function, and is often termed "phosphoregulation". In almost all cases of phosphoregulation, the protein switches between a phosphorylated and an unphosphorylated form, and one of these two is an active form, while the other one is inactive, respectively. Behind this in the late 1990s it was recognized that phosphorylation of some proteins causes them to be degraded by the ATP-dependent ubiquitin/proteasome pathway. These target proteins become substrates for particular E3 ubiquitin ligases only when they are phosphorylated.

Phosphorylation of a protein causes it to be bound to other proteins which have "recognition domains" (adaptor domains) for a phosphorylated tyrosine, serine or threonine motif. As a result of binding a particular protein, a distinct signaling system may be activated or inhibited.

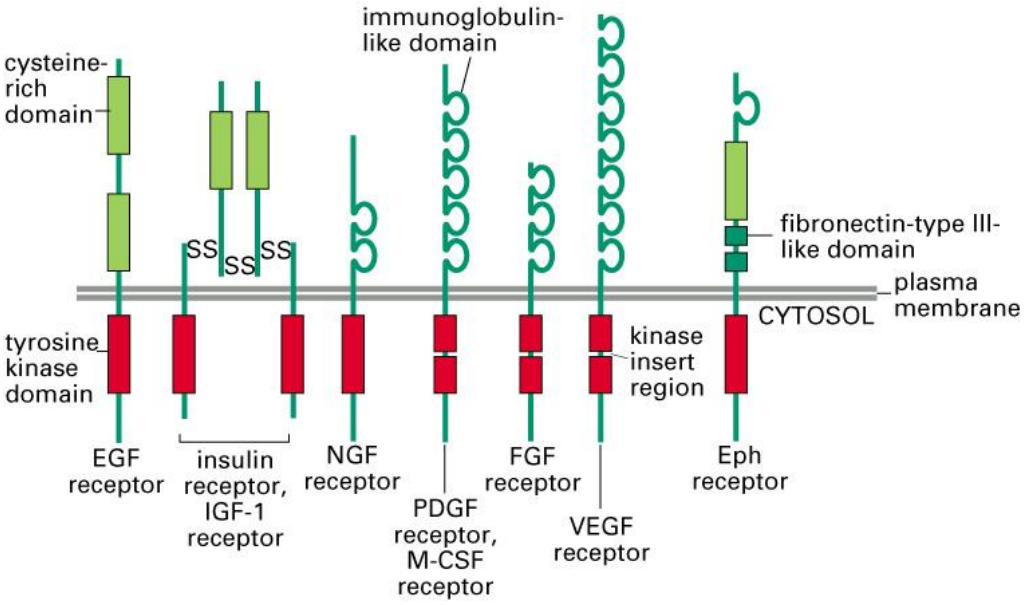
Adaptor domains allow specific interactions with several other specific proteins. **So, pleckstrin homology domains interact with lipids PIP3.** The **SH2** (**S**rc **H**omology 2) **domain** is a structurally conserved protein domain contained within the Src oncoprotein and in many other intracellular signal-transducing proteins. Its presence in a protein helps that protein to bind another protein by recognizing specific phosphorylated tyrosine residues on the other protein.

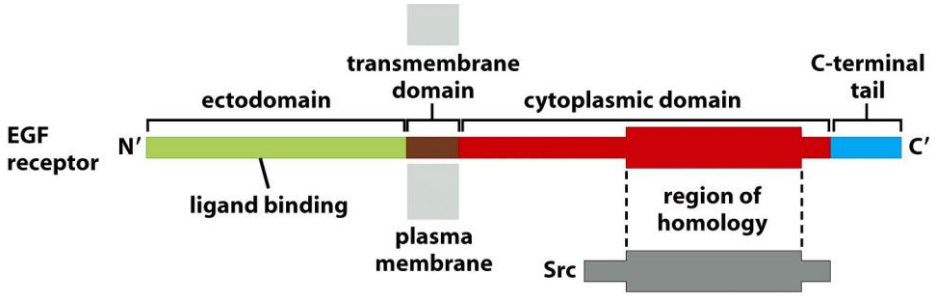
2.1. Tyrosine protein kinases: classification, structure and function in signaling pathways of cell

Protein tyrosine kinases (PTK) catalyze the transfer of the γ-phosphate group from ATP to the hydroxyl group of tyrosine residues, whereas protein tyrosine phosphatases (*PTP)* remove the phosphate group from phosphotyrosine.

There are two classes of PTKs: ***receptor tyrosine protein kinases*** **(RTKs,** transmembrane protein kinases) and ***nonreceptor tyrosine protein kinases*** **(NRTKs,** localisated in cytosol, not bind to cell membrane).

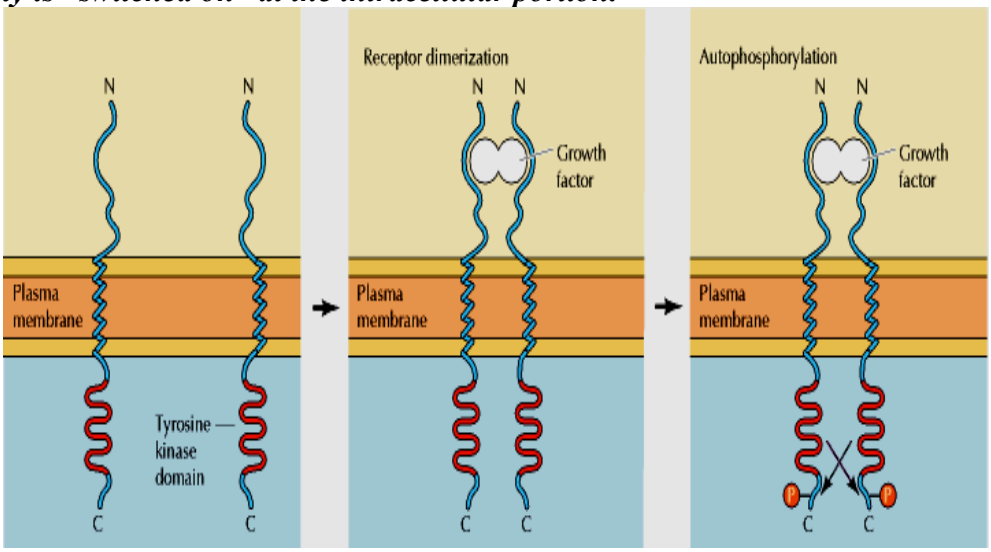
Receptor protein-tyrosine kinases transmit signals across the plasma membrane, from the cell exterior to the cytoplasm.





Perhaps the most noticeable thing about the tyrosine protein kinase system is that there are no second messengers. The system also uses protein kinase activity to transmit and amplify signals intracellularly, however the kinase is part of receptor.

The binding of ligand by the receptor results in receptor dimerization and the activation of the tyrosine protein kinase activity. The ligands for some RTKs, such as the receptor for EGF, are monomeric; ***ligand binding induces a conformational change in receptor monomers that promotes their dimerization.*** The ligands for other RTKs are dimeric; their binding brings two receptor monomers together directly. In either case, ***upon ligand binding, a tyrosine kinase activity is "switched on" at the intracellular portion.***



The kinase activity of each subunit of the dimeric receptor initially phosphorylates tyrosine residues near the catalytic site in the other subunit. So tyrosine protein kinase activity

cross-auto-phosphorylates the RTK complex and phosphorylates tyrosine residues in substrate proteins, acting in a classical protein kinase stimulatory pathway, thus altering the primary structure and activity of the target proteins.

Each tyrosine-phosphorylation site has a unique sequence for interaction with specific signaling molecules containing SH2 or phosphotyrosine-binding (PTB) domains. Subsequently, ***autophosphorylated tyrosine residues in cytosolic domain RTK are the targets to bind SH2-domens of other proteins, which can to be phosphorylated by RTK. Protein phosphorylation leads to altered cell function via the assembly of other signal proteins.***

RTKs can induce 4 important signaling cascades: Ras-, PLCγ-, PI3K- and JAK/STAT – including systems. Classical examples of RTK-dependent cascades are insulin and cytokines signaling.

Inactive insulin receptor consists of α- and β-chain linked by a disulfide bond (monomer unit). Active insulin receptor is dimer of two identical units linked by a disulfide bond too. After insulin binding on the outside of the cell, membrane-associated kinase within the cell is activated and cross-phosphorylationed***.***

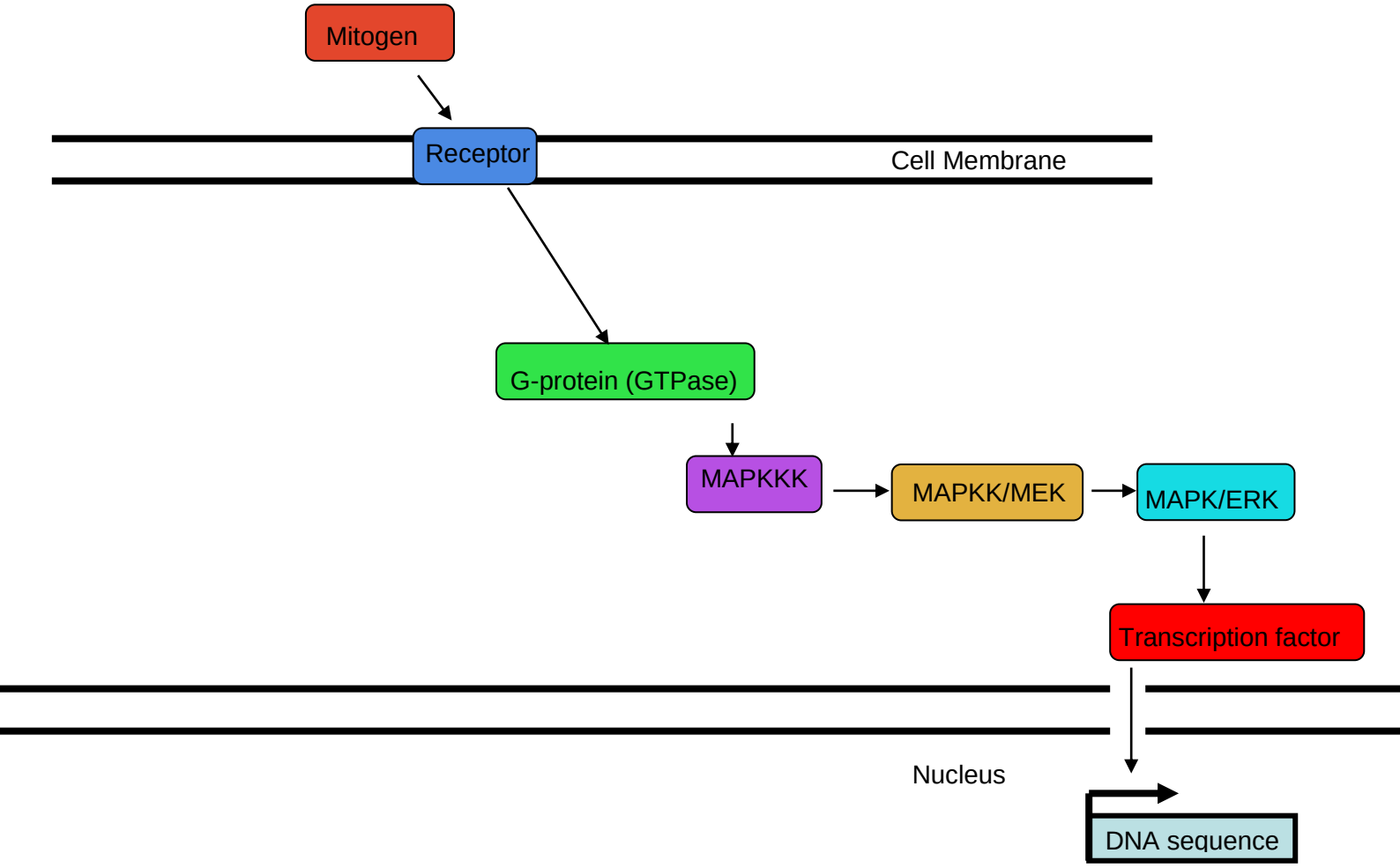
The one of Insulin Receptor Cascades inserts protein Ras and ERK-family of MAPKs. *ras* gene originally discovered as a viral oncogene but also is part of the normal genome. Many tumor cells contain a mutant *ras.* Protein product Ras is a small G-protein that localized at inner surface of plasma membrane and forms an important link between RTK and nucleus via ERK-family *of mitogen activated protein kinases* (MAPKs).

Mitogen - a compound that encourages a cell to commence division, triggering mitosis. Cell division requires the regulation of specific genes (including migration of cell-division specific proteins to key areas of the cell, manipulation of the cell cycle, and at various stages, growth of cell; ie: DNA synthesis, accumulation of energy for division).

The Mitogen-Activated Protein Kinases Pathway has evolved to regulate numerous transcriptional events in practically every eukaryotic organism. The MAPKs Pathway controls everything from cell growth, to cell division, to inducing mating in haploid yeast.

There are 3 types of Map kinases: ***c-Jun NH2-terminal kinases (JNKs)*** - it phosphorylates Jun (in AP-1) and is proapoptotic; ***p38 MAPK***- regulates cell death too (is proapoptotic) and controles inflammatory cytokine expression (may be important in arthritis); ***extracellular signal-related kinases (ERKs)*** **–** can be activated by growth factors and are crucial in cell division, memory and learning (abnormal ERKs may lead to Alzheimers and oncology).

The Mitogen-Activated Protein Kinases cascade exist of three kinase modules: MAPKKK (kinase kinase MAPK), MAPKK (kinase MAPK), MAPK. MAPKKK is activated by small G-protein (Ras, Rho, etc) and MAPK activates transcriptional factors (NF-kB, c-jun, c-fos etc).



Extracellular signal-regulated kinase (ERK) family acts via either of two pathways: RTKs such as the insulin cascade and also via G-protein coupled receptors. ERK activation is coupling to small G-protein Ras and a protein serine/threonine kinase Raf (which is MAPKKK).

Ras is tethered to the membrane by a farnesyl anchor. Its activation is mediated by *guanine nucleotide exchange factors*. A well characterized GEF is Sos, which in inactive form is bound to Grb2. Grb2 is an adaptor protein which has no other intrinsic activity and has SH2 domain with high affinity for phosphorylated tyrosine residues in protein targets (e.g. on IRS). Sos is localized in cytosol and Sos-Grb2 complex is localized to the membrane via Ras. Sos stimulates release of bound GDP and its exchange for GTP. Activation of Ras-GTP is terminated by GTP hydrolysis. GTPase-activating proteins – GAPs - interact and accelerate GTP hydrolysis.

Activated Ras binds to the N-terminal domain of Raf kinase, a serine/threonine kinase (which is MAPKKK in this cascade). Raf then phosphorylates *MAPK/ERK kinase* known as MEK (MAPKK in this cascade). MEK has dual specificity – phosphorylates threonine and tyrosine residues in target MAPK – for example, in ERK2 residues Thr 183 and Tyr 185. Phosphorylated ERK (MAPK in this cascade) then translocates to the nucleus and regulates transcription factors (e.g. NF-kB) by its phosphorylation.

The Insulin Receptor Cascades: PLCγ

The autophosphorylation of RTK provides a surface for a number of proteins with SH2-domain to bind to autophosphorylated RTK. When bound to the membrane surface by RTK, these proteins can catalyze a number of reactions.

Among these proteins is *phospholipase Cγ,* which like other phospholipase - phospholipase Cβ - catalyzes the hydrolysis of PI-4,5-P2 to yield IP3 and DAG. Therefore, ***the RTK system is capable of stimulating the second messengers and subsequent pathways of the IP3/DAG system***.

The Insulin Receptor Cascades: IRS-1, PI3K, PDK and PkB/Akt

IRS-1 (insulin-receptor substrate) binds to autophosphorylated RTK that phosphorylates it. IRS1/IRS2 act as adaptor proteins: N-terminal of this molecule has PH domain, which binds to phosphoinositide lipids, SH2 domain, which binds to autophosphorylated RTK and Tyr-X-X-M sequence that is phosphorylated by the receptor tyrosine kinase.

Phosphoinositide-3-kinase is a lipid kinase that binds to phosphorylated IRS-1 and catalyzes the phosphorylation with ATP as the phosphate donor, of the 3'-OH of inositol residues in phospholipids. PI3K has 2 subunits - 110 kD catalytic subunit and 85 kD regulatory subunit – and containing a SH2 domain, that recognize the phosphotyrosine residues in the IRS.

PI3K generates a family of 3'-O-P inositides which act to stimulate PDK-1. PDK-1 phosphorylates and activates the PkB (Akt). The activate PkB then phosphorylates various substrate proteins, altering their primary structure and thus their function, for example, glucose transporters (GLUT4) that stimulate glycogen synthesis.

Cytokines & JAK-STAT System

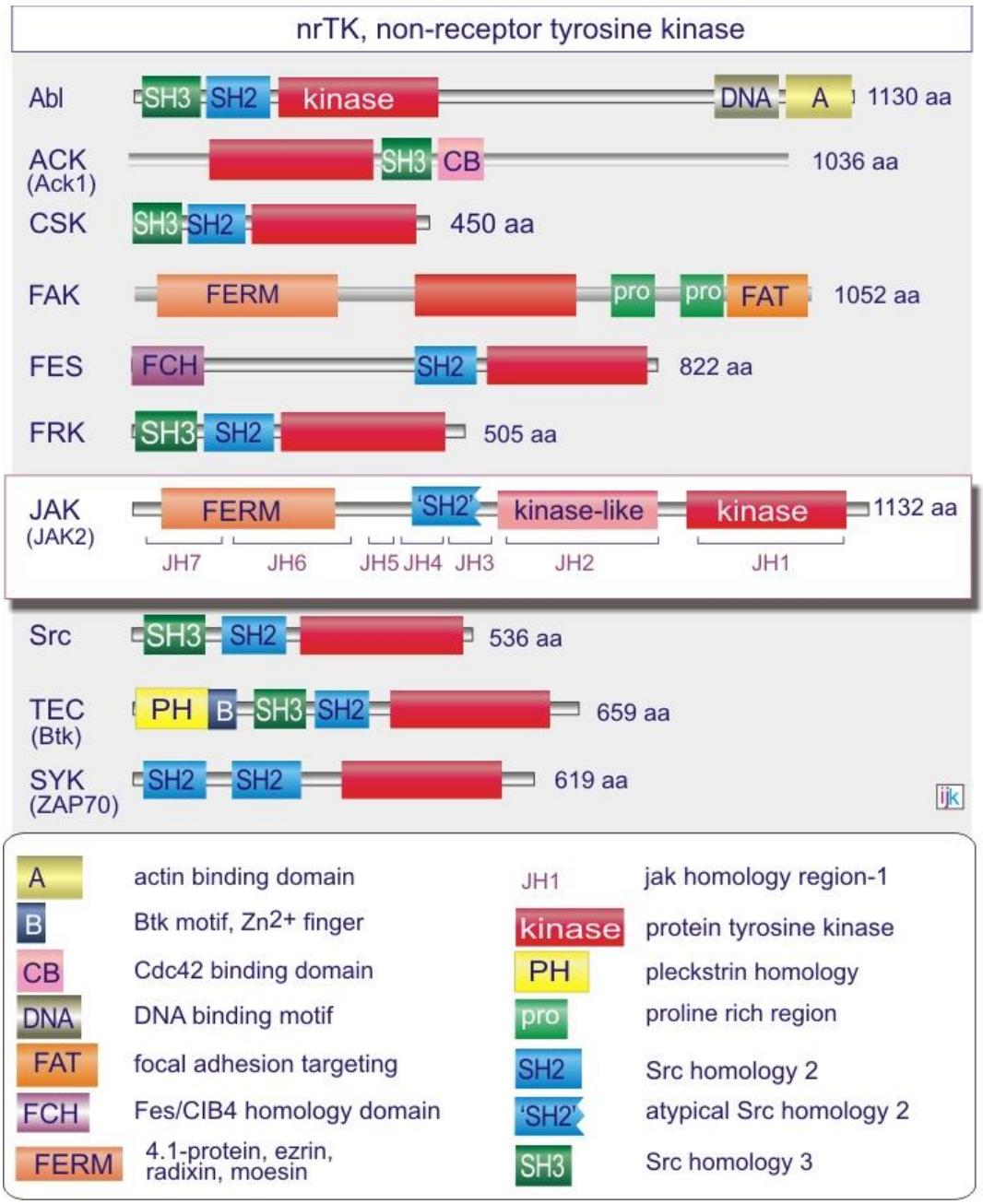
The **Ja**nus **K**inases (JAKs) / **S**ignal **T**ransduction **A**ctivators of **T**ranscription (STATs) system is somewhat similar to the RTK-MAPKs-system.

JAKs are receptor-associated JAK cytosolic tyrosine protein kinases. The binding of ligand causes the RTK to dimerize and its autophosporylation***. JAKs have SH2 domain and bind to the dimerized autophosphorylated receptor and in turn phosphorylated and activated by it.***

The STATs (signal transducers and activators of transcription) proteins are transcription factors that contain SH2 domains that mediate their binding to phosphotyrosine-containing sequences.

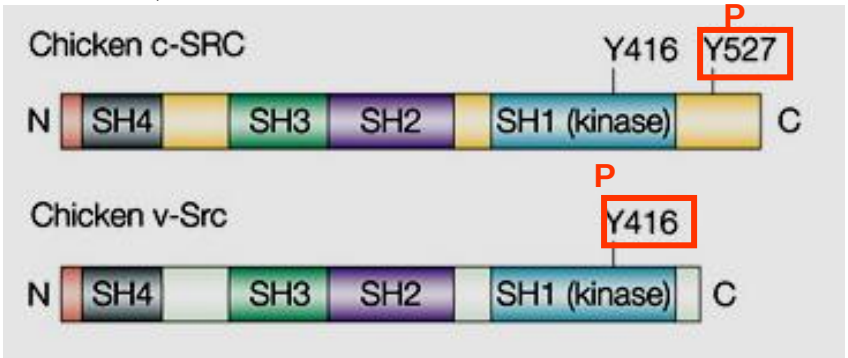
In unstimulated cells, STAT proteins are inactive in the cytosol. ***Stimulation and autophosphorylation of cytokine receptors leads to the binding of STAT proteins, where they are phosphorylated by the JAKs. The phosphorylated STAT proteins then dimerize and translocate to the nucleus, where they activate the transcription of target genes.***

Nonreceptor tyrosine protein kinase (NRTK) families have been associated with malignancies in humans and/or animal models. Most of them contain SH2 and SH3 domains. Classical example of NRTKs is Src.



Nonreceptor protein tyrosine kinase Src is encoded by the gene *src*. The Src protein has three major domains, SH2, SH3, and the kinase catalytic domain (or SH1). SH2 and SH3 both play a part in protein-protein interactions, while the kinase catalytic domain contains the kinase active site.

Src can be switched from an inactive to an active state through control of its phosphorylation state, or through protein interactions. ***There are two major phosphorylation sites on Src: one is at Tyr416 (or Y416), the other at Tyr527, as marked in the drawing above for chicken c-Src).***



Kinase Activity

Weak

Strong

So, c-Src is auto-inhibited by SH2 through p-Tyr (Y527 on C-termines). v-Src hasn't p-Tyr (Y527) on C-termines and is constitutively active Tyr kinase.

Tyr416 can be auto-phosphorylated, which activates Src by displacing the P-Tyr416 from the binding pocket, allowing the substrate to gain access. A more critical site is Tyr527, which can be phosphorylated and dephosphorylated by various proteins, such as CSK kinase (*phosphorylates*), or SHP-1 phosphorylase (*dephosphorylates*).

Phosphorylation of Tyr527 inactivates Src through the interaction of P-Tyr527 with the SH2 domain, which effectively folds Src up into a closed, inaccessible bundle. Dephosphorylation of Tyr527 releases this bond, opening up the molecule to an active state. Interaction between SH3 domain and Polyproline region in Src molecule also can inhibite its activity.

Protein interactions also act to regulate Src by either directly activating Src, or by moving Src to sites of action.

Receptor tyrosine protein kinases are able to bind to the SH2 domain, causing Src to open up into the active form.

Src proteins are targeted to the plasma membrane by covalently attached lipids. Many growth factors bind to and activate specific receptor tyrosine kinases. Activated RTKs activates Src by releasing catality domain (SH2 domain of Src disconnected from p-Tyr (Y527) on C-termines and connecte with autophosphorylated Tyr on receptor cytozolic part) and phosphorylates Src.

Src is involved in the control of many functions, including cell adhesion, growth, movement and differentiation. Src is widely expressed in many cell types, and can have different locations within a cell. It appears that the subcellular location of Src can affect its function. Src can associate with cellular membranes, such as the plasma membrane, the perinuclear membrane and the endosomal membrane. So, at the plasma membrane, Src can transduce signals from a variety of receptors to internal signalling pathways that convey these signals to the nucleus, cytoskeleton and other cellular components. For example, Src can act through the growth factor receptors to affect cell growth and proliferation. Within the nucleus, Src is thought to help regulate the cell cycle and cell division by its interactions with other proteins. Src can also be found in the cytoplasm, and between cells at adherens junctions, where it takes on different roles.

Overexpression or high activation of Src occurs frequently in tumor tissues and they are central mediators in multiple signaling pathways that are important in oncogenesis. Src can interact with tyrosine kinase receptors, such as EGFR and the VEGF receptor. Src can affect cell proliferation via the Ras/ERK/MAPK pathway and can regulate gene expression via transcription factors such as STAT molecules. Src can also affect cell adhesion and migration via interaction with integrins, actins, GTPase-activating proteins, scaffold proteins, such as p130(CAS) and paxillin, and kinases such as focal adhesion kinases. Src can regulate angiogenesis via gene expression of angiogenic growth factors, such as fibroblast growth factor, VEGF, and interleukin 8.

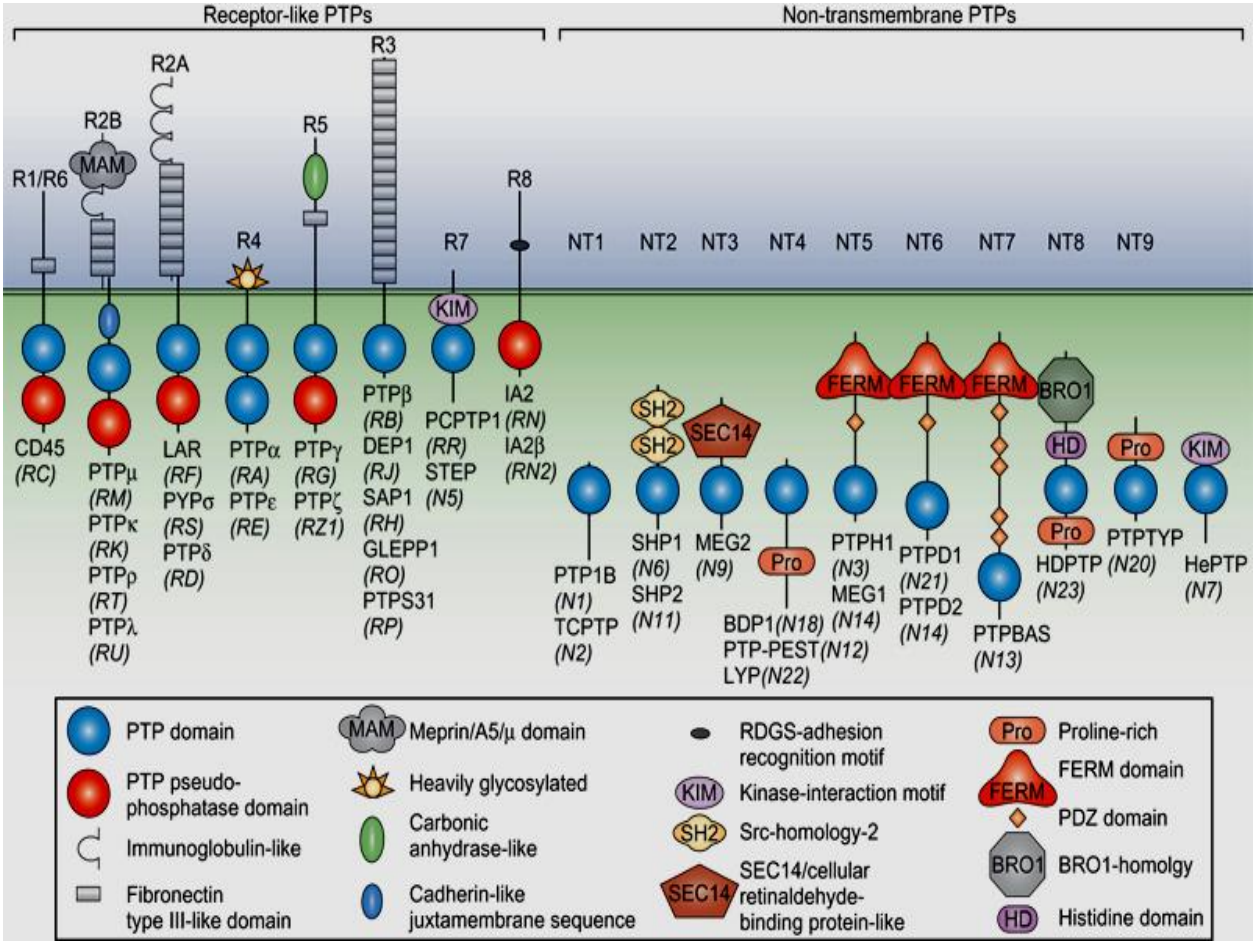
On the basis of these important findings, small-molecule Src-inhibitors have been developed and are undergoing early phase clinical testing. In preclinical studies these agents can suppress tumor growth and metastases. The agents seem to be safe in humans and could add to the therapeutic arsenal against subsets of cancers.

2.2. Protein tyrosine Phosphatases (PTPs)

Protein tyrosine phosphatases (PTPs) regulate of cell proliferation by reversing the action of protein tyrosine kinases.

There are two groups of PTPs: <u>"Classical" PTPs</u> (hydrolyze phosphotyrosine and divided into receptor protein tyrosine phosphatases (RPTPs) and nonreceptor protein tyrosine phosphatases (NRPTPs)) and DSPs (<u>PTPs dual specifity</u> – hydrolyze phosphotyrosine and

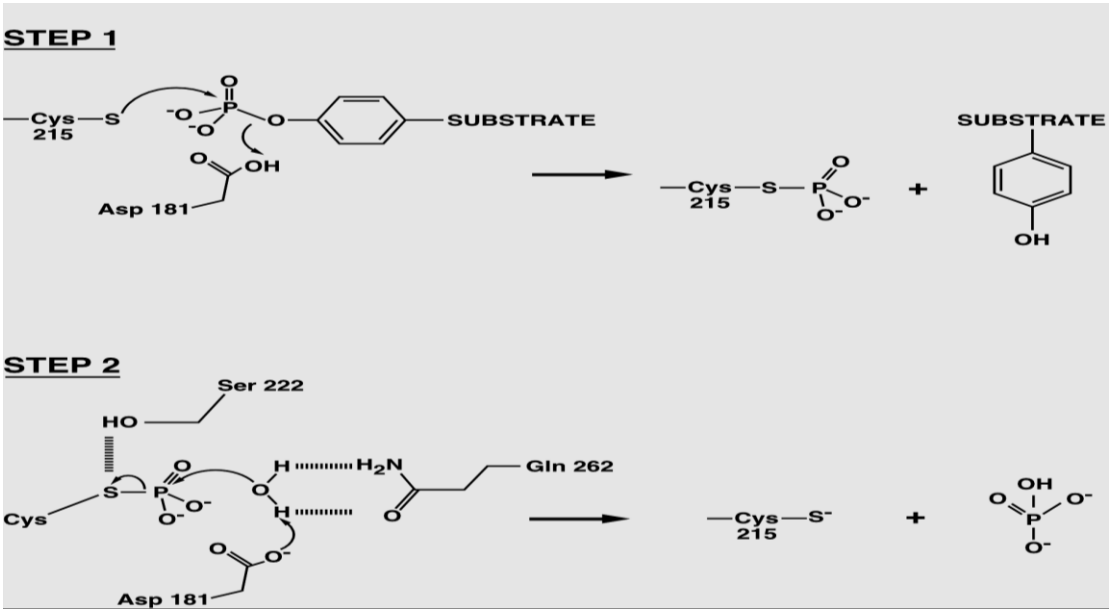
phosphoserine/phosphothreonine). Individual PTPs are designated by a name that is commonly used in the literature. Where necessary, the gene symbol is given in parentheses.



RPTPs are composed of an *extracellular domain*, a *transmembrane domain* and an *intracellular domain* with one or two catalytic domains (~240-250 amino acids). NRPTPs contain a *catalytic domain* and *various regulatory domains*.

For both RPTPs and NRPTPs, catalytic domain is characterized by the presence of a signature motif (H-C-X-X-G-X-X-R) and non-catalytic domains contain motifs that serve a structural or regulatory function as well as help to define various subfamilies based on sequence similarity.

Cysteine in signature motif executes nucleophilic attack on phosphate group in the substrate and invariant asparagine acid residue plays critical role in catalysis:



Structrual features inspired the development of "substrate-trapping" mutant forms of PTPs (Asp → Ala and Cys → Ser), which retain their ability to bind substrates but display impaired catalytic activity.

En example of "classical" PTP is PTP1B. Its substrates include the EGF receptor (EGFR) and the insulin receptor (IR). PTP1B action increases IR auto-phosphorylation, enhances sensitivity to insulin in skeletal muscle and liver, shows protection from diet-induced obesity and normal in all other ways tested. Together, PTP1B acts as a major negative regulator of insulin signaling and small-molecule inhibitors of PTP1B might be an effective anti-diabetes/obesity agent.

2.3. Test questions

1. The binding of hormone by the receptor tyrosine protein kinase results in receptor _____ and _____ of the tyrosine protein kinase activity.

(- monomerization; dimerization; endocytosis; nuclear translocation;
- activation/inhibition)

2. Which of Phospholipase C isoform is activated by receptor tyrosine protein kinase?

a) Phospholipase Cα; b) Phospholipase Cβ; c) Phospholipase Cγ; d) Phospholipase Cδ

3. SH_2 domains specifically bind to:

A. phosphorylated serine residues
B. phosphorylated tyrosine residues
C. GDP
D. Ca^{2+}

4. ***Which of these Protein kinases are tyrosine Protein kinases?***

a) Protein kinase A; b) Protein kinase C; c) Ras; d) Src; e) insuline receptor; f) adrenaline receptor; g) Jac; h) CD45; i) STATs

5. What type of protein is Ras?

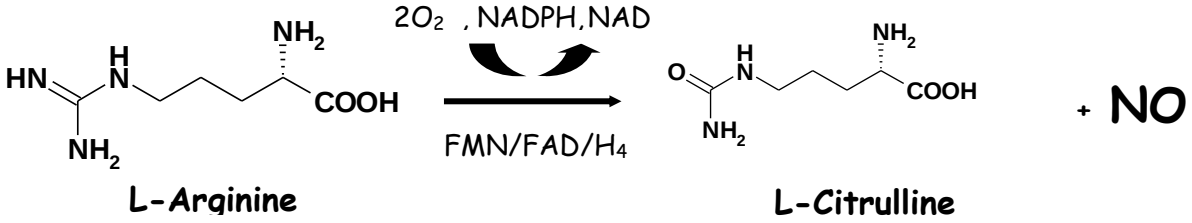
A) A tyrosine kinase
B) A serine-threonine kinase
C) A small monomeric GTPase switch protein
D) A G protein switch

3. Nitric oxide as a signaling molecule

NO is a radical that is water soluble and can cross membranes fairly freely by diffusion. Because of its radical nature, NO has a lifetime in aqueous solution of only 4 s. Important reaction partners of NO in biological systems are oxygen O_2, the $O_2^{\bullet-}$–radical and transition metals in free or complex form, e. g. Fe^{2+} in heme. NO also readily reacts with nucleophilic centers in peptides and proteins, in particular with the SH groups of Cys residues.

3.1. NO formation: nitric oxide synthases.

NO is formed enzymatically from arginine with the help of NO synthase, producing citrulline. Citrulline and arginine are intermediates of the urea cycle, and arginine can be regenerated from citrulline by urea cycle enzymes.



Nitric Oxide Synthase is a homodimer. Each subunit consists of reductase domain (it has places for binding NADPH, FMN, FAD), calmodulin binding cite and oxygenase domain (with places for binding O_2, arginine, haem, BH4).

So**,** NOS requires five cofactors: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH4) and calmodulin.

The electron flow in the NO synthase reaction is:

NADPH →FAD → FMN → heme → O_2

Tetrahydrobiopterin provides an additional electron during the catalytic cycle which is replaced during turnover.

There are three NOS isomorms in mammalian cells: neuronal NOS (nNOS), endothelial (macrofagal) NOS (eNOS) and induced NOS (iNOS).

Neuronal NOS produces NO in nervous tissue in both the central and peripheral nervous system and involved in long term memory, stroke. The gene coding for nNOS is located on Chromosome 12. This NOS isoform is constitutively expressed and Ca^{2+} dependent. Neuronal NOS is associated with plasma membranes by an additional N-terminal domain, the PDZ domain, which binds to cell cytoskeleton. The subcellular localisation of nNOS in skeletal muscle is mediated by anchoring of nNOS to dystrophin.

Endothelial NOS (eNOS) generates NO in blood vessels and is involved in regulating vascular function, for example, in vasodilatation - inhibition eNOS causes increased blood pressure. Gene encoding for eNOS is located on Chromosome 7. This NOS isoform is constitutively expressed and Ca^{2+} dependent too. In cell it is associated with plasma membranes surrounding cells (in caveolaes) and the membranes of Golgi bodies within cells. eNOS localisation to endothelial membranes is mediated by cotranslational N-terminal myristoylation and post-translational palmitoylation.

Inducible NOS (iNOS) as opposed to the critical calcium-dependent regulation of constitutive NOS enzymes (nNOS and eNOS) has been described as calcium-insensitive, likely due to its tight non-covalent interaction with calmodulin and Ca^{2+}. The gene coding for iNOS is located on Chromosome 17. The most famous inductor of inducible NOS is bacterial LPS (lipopolysaccharides), which acts throw IRF1 and NF-κB and causes inflammation and pain.

Induction of the high-output iNOS usually occurs in an oxidative environment, and thus high levels of NO have the opportunity to react with superoxide leading to peroxynitrite formation and cell toxicity. These properties may define the roles of iNOS in

host immunity, enabling its participation in anti-microbial and anti-tumor activities as part of the oxidative burst of macrophages.

3.2. Chemical Biology of Nitric Oxide.

The main effects of NO are following:

- NO directly inhibits respiration and P450 but stimulates guanylyl cyclase and scavenges radicals;
- NO indirectly forms N_2O_3 leading to ***nitrosation (SH-group of Cysteine)***;
- NO is converted to $ONOO^-$ and NO_2 leading to oxidation and ***nitration (OH-group of Tyrozine).***

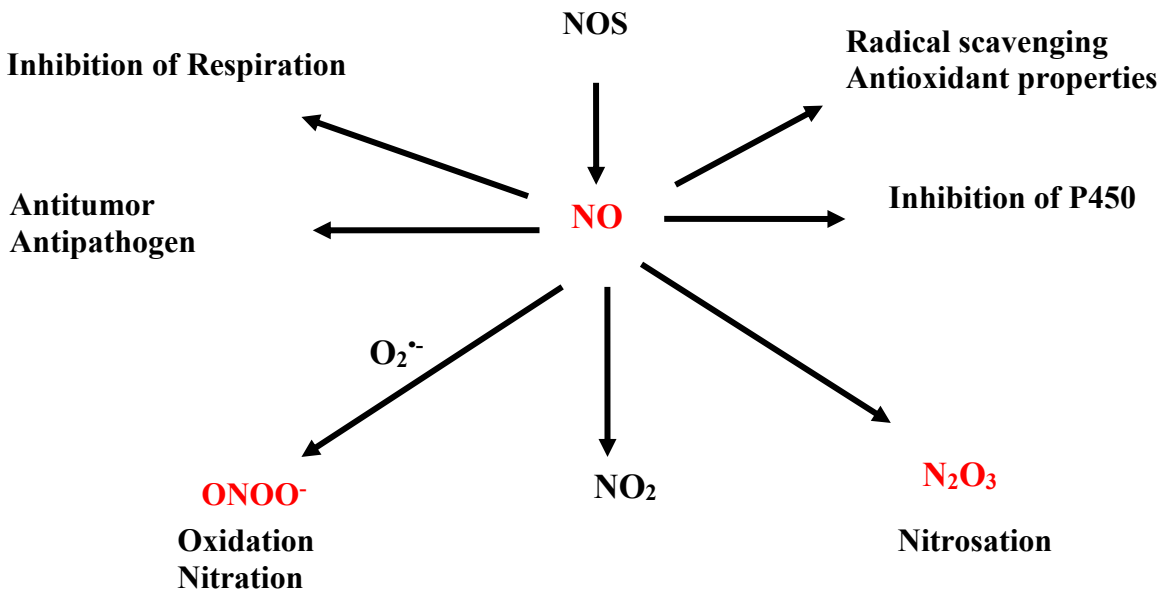
NO represents these different effects because it has three redox forms: the nitrosonium anion (NO^+), the free radical ($NO^\bullet$) and the nitroxyl anion (NO^-) (Stamler et al., 1992). These three forms have different physiological functions, including a differential ability to stimulate generation of the classic second messenger cGMP.

Some reports have suggested that **$NO^\bullet$** is the only redox form to stimulate soluble guanylate cyclase. $NO^\bullet$ also reacts with anion of superoxide, which draws formations of reactive nitrous intermediate products as *peroxynitrite anion*, that then attracted to reaction of nitration (ex. Tyrosine nitration). NO in form $NO^\bullet$ it is high-reactive in relation to cations of transitional metals, and that is why able to contact with iron of hemoproteins (hemoglobin, myoglobin, guanilatcyclase, catalase, cytochromes) and Fe-containing proteins that haven't heme structure, in thereby and Fe-S-centers, here changing valency of it cation ($Fe(2+) \rightarrow Fe(3+)$). Examples of proteins with Fe-S-centers: NADH:ubichinon-oxydoreductase (complex I) and cuccinat:ubichinon-oxydoreductase (complex II) of respiratory chain of mitochondria and akonitaze Cycle Krebs reactions.

Nitroxyl anion reacts with molecular oxygen to form peroxynitrite anion:

$$NO^- + O_2 \rightarrow ONOO^-$$

NO in NO^+ form is attracted to reaction of nitrosation (=nitrosylation, ex. nitrosylation of Cysteine SH-groups).



3.3 Physiological Functions and Attack Points of NO

When NO is produced in excess amounts and in a less than regulated fashion, nonspecific reactions with various cell constituents including proteins, lipids and DNA are observed. This situation has been termed *nitrosative stress* in analogy to *oxidative stress* caused by the generation of reactive oxygen species, ROS. Nitration, nitrosation and oxidation of proteins, lipids and DNA can occur under these conditions and can lead to damage of cellular functions and eventually to cell death.

NO produced in a regulated way by enzymatic synthesis is involved in the control of a wide array of cellular functions including relaxation of blood vessels, neurotransmission, cellular immune response and apoptosis.

Because of its high reactivity, NO can interact and react with many effector proteins.

The first cellular target of NO to be identified was a specific isoform of guanylyl cyclase: stimulation of NOS leads to activation of a cytoplasmic NO-sensitive guanylyl cyclase. This activation is achieved by binding of NO to a heme group of the enzyme. The associated increase in the cGMP level has multiple consequences.

The cGMP can stimulate cGMP-dependent protein kinases (PkG); it can also open cGMP controlled ion channels. As a consequence, an increase in the intracellular Ca^{2+} concentration takes place and a Ca^{2+} signal is produced. NO can influence both protein phosphorylation and IP3/diacylglycerol and Ca^{2+} metabolism by this mechanism and activate a broad palette of biochemical reactions in the cell.

Reaction of nitrosation and nitrosothiols (R-S-NO)

The addition of a nitroso group to a sulfur atom of an amino acid residue of some protein is known as ***S-nitrosation*** or ***S-nitrosylation*.**

NO readily reacts with physiological thiols to produce S-nitrosothiols:

$$RS^{\cdot} + {}^{\cdot}NO \rightarrow R\text{-}S\text{-}NO$$

$$R\text{-}S\text{-}NO + R'\text{-}SH \rightarrow R\text{-}SH + R'\text{-}S\text{-}NO \text{ (transnitrosation)}$$

S-nitrosothiols - organic compounds or functional groups containing a nitroso group attached to the sulfur atom of a thiol. S-nitrosothiols have the general formula R-S-NO, where R denotes an organic group.

S-nitrosothiols release NO^+ upon treatment with acids:

$$R\text{-}S\text{-}NO + H^+ \rightarrow R\text{-}SH + NO^+$$

or release NO with thioldisulfides:

$$2R\text{-}S\text{-}NO \rightarrow R\text{-}S\text{-}S\text{-}R + 2NO$$

The degradation of S-nitrosothiols with release NO is believed to occur both enzymatically and nonenzymatically. *Nonenzymatic decomposition* is known to be influenced by factors such as heat, UV light, certain metal ions, superoxide anion, ascorbic acid and thiols, where as *enzymatic decomposition* can be influenced by the enzymes glutathione peroxidase, γ-glutamyl transpeptidase and xanthine oxidase.

Physiological roles of nitrosothiols is that these compounds (for ex., *S*-nitrosoglutathione (GSNO), *S*-nitrosoalbumin (AlbSNO), *S*-nitrosohemoglobin (HbSNO)) are potent vasorelaxants, have antiplatelet and antimicrobial activity, take part in regulation of vasodilation/oxygenation (hemoglobin), and can cause enzymes inhibition/activation.

Nitrated proteins (R-Tyr-NO_2)

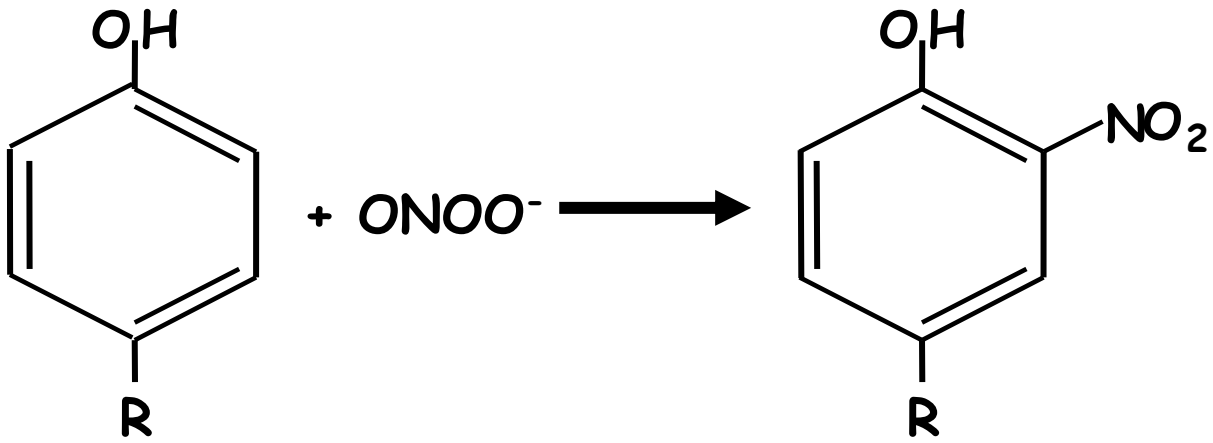
NO in different redox forms can reacts with anion of superoxide or with oxygen forming reactive nitrous intermediate products as *peroxynitrite anion* that then attracted to reaction of nitration.

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^-$$

$$NO^- + O_2 \rightarrow ONOO^-$$

Nitration of a single tyrosine residue in proteins is not reversible and is sufficient to accelerate the degradation of these proteins **(R-Tyr-NO_2)** by the proteasome. 75% of specific proteins modified by nitration have been detected in more than 50 human disorders including atherosclerosis, myocardial infarction, myocarditis, heart failure, shock, diabetic complication and neurodegenerative and inflammatory disorders. Approximately 80 key proteins have been reported to be modified by peroxynitrite with potential relevance to various human diseases.

Peroxynitrite may also be an important trigger of cell death, both apoptotic and necrotic.



Nitric Oxide in the Nervous System

NO is a signaling molecule, but is Nitric Oxide a "neurotransmitter?"

NO serves in the body as a neurotransmitter, but it has differences compared to other neurotransmitters used commonly in the body. NO synthesis mechanism in the nervous system involving Ca^{2+}/Calmodulin activated nNOS, diffuses out of the cells making it witout storage in vesicles and release by exocytosis. NO does not bind to surface receptors, but instead exits cytoplasm, enters the target cell, and binds with intracellular guanylyl cyclase. It can also travels from postsynaptic neuron back to presynaptic neuron which activates guanylyl cyclase, the enzyme that catalyzes cGMP production. This starts a cycle of nerve action potentials driven by NO.

NO also has some similarities to normal neurotransmitters, for example, it is present in presynaptic terminal and natural removal from synaptic junction.

Nitric oxide is believed to play a role in long term memory being a retrograde messenger that facilitates long term potentiation of neurons (memory). Behind this NO can cause development of ***excitotoxicity*** - the pathological process by which nerve cells are damaged and killed by excessive stimulation by neurotransmitters such as glutamate and similar substances. This occurs when receptors for the excitatory neurotransmitter glutamate such as the NMDA receptor are overactivated. Excitotoxins like NMDA bind to these receptors, as well as pathologically high levels of glutamate, and can cause excitotoxicity by allowing high levels of calcium ions to enter the cell. Ca^{2+} influx into cells activates a number of enzymes: phospholipases, endonucleases, and proteases such as calpain. These enzymes go on to damage cell structures such as components of the cytoskeleton, membrane, and DNA. Ca^{2+} influx into cells also activates nNOS, which produce NO. NO can transformates to peroxynitrite, which damages cell structures.

Excitotoxicity may be involved in spinal cord injury, stroke, traumatic brain injury, hearing loss and in neurodegenerative diseases of the central nervous system (multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, alcoholism or alcohol withdrawal, and Huntington's disease). Other common conditions that cause excessive glutamate concentrations around neurons are hypoglycemia and *epilepsy.*

Nitric Oxide as a vasodilator in the Circulatory and Muscular Systems

In the circulatory system NO prevents the formation of thrombi and serves as a vasodilator (it was originally called *EDRF - endothelium derived relaxation factor*) that released in response to high blood flow rate and signaling molecules (acetylcholine and bradykinin). NO also aids in gas exchange between hemoglobin and cells. In blood NO binds to hem of hemoglobin which so is a vasoconstrictor. NO is protected by cysteine group of this hemoprotein, when O_2 binds to hemoglobin. During O_2 delivery, NO locally dilates blood vessels to aid in gas exchange.

NO-dependent signals inhibit of smooth muscle contraction by eNOS activation in vascular endothelial cells. NO is synthesized by eNOS and causes guanylyl cyclase to produce cGMP. A rise in cGMP causes Ca^{2+}-ATPase to be activated, thus reducing Ca^{2+} concentration in the cell, that due to muscle relaxation.

Nitroglycerol, amyl nitrite, "poppers" (isobutyl nitrite or similar) and other nitrite derivatives are used in the treatment of heart disease. These compounds are converted to NO (by a process that is not completely understood), which in turn dilates the coronary artery (blood vessels around the heart), thereby increasing its blood supply. These drugs, however, are predominantly venodilators, dilating peripheral veins and hence reducing venous return and preload to the heart. This reduces the oxygen requirement of the myocardium and subsequently lessens the anginal pain felt with myocardial ischemia.

Nitric Oxide in the Immune System

In the immune system iNOS catalyzes synthesis of NO used in host defense reactions. Activation of iNOS is independent of Ca^{2+} in the cell. Synthesis of NO happens in most nucleated cells, particularly macrophages. This NO pool is a potent inhibitor of viral replication and bactericidal agent.

iNOS overactivity can cause development of *septic shock*: after powerful bacterial invasion (for ex., sepsis) microbal LPS activate iNOS with excessive production of NO, which dilate of blood vessels and down of blood pressure.

3.4. Test questions

1. ***NO in form _____ is high-reactive in relation to cations of transitional metals, and that is why able to contact with iron of hemoproteins and Fe-containing proteins that haven't heme structure, in thereby and Fe-S-centers, here changing valency of it cation ($Fe^{2+} \rightarrow Fe^{3+}$).***
($NO^{\bullet}$, NO^{+}, NO^{-})

2. ***Which of these enzymes can causes septic shock if it is overactive?***

a) nNOS;
b) eNOS;
c) iNOS

3. ***The electron flow in the NO synthase reaction is: _____***

4 .

Reaction	***NO form ($NO^{\bullet}$, NO^{+}, NO^{-})***	***Modified Group/Amino acid***	***General formula***	***Reversible/ irreversible***
nitrosation (nitrosylation)			***Nitrosothiols (_____)***	
nitration			***Nitrated proteins (_____)***	

5. NO triggers the formation of _____ in cell, which is a mediator of smooth muscle cell relaxation:

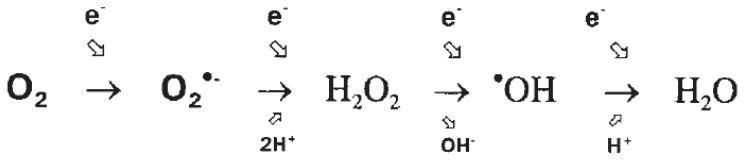
A) cAMP
B) cGMP
C) ppGppp
D) CDP
E) none of the above

4. Reactive Oxygen Species (ROS)

ROS are the group of ***reactive radical*** ($O_2^{\cdot -}$ - superoxide; $OH^{\cdot}$ – hydroxyl; $RO_2^{\cdot}$ - peroxyl; $RO^{\cdot}$ – alkoxyl; $HO_2^{\cdot}$ – hydroperoxyl etc) and ***non-radical*** (H_2O_2 - hydrogen peroxide; $HOCl^-$ - hypochlorous acid; O_3 – ozone; $^1O_2$ - singlet oxygen; $ONOO^-$ - peroxynitrite etc.) compounds.

Reactive species differ by their "longevity": hydrogen peroxide, organic hydroperoxides and hypohalous acids have half-life about minutes, peroxyl radicals and nitric oxide – about seconds, peroxynitrite – about milliseconds, superoxide anion, singlet oxygen and alkoxyl radicals about microsecond and hydroxyl radical near nanosecond.

The first oxidative agent in cells is oxygen. All main ROS are formed from it in next reactions:



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

The hydroxyl radical, which has a very short in vivo half-life of approximately 10^{-9} seconds, is highly reactive. This makes it a very dangerous compound to the organism. Two main sources of hydroxyl radical in cells are ***Fenton*** (a) and ***Haber-Weiss*** (b) ***reactions***.

$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$ **(ferrous)** $H_2O_2 + Fe^{2+} \rightarrow OH^- + {}^{\cdot}OH + Fe^{3+}$ **(ferric)** (a)	$O_2^{\bullet -} + H_2O_2 \rightarrow OH^- + O_2 + {}^{\cdot}OH$ (b)

The hydroxyl radical can damage virtually all types of macromolecules: carbohydrates, nucleic acids (mutations), lipids (lipid peroxidation) and amino acids (e.g. conversion of Phenylalanine to m-Tyrosine and o-Tyrosine). Unlike superoxide, which can be detoxified by superoxide dismutase, the hydroxyl radical cannot be eliminated by an enzymatic reaction. Mechanisms for scavenging peroxyl radicals for the protection of cellular structures include endogenous antioxidants such as melatonin and glutathione, and dietary antioxidants such as mannitol and vitamin E.

4.1. Endogenous sources of ROS

All ROS sources can be classificated on enzymatic and nonenzymatic. In turn enzymatic ROS sources can form ROS as the main product or as side compound.

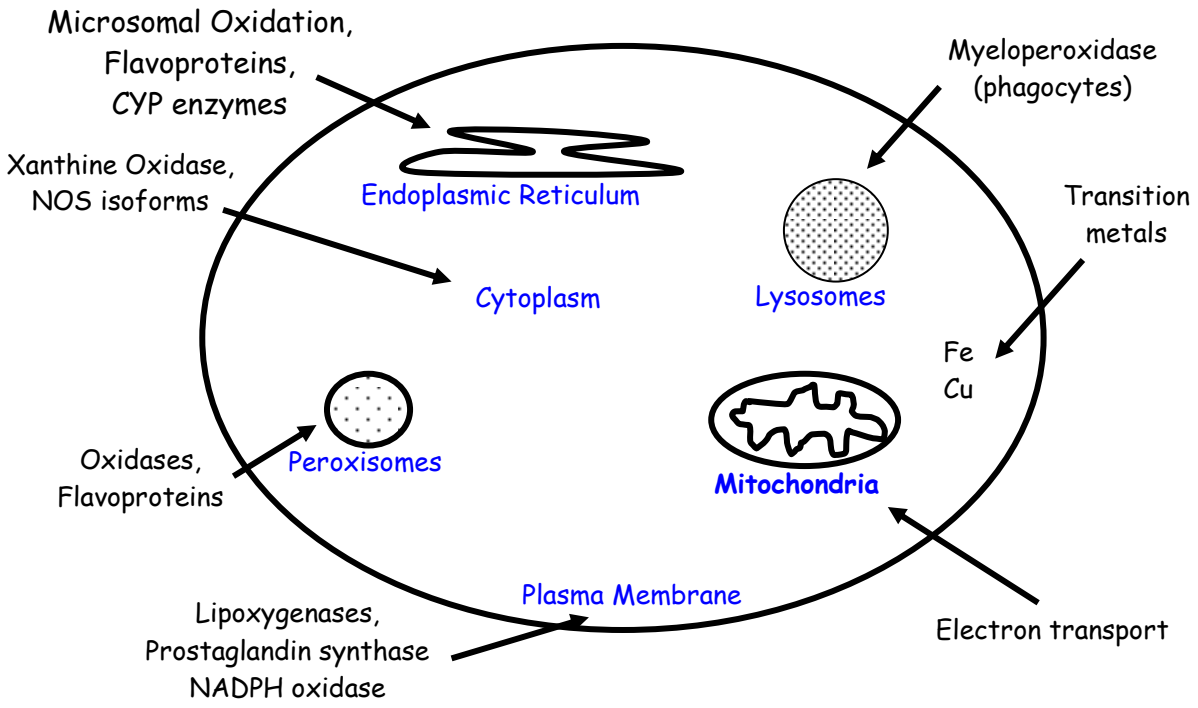
Nonenzymatic sources ROS are processes of glucose autooxidation, advanced glycation, and polyol pathway.

Examples of ***enzymatic ROS-formation*** in which ROS isn't a main product are:

- arachidonic acid metabolism enzymes (5'-lipoxygenase (5'-LO) and cyclooxygenase (COX));
- electronic transport chains (mitochondrial, microsomal, nuclear);
- xanthine oxidase (XO), that catalyze two reactions of purine metabolism:

$$O_2 + \text{Xanthine} \rightarrow \text{Urate} + O_2^{\bullet -}/H_2O_2$$

$$O_2 + \text{Hypoxanthine} \rightarrow \text{Xanthine and } O_2^{\bullet -}/H_2O_2$$

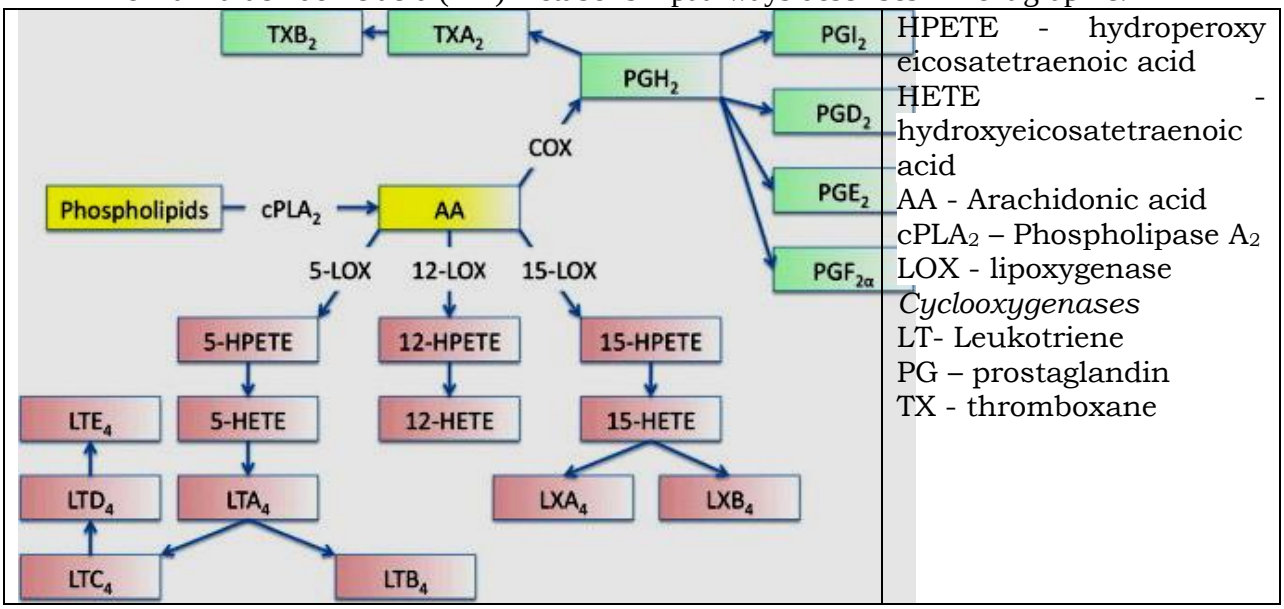


Ros is main product only forming by NADPH oxidase (NOX) in reaction:

$$NADPH + 2\ O_2 \rightarrow\ 2\ O_2^{\bullet -} + NADP^+$$

4.1.1. Arachidonic acid metabolism enzymes (5'-lipoxygenase and cyclooxygenase)

The main arachidonic acid (AA) metabolism pathways describes in next graphic:



Cyclooxygenases convert arachidonic acid to prostaglandin H2 (PGH2), the precursor of the series-2 prostanoids.

The enzyme contains two active sites: a *heme with peroxidase activity*, responsible for the reduction of PGG2 to PGH2, and a *cyclooxygenase site*, where arachidonic acid is converted into the hydroperoxy endoperoxide prostaglandin G2 (PGG2).

At present, three *COX* isoenzymes are known: *COX-1*, *COX-2* and *COX-3*. *COX-3* is a splice variant of COX-1, which retains intron one and has a frameshift mutation; thus some prefer the name *COX-1b* or *COX-1 variant* (*COX-1v*). *COX-1* is considered a constitutive enzyme, being found in most mammalian cells. *COX-2*, on the other hand, is undetectable in most normal tissues. It is an inducible enzyme, becoming abundant in activated macrophages and other cells at sites of inflammation. More recently, it has been shown to be upregulated in various carcinomas and to have a central role in tumorigenesis.

The main *COX* inhibitors are the non-steroidal anti-inflammatory drugs (NSAIDs).

Lipoxygenases are a family of iron-containing enzymes that catalyze the dioxygenation of polyunsaturated fatty acids in lipids containing a cis, cis-1,4-pentadiene structure. It catalyses the following reaction:

Fatty acid + O_2 = Fatty acid hydroperoxide

Arachidonate 5'-lipoxygenase is a member of the lipoxygenase family of enzymes. It transforms arachidonate into leukotrienes throw 5-HPETE (5-hydroperoxyeicosatetraenic acid) and is a current target for pharmaceutical intervention in a number of diseases. 5'-LO is activated by 5-lipoxygenase activating protein (FLAP).

4.1.2. Electron transport systems, peroxysomes and lysosomes as a source of ROS

Mitochondria is considered to be the main cellular source of superoxide formed by one-electron reduction of oxygen, a side reaction of the respiratory chain.

The main sources of one-electron leakage are Complexes I (by NADH-dehydrogenase) and III (by ubiquinol-cytochrome c-reductase) of the respiratory chain.

In Complex I, the iron-sulfur centers and the active-site flavin of Complex I have been proposed as the main sites of superoxide production. While Complex III releases superoxide both to the matrix and to the intermembrane space, superoxide production by Complex I is directed towards matrix.

Other sources of ROS in the mitochondria have also been considered. Complex II (succinate dehydrogenase) may perhaps generate superoxide when damaged or in hypoxia. Autoxidation of reduced flavins in other mitochondrial flavoproteins may also generate superoxide.

Mitochondrial MnSOD transforms $O_2^{\bullet-}$ to H_2O_2, which diffuses from mitochondria into cytosol.

Smooth endoplasmic reticulum contains enzymes detoxifying xenobiotics, including drugs, among them cytochrome P-450 isozymes, especially the ethanol-inducible CYP2E1. These enzymes are able to reduce molecular oxygen and produce $O_2^{\bullet-}$ and H_2O_2. Nuclear membranes contain cytochrome oxidases and electron transport systems of unknown functions which may also release ROS.

Peroxisomes can both produce and scavenge hydrogen peroxide. They contain a number of H_2O_2-generating enzymes (e.g. D-amino acid oxidase, urate oxidase, L-α-hydroxyacid oxidase, fatty acyl-CoA oxidase, glycolate oxidase) but also catalase. Therefore, only a small fraction of H_2O_2 produced in peroxisomes may escape from these organelles under normal conditions

Lysosomal myeloperoxidase (MPO) 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:

$$Cl^- + H_2O_2 + H^+ \rightarrow HOCl + H_2O$$

4.1.3. NADPH oxidases

There is a family of enzymes, Noxs, designed for generation of $O_2^{\bullet-}$ as the main product. The phagocyte enzyme (now classified as Nox2 or **Phox**), responsible for the respiratory burst, was the first discovered. Nox2 produces large amounts of superoxide (and, indirectly, other

ROS) for defensive purposes as microbicidal agents. The same enzyme (albeit at lower levels) and its analogs (NOX-1 - colon VSMC, prostate; NOX-3 - inner ear; NOX-4 – kidney; NOX-5 - spleen (human only)) are present ubiquitously in various cells and there is little doubt that small amounts of superoxide are produced by these proteins for the sake of cellular signaling and regulation of physiological function. The existence of the many Nox isoforms suggests the in vivo relevance of redox-sensitive signaling cascades. Excessive stimulation of Noxs by cytokines and other mediators is implicated in various disease conditions.

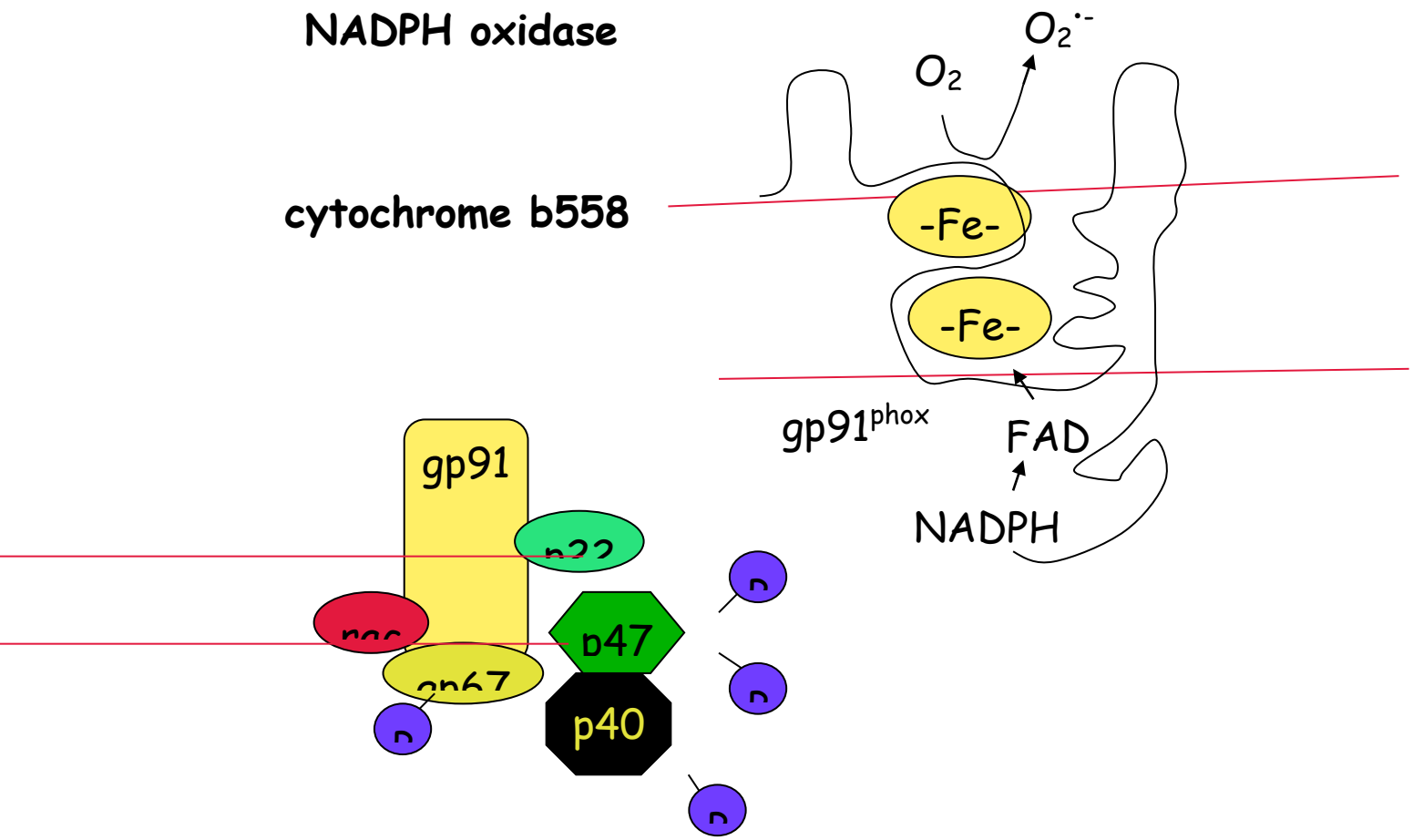
The active complex of Phox consists of several proteins: the heterodimeric flavocytochrome (cytochrome b558) composed of two subunits and cytosolic components ($p47_{phox}$, $p67_{phox}$, $p40_{phox}$, Rac, p21Ras) which associate with them, activating the complex.

The cytochrome b558 subunits ($gp91_{phox}$ and $p22_{phox}$) are integral membrane polypeptides spanning several times the lipid bilayer. $gp91_{phox}$ has six transmembrane domains, two low-potential hemes located near the internal and external surfaces of the membrane, respectively, and a cytosolic domain containing the FAD-binding region, interacting with the substrate (NADPH). $gp91_{phox}$ constitutively associates with $p22_{phox}$. *Activation of NADPH oxidase is initiated by the Rac protein which, upon exchange of bound GDP for GTP, induces phosphorylation of a cytosolic $p47_{phox}$ subunit*. Conformational change of $p47_{phox}$ occurring upon phosphorylation enables it to interact with the "organizer" $p22_{phox}$ subunit.

Another GTP-binding protein - *p21Ras* - functions in oxidant-dependent signaling upstream of Rac. The Rac protein is involved in the activation of most if not all Nox enzymes. In mammalian cells there are three highly homologous Rac proteins: Rac1 distributed ubiquitously, Rac2, expressed mostly in myeloid cells and Rac3, found mainly in the nervous system.

Phox produces superoxide by transmembrane electron transfer from cytosolic NADPH, via FAD and hemes, to oxygen at the extracytoplasmatic side of the membrane. NADPH is the preferred substrate; affinity of $gp91_{phox}$ for NADH is about 100 times lower.

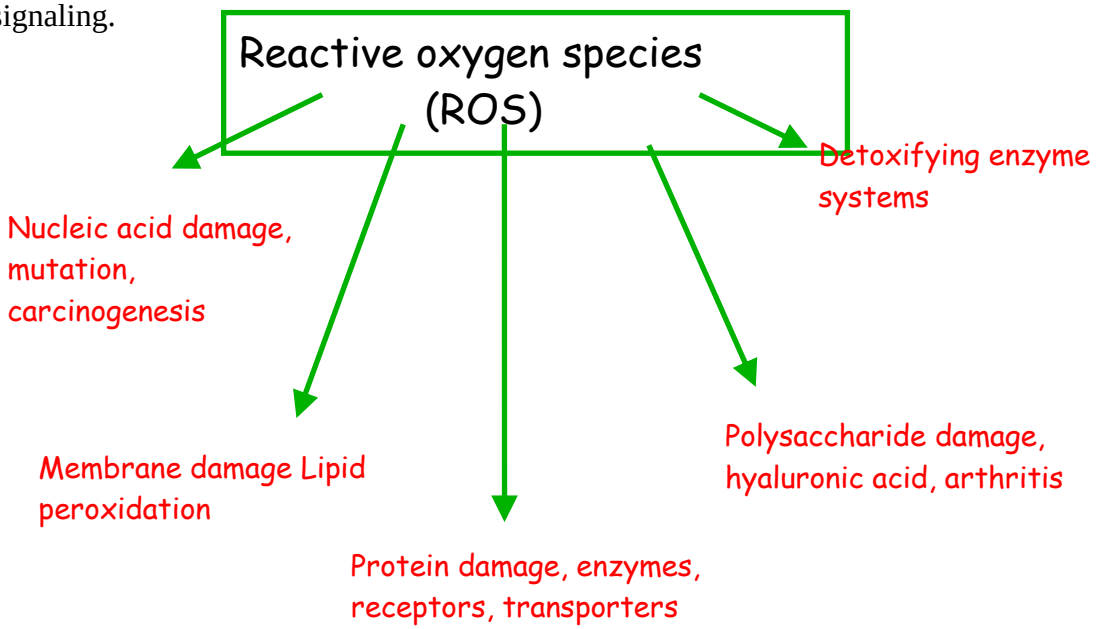
Nox2 analogs differ in the structure of the main subunit and in the composition of and even requirements for the regulating subunits. Some of Noxs are called Duoxs ("dual function oxidases") since, in addition to the Nox domain, they have a domain homologous to that of thyroid peroxidase (lacking a peroxidatic activity but generating H_2O_2 instead). This H_2O_2 is required for thyroperoxidase-mediated iodination of tyrosyl residues of thyreoglobulin in the thyroid gland. In the digestive tract, Duox2 in combination with lactoperoxidase plays a role in bacterial killing. It has been a question of dispute whether Duoxs produce superoxide or directly hydrogen peroxide. The prevalent view is that the primary product is superoxide although rapid dismutation may preclude its detection.



4.2. Biological targets of ROS

Oxidative stress is "an imbalance favoring prooxidants and/or disfavoring antioxidants, potentially leading to damage" (H. Sies). So, high doses of ROS directly damage/kill cells by damaging cellular proteins, DNA and lipids, but low doses/chronic overproduction of oxidants cause activation of cellular signaling pathways.

ROS have their targets in all cells. They cause heme oxidation, oxidation of iron-sulfur centers in proteins, changes in thiol/disulfide redox state of the cell, changes in conformation and so in activity of enzymes, receptors, ion channels etc, oxidative modification of proteins: degradation, loss of function, or gain of function, oxidative modification of DNA (activation of repair, and/or apoptosis) and oxidative modification of lipids with disruption of membrane-associated signaling.



Oxidative modification of lipids (***lipid peroxidation***) refers to the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie methylene bridges ($-CH_2-$) that possess especially reactive hydrogens.

Lipid peroxidation causes structural changes in membranes which 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, have direct toxicity (e.g., 4-hydroxynonenal toxicity), cause disruptions in membrane-dependent signaling, DNA damage and mutagenesis.

The reaction of lipid peroxidation consists of three major steps: initiation, propagation, and termination.

1. Initiation **$LH + HO^{\bullet} \rightarrow L^{\bullet} + H2O$**	
2. Propagation **$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$** **$LOO^{\bullet} + L''H \rightarrow L''^{\bullet} + LOOH$**	**2a. Catalyzed by metals** **$LOOH + Fe^{2+} \rightarrow OH^- + LO^{\bullet} + Fe^{3+}$**
3. Termination **$2\ LOO^{\bullet} \rightarrow$ non-radical products** **$L^{\bullet} + LOO^{\bullet} \rightarrow$ non-radical products** **$L^{\bullet} + L^{\bullet} \rightarrow$ non-radical products**	

Initiation is the step in which a fatty acid radical is produced. The most notable initiators in living cells are reactive oxygen species (ROS), such as $OH^{\bullet}$, which combines with a hydrogen atom to make water and a *fatty acid radical* ($L^{\bullet}$).

In ***propagation step*** the fatty acid radical, which is not a very stable molecule, reacts readily with molecular oxygen, thereby creating a *peroxyl-fatty acid radical* ($LOO^{\bullet}$). This too is an unstable species that reacts with another free fatty acid, producing a *different fatty acid radical* and *lipid peroxide* (LOOH), or cyclic peroxide if it had reacted with itself. This cycle continues, as the new fatty acid radical reacts in the same way.

Reduced metal complexes [e.g., iron(II) or copper(I)] react with lipid peroxides to give *alkoxyl radicals* ($LO^{\bullet}$):

$$LOOH + Met^n \rightarrow LO^{\bullet} + M^{n+1} + OH^-$$

Alkoxyl radicals then stimulate the chain reaction of lipid peroxidation by abstracting further hydrogen atoms.

When a radical reacts with a non-radical, it always produces another radical, which is why the process is called a "chain reaction mechanism". The radical reaction stops when two radicals react and produce a non-radical species (***termination step***). This happens only when the concentration of radical species is high enough for there to be a high probability of collision of two radicals. Living organisms have evolved different molecules-antioxidants that speed up termination by catching free radicals and, therefore, protecting the cell membrane.

4.3. ROS as signaling molecule

ROS (H_2O_2 & $O_2^{\bullet-}$) are the second messengers because they have:

1. *A regulatable source for generating ROS*: NADPH-oxidase (cytokine- or growth factor-activated Rac activates and recruits p47 and p67) or another sources of ROS;
2. *A means to couple ROS to an external stimulus*: GFR, TNFαR, etc

3. *Signal termination*: SOD, catalase, peroxydase, peroxyredoxines

4. *Effector molecules*: Ca^{2+}-signaling, proteinkinases (PKC, MAPKs, PkRaf, RTPKs, NRTPKs), proteinphosphatases, Ros-induced transcription factors (Nf-kB, AP-1, Hif-1, p53 etc), ATPases, ion channels, phospholipases, adenylate cyclase...

Current concepts of ROS signaling can be divided into two general mechanisms of action:

1) oxidative modifications of proteins;

1) alterations in intracellular redox state.

4.3.1. Redox-regulation by oxidative modifications of proteins: mechanisms

ROS can alter protein structure and function by modifying critical amino acid residues, inducing protein dimerization, and interacting with Fe-S moieties or other metal complexes. Oxidative modifications of critical amino acids within the functional domain of proteins may occur in several ways. The best described of such modifications involves ***cysteine residues***.

The sulfhydryl group (-SH) of a single cysteine residue may be oxidized to form ***sulfenic*** (-SOH), ***sulfinic*** ($-SO_2H$), ***sulfonic*** ($-SO_3H$), or ***S-glutathionylated*** (-SSG) ***derivatives***. Such alterations may alter the activity of an enzyme if the critical cysteine is located within its catalytic domain or the ability of a transcription factor to bind DNA if it is located within its DNA-binding motif. Cysteine oxidation may inactivate proteins (e. g. PTPs) but sometimes may activate them (ryanodine receptors), change their conformation to induce dimerization (chaperone proteins) or release a complexed molecule (ASK-1 from oxidized thioredoxin). For example, protein phosphatase PTP-1B is directly inactivated by ROS-induced reversible oxidation of its catalytic site, Cys-215, and this has been proposed as a mechanism for EGF-mediated mitogenic signaling. ***Sulfenic acids may be converted to disulfides upon reaction with other thiols. The disulfide bonds can be reduced by glutaredoxins (Grxs), thioredoxins (Trxs) or glutathione so this modification is reversible. Further oxidation of a thiol group leads to a sulfinic acid ($–SO_2H$) or sulfonic acid ($–SO_3H$). Both these reactions were considered irreversible;*** however, sulfiredoxins (Srxs), enzymes able of ATP-dependent reduction of sulfinic acid residues have been identified. In addition to Srxs, sestrins are also able to reduce sulfinic acids

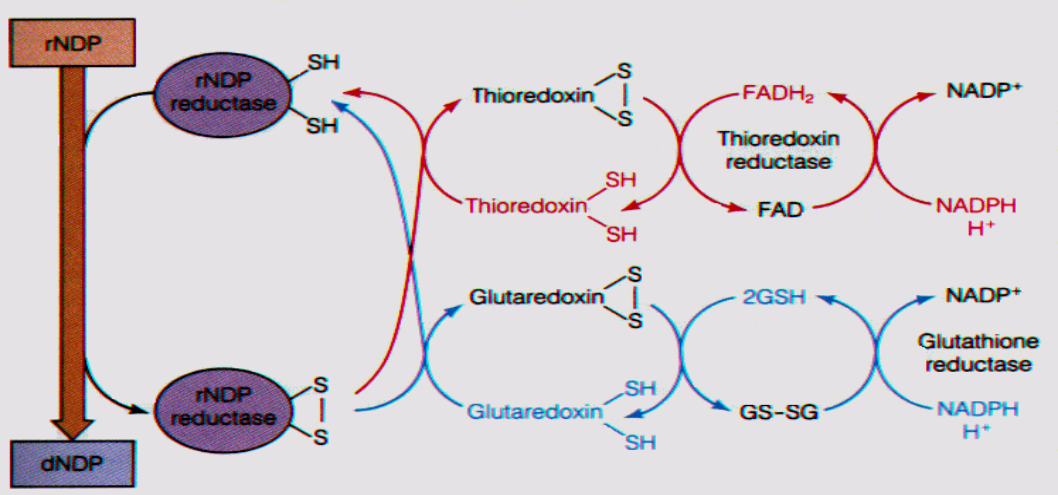
The specific cysteine modification involves S-glutathionylation, which is readily reversed to the active sulfhydryl group by thioltransferases. Reversible S-glutathionylation also appears to form the basis for redox regulation of c-Jun DNA binding. ***S-Glutathionylation (formation of a mixed protein-glutathione disulfide) is thought to prevent further, irreversible oxidation of cysteine sulfur.*** It is often viewed as a general reversible protein modification, comparable to protein phosphorylation. Glutathionylation, thought previously to be due to exchange reaction between ***glutathione disulfide (GSSG)*** and protein –SH, is now believed to occur mainly in reaction of protein cysteine sulfenic acids with ***glutathione (GSH)***. Glutathionylation and deglutathionylation are catalyzed by thioredoxins and glutaredoxins.

Thioredoxins (Trx) are proteins found in nearly all known organisms (from plants and bacteria to mammals). They are an antioxidant that prevents oxidative stress, since an accumulation of oxidative stress can lead to certain diseases and certain cancer.

Thioredoxin systems involve two redox conditions of a dithiol/disulfide group. ***This small protein contains an active site that involves residue of cysteine. These -cys residues reverse from a dithiol (-SH HS-) group to a disulfide bridge (-S-S-). The oxidized protein is a disulfide with one a bridge between two cysteines whereas the reduced protein is a dithiol with two cysteines.***

Reduced Trx participates in many cellular functions by donating its electrons and restoring oxidative inactive enzymes back to its reduced and active state (*through the cysteine thiols-disulfide exchange*). ***Thioredoxin reductase is the only known enzyme to reduce oxidized trx. Trx reductase catalyzes the NADPH-dependent reaction that supplies the electrons to reduced oxidized trx.***

Glutaredoxin is the sulfhydryl protein too. ***The oxidized protein is a disulfide with one bridge between two cysteines whereas the reduced protein is a dithiol with two cysteines. Glutathione reductase and glutathione are nessesary for reduce oxidized Grx in the NADPH-dependent reaction that supplies the electrons to reduced oxidized Grx.***



Iron-sulfur proteins, for example, mammalian (4Fe-4S) aconitase (enzyme of citric acid cycle), contains non-heme iron complex Fe-S-Cys. ROS and RNS disrupt Fe-S clusters and inhibit aconitase activity by influence on sulfide, cysteine thiolate groups in non-heme iron proteins.

Signaling by methionine oxidation (reversible thanks to methionine sulfoxide reductases) is also possible and has been reported for calmodulin and membrane transporters.

4.3.2. Pathways of redox-regulation by oxidative modifications of proteins

Redox signaling: tyrosine protein kinases and protein phosphatases

Receptor kinases and phosphatases themselves may be targets of oxidative stress. Growth factor receptors are most commonly activated by ligand-induced dimerization or oligomerization that autophosphorylates its cytoplasmic kinase domain. Ligand-independent clustering and activation of receptors in response to ultraviolet light have also been well demonstrated, and this effect appears to be mediated by ROS.

Exogenous H_2O_2 (usually in the millimolar range) has been shown to induce tyrosine phosphorylation and activation of the PDGF-a, PDGF-b, and EGF receptors. ***The mechanism of these effects may be related to ROS-mediated inhibition of the dephosphorylation of RTKs by inactivation of membrane-bound protein tyrosine phosphatase(s). Since the level of phosphorylation of substrates is a resultant of the rates of phosphorylaton and dephosphorylation, oxidative inactivation of PTPs increases the level of tyrosine phosphorylation of protein substrates.***

Non-RTKs belonging to the Src family (Src kinases) and Janus kinase (JAK) family have been reported to be regulated by various forms of oxidative stress (activation).

Redox signaling: the levels of intracellular Ca^{2+}

H_2O_2 induces a rapid **increase in intracellular Ca^{2+},** that appears to be related to inositol 1,4,5-triphosphate-sensitive Ca^{2+}-stores in the sarcoplasmic reticulum (SR) followed by a slower increase in intracellular Ca^{2+} that is most likely derived from the extracellular space. ROS have been shown to inhibit the activity of Ca^{2+}-ATPase of the SR, which would result in passive diffusion of SR Ca^{2+} into the cytosol.

Redox signaling: MAPK cascades

Because the MAPK pathways mediate both mitogen- and stress-activated signals, there has been significant interest in the redox regulation of these pathways.

Exogenous oxidants can **activate the ERK/**MAPK pathway. Some studies suggest that ROS-mediated ERK-activation may be an upstream event at the level of growth factor receptors, Src kinases, and/or p21 Ras. Another potential mechanism for this effect may be oxidant-induced inactivation of protein tyrosine phosphatases (PTPs) and/or protein phosphatase A.

Other members of the MAPK family have also been implicated as potential targets of ROS.

Redox signaling: transcription factors.

Nuclear factor kappa B (NF-κB) is a redox-sensitive transcription factor that coordinates regulators of immunity, inflammation, development, cell proliferation, and survival.

In mammals, the NF-*κ*B family consists of NF-*κ*B1 (p50/p105), NF-*κ*B2 (p52/p100), RelA (p65), c-Rel, and RelB. All members are characterized by the presence of the Rel homology domain (RHD). The RHD mediates DNA binding, dimerization between the family members, and the association of NF-*κ*B dimers with the inhibitors of kappa B (I*κ*B).

Normally, the NF-*κ*B components are sequestered and inactivated by I*κ*B *(inhibitor of NF-KB*) in the cytosol. A wide range of stimuli, including cytokines and ROS stress, are capable of activating NF-*κ*B through activation of IKK (*IκB kinase*). Active IKK phosphorylates I*κ*B, leading to dissociation of NF-*κ*B from the inhibitor and the attraction of I*κ*B to degradation by ubiquitin/proteasome system. Free NF-*κ*B translocates to the nucleus, binds to DNA at the promoter region, and activates the transcription of target genes.

The function of NF-*κ*B can be activated or inhibited through various redox-mediated mechanisms at multiple levels of the activation pathways.

In the nucleus, direct oxidation of Cys in the DNA-binding domain can inhibit NF-κB-DNA-binding activity.

In cytosol, activation of NF-κB can be regulated through phosphorylation of NF-κB itself or phosphorylation of its inhibitor IκB.

Normally, NF-*κ*B and I*κ*B form a complex, which is sequestered in cytosol. ***Increased ROS can activate IκB-kinase (IKK) either directly through redox modification of IκK, or indirectly through activation of Akt and/or ERK-cascade, which then phosphorylates and activates IκK. Active IκK phosphorylates IκB and liberates active NF-κB from the complex to translocate to the nucleus. Phosphorylated IκB undergoes ubiquitination and degradation by proteosomes. Because the proteosome system is also redox sensitive, ROS can also regulate NF-κB activity by affecting the stability of IκB. Furthermore, phosphorylation of NF-κB by certain kinases may dissociate NF-κB from IκB and promote its nuclear translocation.***

The AP-1 family of proteins *(from "activated protein-1")* represents an example of transcription factors whose functions involve control of both cell growth and apoptosis. Under certain conditions, AP-1 activation could lead to cell death, whereas under other circumstances, AP-1 may promote cell proliferation and survival.

The AP-1 family consists of several groups of basic leucine zipper domain (bZIP) proteins, including Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, and Fra2), Maf (*from "avian musculoaponeurotic fibrosarcoma virus"* - c-Maf, MafB, MafA, MafG/F/K, and Nrl), and ATF (from "*activating transcription factor-2*" - ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) subfamilies.

AP-1 proteins form heterodimers and bind to the target DNA sequence.

Activation of AP-1 is regulated at both transcript and protein levels. The intracellular levels of c-jun and c-fos are controlled mainly by their transcription rates, which are tightly regulated by a variety of stimuli. The mitogen-activated protein kinase (MAPK) plays a major role in controlling activation of AP-1 proteins through phosphorylation. All three classes of MAPKs are involved in regulation of AP-1 activity (*i.e.*, c-jun is regulated mainly by JNK and ERK in some cell types). c-Fos is a substrate of ERK, and ATF2 is regulated by JNK and p38 kinases. JNK and p38 are both activated by stress stimuli.

Oxidative stress can activate c-Jun and ATF2 through phosphorylation by JNK and p38, respectively.

In the nucleus, direct oxidation of Cys in the DNA-binding domain can inhibit NF-κB-DNA-binding activity.

Protein p53 serves as a master transcription factor to activate the expression of proteins involved in maintaining genomic stability and cellular homeostasis. As a tumor suppressor, p53 exerts its genome guardian effect by controlling various cell-cycle checkpoints and regulating DNA-damage repair, senescence, and apoptotic machineries. Recent studies suggested a novel function of p53 in maintaining redox homeostasis through regulating energy metabolism, mitochondrial biogenesis, and the expression of antioxidant enzymes.

Loss of p53 function contributes to the development of many types of human cancer. A number of studies suggest that p53 plays an important role in controlling cell fate through regulation of cellular ROS level.

Although p53 functions as a transcription factor to control the expression of several redox-regulating molecules, p53 itself is redox sensitive. ***p53 is a zinc-binding protein containing 10 cysteine residues susceptible to ROS oxidation. The mechanisms that regulate the redox status of p53 remain to be fully elucidated, but much evidence suggests that p53-DNA–binding ability can be strongly inhibited by oxidation and nitrosylation.***

Hypoxia-inducible factor-1 (HIF-1) mediates the transcriptional activation of many oxygen-sensitive genes such as erythropoietin, heme oxygenase-1 (HO-1), inducible nitric oxide synthase, vascular endothelial growth factor, transferrin, and several glycolytic enzymes.

HIF-1 was found to be a heterodimer composed of two proteins, HIF-1α and HIF-1β. Of these, HIF-1β had previously been identified as the aryl hydrocarbon nuclear receptor translocator (ARNT), which is dimerized with the aryl hydrocarbon receptor. HIF-1α was a newly defined protein and uniquely associated with the transcription of the hypoxia-inducible genes.

The HIF-1 heterodimer complex recognizes a DNA consensus sequence 5’-CGTG-3’ in enhancer or promoter regions of many hypoxia-responsive genes, interacts with these binding sites in the major groove, and activates the transcription of these genes

Strong oxidizing reagents impair the expression of HIF-1α in hypoxic cells, activate its proteolisys in proteasome and decrease the DNA-binding activity of HIF-1.

4.3.3. Redox-regulation by alterations in intracellular redox state.

The term “redox state of a cell” can be defined by a set of values of actual redox potentials of individual redox couples (e. g. GSSG/GSH) in a given cellular compartment. It is well documented that the redox state of a cell, as probed by the redox potential of the glutathione couple, is different for actively growing, confluent, differentiated and apoptotic cells.

Production of ROS which are consumed in redox reactions must change the redox state of a compartment in which this production occurs. This change may be reversible as the cell attempts to keep the redox homeostasis. Oxidation of protein thiols means, of course, a change in the redox potential of a thiol redox couple of individual protein species. However, what is meant by this term is usually a change of redox potentials of main intracellular “redox buffers” (GSSG/GSH, $NADP^+$/NADPH, thioredoxins (Trx), glutaredoxins (Grx)) and effects of these changes on cellular phenomena.

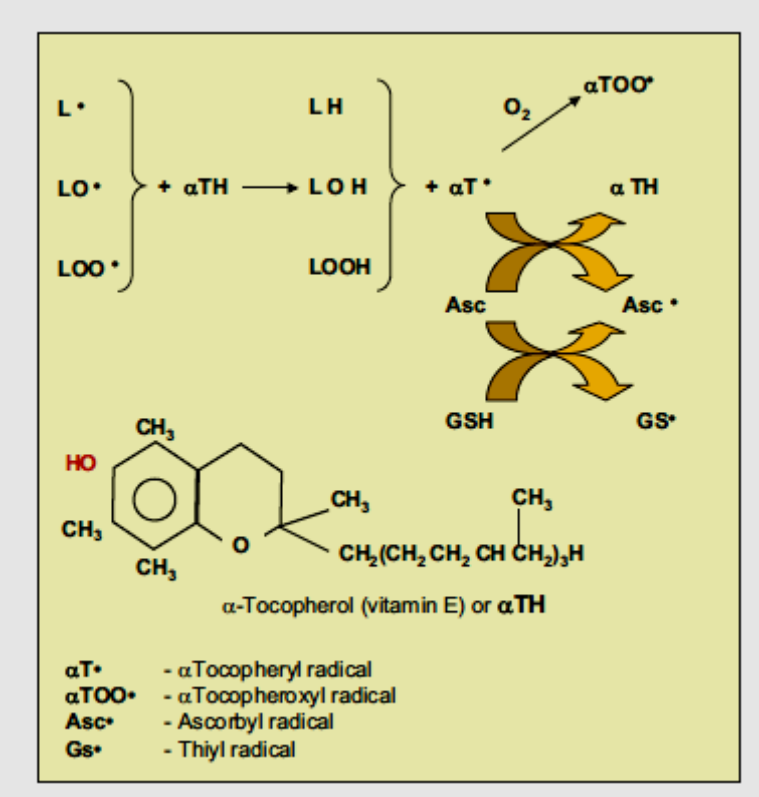
In comparison with the extracellular environment, the cytosol is normally maintained under strong “reducing” conditions. The high ratios of reduced to oxidized GSH and Trx are maintained by the activity of GSH-reductase (glutathione-reductase) and Trx-reductase (thioredoxin-reductase), respectively.

Antioxidant Defense

Preventing of prooxidant formation can occur by ***physical prevention*** on organismal, organ and cellular levels, ***biochemical prevention*** **(**accomplish by *control of prooxidant molecules* with transition metal chelators and enzymatic control of $O_2^{\bullet-}$ reduction or *control of prooxidant enzymes* using blockade of stimuli or inhibition of enzymes). Anti-inflammatory agents, NOS inhibitors, metal chelators (metallothionein, transferring, lactoferrin), NADPH oxidase and xanthine oxidase inhibitors are examples of ***preventative 'antioxidants'***.

Antioxidant is 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. There are two groups of antioxidants: ***nonenzymatic antioxidants,*** which intercepts species, once formed and excludes them from further damaging activity by transfering species from critical parts of cell (radical scavengers, = chain breaking antioxidants) or takes part in reduces disulphide bond, and ***antioxidant enzymes***.

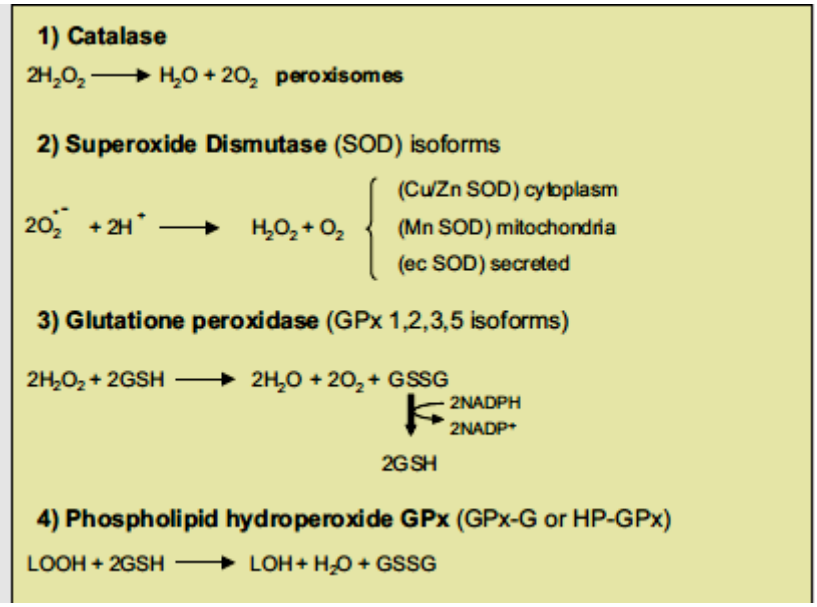
Chain breaking nonenzymatic antioxidants break the chain of radical reactions, in part, the chain of lipid peroxidation. ROO• (peroxyl radicals) are often the chain-carrying radicals, so chain-breaking antioxidants act by reacting with peroxyl and other radicals. Examples of this group of antioxidants are *"donor" antioxidants* (tocopherol, ascorbate, uric acid etc):



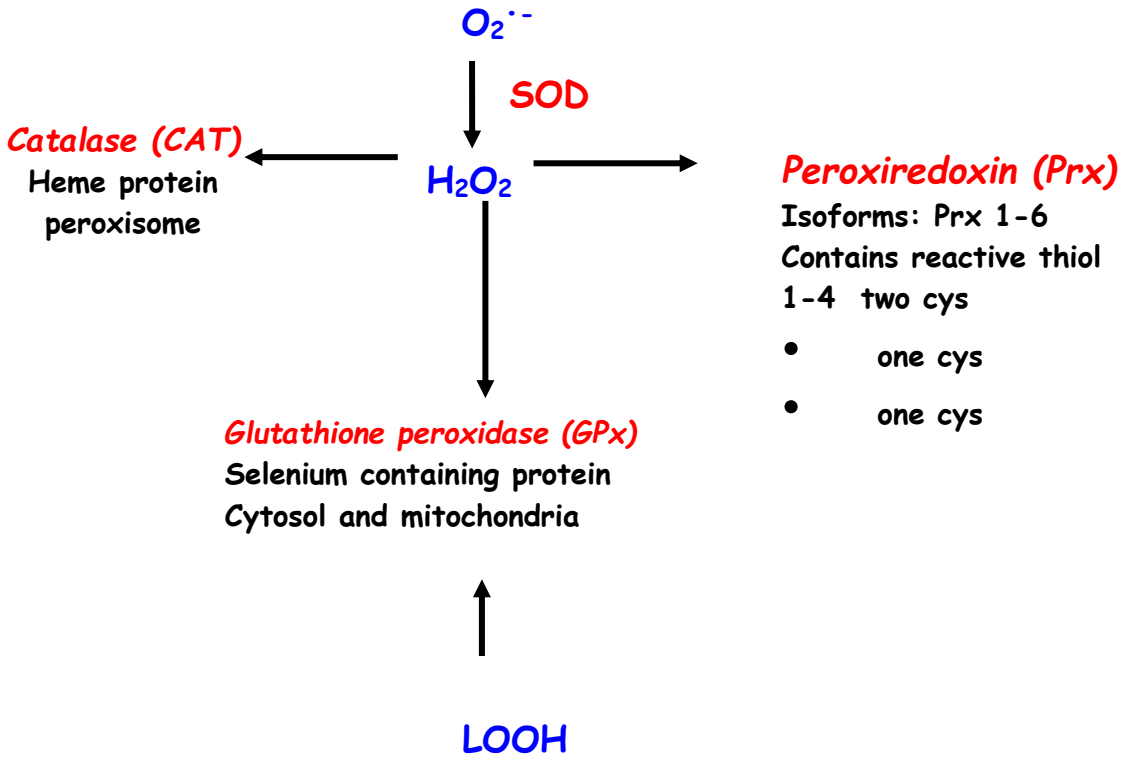
Good chain-breaking antioxidants should be relatively unreactive (both antioxidant and its radical) and be renewed (recycled).

Nonenzymatic antioxidants can function in cytosol **(***water soluble compounds* -glutathione, uric acid, ascorbate, selenium, albumin, ceruloplasmin, lipoic acid, Trx, Grx, peroxiredoxins etc) or in biological membranes (*lipid soluble compounds* as α-tocopherol, b-carotene, coenzyme Q).

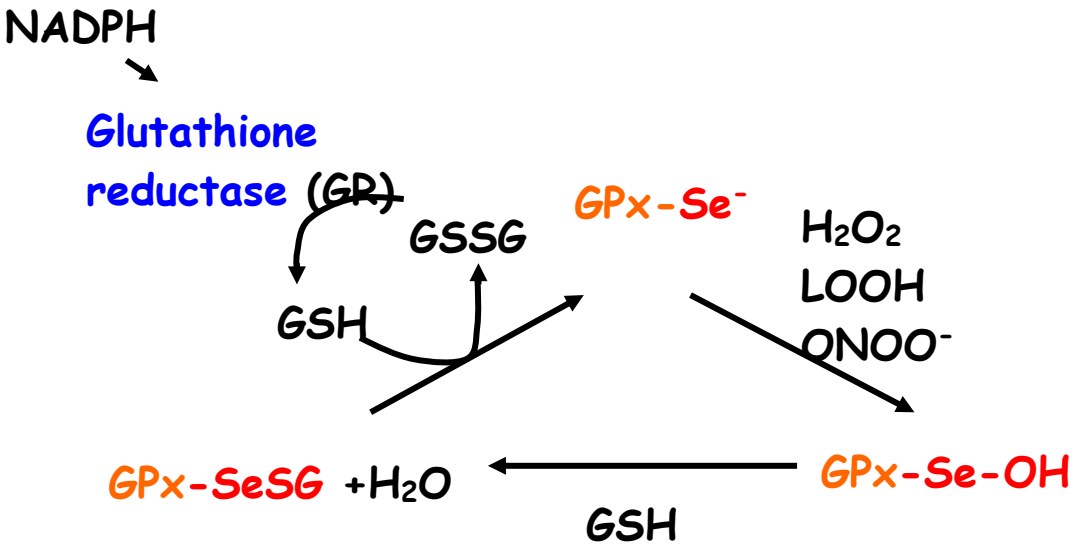
The most important antioxidant enzymes are superoxide dismutase (SOD I, II and III), glutathione peroxidase (GPx), catalase (CAT), phospholipid hydroperoxide GPx (PHGPx), GSH-reductase, glutathione transferase:



The primary antioxidant enzymes in mammalian tissues are superoxide dismutase (SOD), CAT and GPx.



The GSH-dependent antioxidative system consists of two enzymes: Selen-dependent GPx and GSH-reductase. GSH-reductase catalyses the reduction of GSSG to GSH. By the contrast to GPx, this enzyme is involved in the maintainance of glutathione in reduced form and owing to this, GSH plays its antioxidant functions. In maintaining the intracellular pool of GSH, NADPH - the cell principal reductant, plays a critical role. The pentose phosphate pathway is considered to be a major source of cellular reducing power.



GPX – Glutathione Peroxidsase; GSH – Glutathione reduced; GSSG – Glutathione oxidized

Peroxiredoxins (Prxs) are a ubiquitous family of antioxidant enzymes that also control cytokine-induced peroxide levels which mediate signal transduction in mammalian cells. Prxs can be regulated by changes to phosphorylation, redox and possibly oligomerization states.

Prxs use redox-active cysteines to reduce peroxides and were originally divided into two categories, the 1-Cys and 2-Cys Prxs, based on the number of cysteinyl residues directly involved in catalysis. Structural and mechanistic data now support the further division of the 2-Cys Prxs into two classes called the 'typical' and 'atypical' 2-Cys Prxs.

All Prxs share the same basic catalytic mechanism, in which an active site cysteine (the ***peroxidatic cysteine***) is oxidized to a sulfenic acid by the peroxide substrate. The recycling of the sulfenic acid back to a thiol is what distinguishes the three enzyme classes.

The peroxidase reaction is composed of two steps centered around a redox-active cysteine called the ***peroxidatic cysteine (S_PH)***. All three Prx classes appear to have the first step in common, in which the peroxidatic cysteine (Cys–SH) attacks the peroxide substrate and is oxidized to a cysteine sulfenic acid (Cys–SOH). All Prxs to date conserve an active-site Arg, which would lower the pKa of the peroxidatic cysteine somewhat by stabilizing its thiolate form.

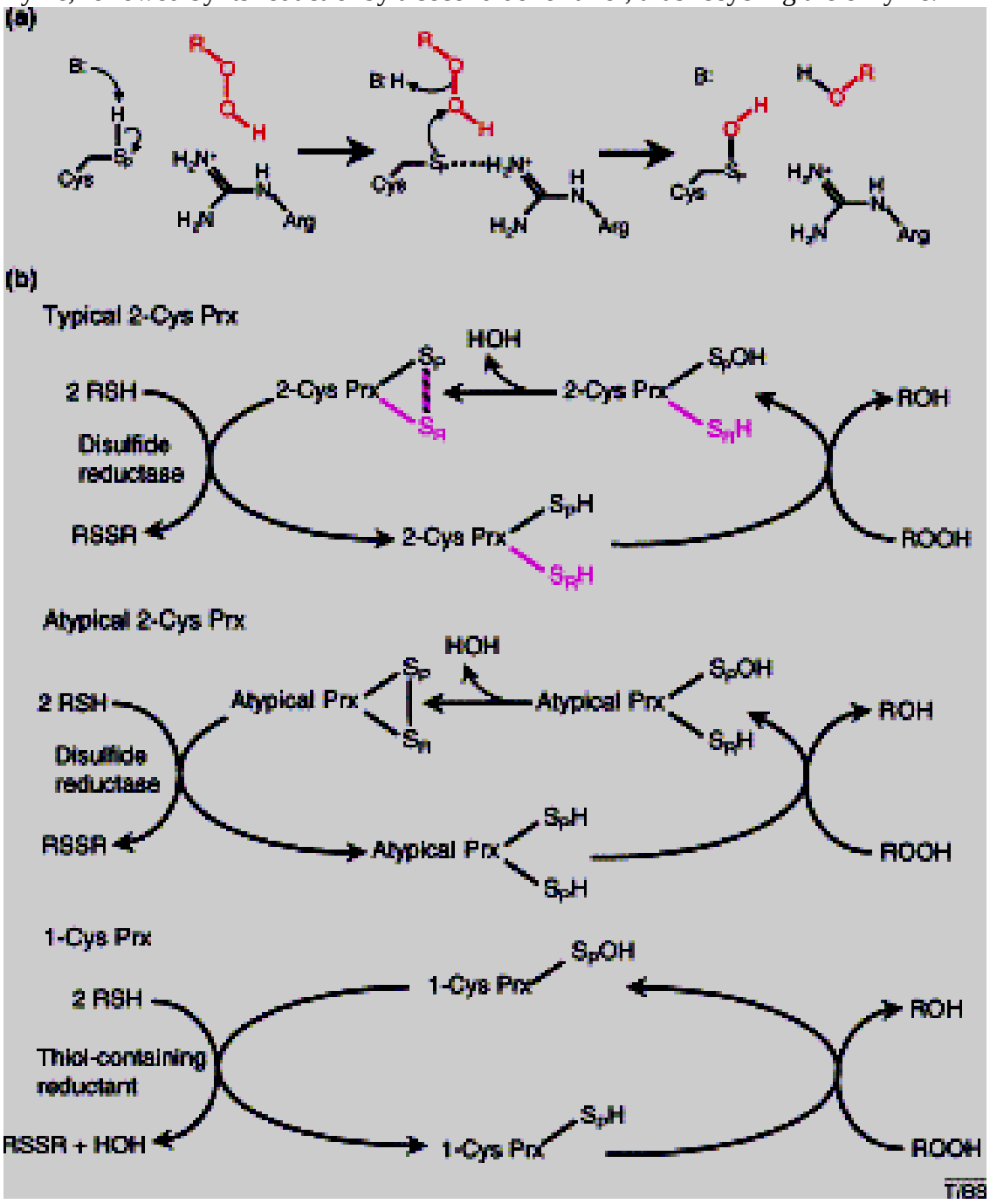
The second step of the peroxidase reaction, the resolution of the cysteine sulfenic acid, distinguishes the three Prx classes**.**

The typical 2-Cys Prxs are the largest class of Prxs and are identified by the conservation of ***their two redox-active cysteines, the peroxidatic cysteine and the resolving cysteine (S_RH).*** Typical 2-Cys Prxs are obligate homodimers containing two identical active sites. In the second step of the peroxidase reaction, ***the peroxidatic cysteine sulfenic acid (Cys–SOH) from one subunit is attacked by the resolving cysteine (Cys–SH) located in the C terminus of the other subunit. This condensation reaction results in the formation of a stable intersubunit disulfide bond***, which is then reduced by one of several cell-specific disulfideoxidoreductases (e.g. thioredoxin), completing the catalytic cycle.

The second class of Prxs are the atypical 2-Cys Prxs, which have the same mechanism as typical 2-Cys Prxs but are functionally monomeric. In these Prxs, ***both the peroxidatic cysteine and its corresponding resolving cysteine are contained within the same polypeptide, with the condensation reaction resulting in the formation of an intramolecular disulfide bond***. Although the resolving cysteines of typical and atypical 2-Cys Prxs are not conserved in sequence, they are functionally equivalent. To recycle the disulfide, known atypical 2-Cys Prxs appear to use thioredoxin as an electron donor.

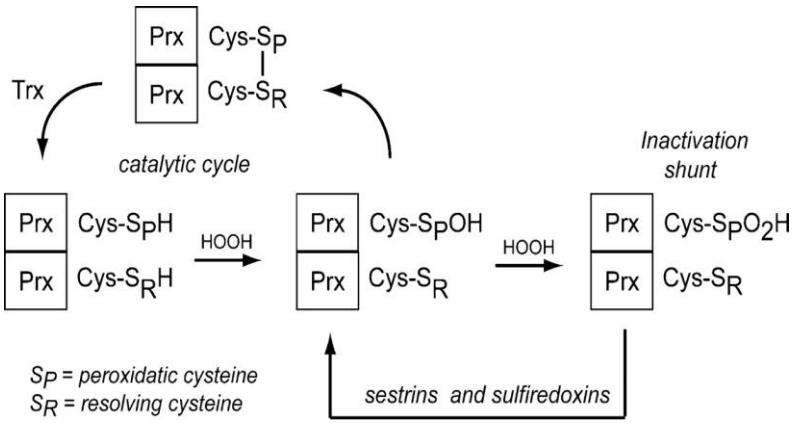
The last class of Prxs, the 1-Cys Prxs, conserves only the peroxidatic cysteine and do not contain a resolving cysteine. Their cysteine sulfenic acid generated in reaction with peroxides is

presumably reduced by a thiol-containing electron donor, but the identity of this redox partner is not yet clear (although proposed electron donors have included glutathione, lipoic acid and cyclophilin). By analogy, one donor thiol probably forms a transient mixed disulfide bond with the enzyme, followed by its reductionby a second donor thiol, thus recycling the enzyme.



So, when exposed to H_2O_2, the ***peroxidatic cysteine of 2-Cys Prxs is oxidized to sulfenic acid (Prx-SOH). Upon reaction with the resolving cysteine, a Prx dimer with an intermolecular disulfide bond is formed, which is then reduced by Trx to regenerate active enzyme***.

Because of a pause in the catalytic cycle, the peroxidatic cysteine of eukaryotic 2-Cys Prxs is susceptible to hyperoxidation, resulting in the formation of a sulfinic acid form (Prx-SO_2H) that is catalytically inactive. Sulfiredoxins and sestrins are ATP-dependent sulfinyl reductases that participate in retroreduction of Prx-SO_2H, regenerating active enzyme.



These enzymes are truly ubiquitous having been identified in yeast, plant and animal cells, including both protozoan and helminth parasites, and most, if not all, eubacteria and archaea. Although located primarily in the cytosol, Prxs are also found within mitochondria, chloroplasts and peroxisomes, associated with nuclei and membranes.

4.5. Test questions

1. Which of these reactive oxygen species are formed in Haber-Weiss and Fenton reactions?
a) $O_2^{\cdot-}$; b) $OH^{\cdot}$; c) $ROO^{\cdot}$; d) NO; e) H_2O_2; f) O_3; g) $ONOO^-$

2. *The NADPH-oxidase structure:*

The membrane bound component (cytochrome b558)	***Cytosolic regulatory component***

3. Which of these proteins can reverse formation of disulfide bonds?
a) catalase; b) glutathion peroxidase; c) peroxiredoxines; d) thioredoxin; e) superoxiddismutase; f) glutaredoxin

4. *The sulfhydryl group (-SH) of a single cysteine residue may be oxidized to form sulfenic (-SOH), sulfinic (-SO2H), sulfonic (-SO3H), or S-glutathionylated (-SSG) derivatives. Which of these modifications are reversible and which are irreversible?*

Cysteine derivatives	***Reversible/ irreversible***
sulfenic acid (-SOH)	
sulfinic acid (-SO_2H)	
sulfonic (-SO_3H)	
S-glutathionylated (-SSG) derivatives	
Intra-molecular disulfide linkages	
Inter-molecular disulfide linkages	

5. What three proteins are able to disrupt H_2O_2?

5. Sphingolipid derivatives signaling

Sphingolipid metabolites, such as ceramide, sphingosine and sphingosine-1-phosphate, are lipid signaling molecules involved in diverse cellular processes.

5.1. Ceramide

Ceramide is a lipophilic messenger that regulates diverse signaling pathways involving apoptosis, stress response, cell senescence, cell growth arrest, differentiation, cell migration and cell adhesion. For the most part, ceramide's effects are antagonistic to cell growth and survival.

Three major pathways of ceramide generation: *the sphingomyelinase pathway* (uses an enzyme to break down sphingomyelin in the cell membrane and release ceramide), *the de novo pathway* and *ceramide generation through breakdown of complex sphingolipids* that are ultimately broken down into sphingosine, which is then reused by reacylation to form ceramide. This latter pathway is termed the Salvage pathway. All of these processes can be induced by diverse stimuli. So, substances known to induce ceramide generation are anandamide, tetrahydrocannabinol and other cannabinoids, TNF-α, Fas-ligand, endotoxin, chemotherapeutic agents, 1,25-dihydroxyvitamin D, interferon γ, heat, ionizing radiation etc. Sphingosine and sphingosine-1-phosphate (S-1-P) can be generated from ceramide by ceramidases (CDases) and sphingosine kinases (SKs).

Membrane sphingomyelin hydrolysis is catalyzed by the enzyme ***sphingomyelinase***. Because sphingomyelin is one of the four common phospholipids found in the plasma membrane of cells, the implications of this method of generating ceramide is that the cellular membrane is the target of extracellular signals leading to programmed cell death.

Sphingomyelinase has similar cleavage specificity to phospholipase C, in that it cleaves an alcohol-phosphate bond. Activation of sphingomyelinase is observed in response to diverse stress challenges including irradiation, exposure to DNA-damaging agents or treatment with pro-apoptotic ligands like tumor necrosis factor a (TNFα). Because of these properties, ceramide is a potent apoptogenic agent (anticancer effects of irradiation and chemotreatment).

De novo synthesis of ceramide begins ***with the condensation of palmitate and serine*** to form *3-keto-dihydrosphingosine*. This reaction is catalyzed by the enzyme ***serine palmitoyl transferase*** and is the rate-limiting step of the pathway. In turn, 3-keto-dihydrosphingosine is reduced to *dihydrosphingosine*, which is then followed by acylation by the enzyme *(dihydro)ceramide synthase* to produce *dihydroceramide*. The final reaction to produce ceramide is catalyzed by *dihydroceramide desaturase*. De novo synthesis of ceramide occurs in the endoplasmic reticulum. Ceramide is subsequently transported to the Golgi by either vesicular trafficking or the ceramide transfer protein CERT. Once in the Golgi apparatus, ceramide can be further metabolized to other sphingolipids, such as sphingomyelin and the complex glycosphingolipids.

The salvage pathway - constitutive degradation of sphingolipids and glycosphingolipids - takes place in the acidic subcellular compartments, the late endosomes and the lysosomes. In case of glycosphingolipids, ***exohydrolases***, acting at acidic pH optima, cause the stepwise release of monosaccharide units from the end of the oligosaccharide chains one after the other leading to the generation of ceramide whereas sphingomyelin is converted to ceramide by acid sphingomyelinase. Ceramide can be further hydrolyzed by *acid ceramidase* to form sphingosine and a free fatty acid, both of which are able to leave the lysosome in contrast to ceramide.

The long-chain sphingoid bases released from the lysosome may then re-enter pathways for synthesis of ceramide and/or sphingosine-1-phosphate. The salvage pathway re-utilizes long-chain sphingoid bases to form ceramide through the action of ceramide synthase. Thus, ceramide synthase family members probably trap free sphingosine released from the lysosome at the surface of the endoplasmic reticulum or in endoplasmic reticulum-associated membranes. It

should also be noted that the salvage pathway has been estimated to contribute from 50% to 90% of sphingolipid biosynthesis.

The most molecular targets of ceramide are ***CAPP*** (*ceramide activated protein phosphatase (PP2A)*), ***PP1*** (*protein phosphatase 1*), ***CAPK*** (*ceramide-activated protein kinase*), ***Protein kinase C***. ***MAPKs*** also are ceramide effectors. So, sphingosine and ceramide has been demonstrated to ***inhibit the Erk-cascade*** and ***simultaneously activate JNK- and p38-cascades*** in a variety of cell types. This same imbalance of ERK- and JNK and p38-cascades is also associated with apoptosis induced by growth factor withdrawal.

Ceramide and its downstream metabolites plays role in a number of pathological states: cancer, neurodegeneration, diabetes, microbial pathogenesis, obesity, inflammation.

5.2. Ceramide 1-phosphate

Ceramide 1-phosphate (C1P) is formed from ceramide by the action of a specific ***ceramide kinase (CerK),*** which is distinct from the sphingosine kinases that synthesize sphingosine-1-phosphate. CerK can be activated by different agonists, including interleukin 1-β, macrophage colony stimulating factor, or calcium ions.

C1P has antiapoptotic properties and also is an ***important mediator of the inflammatory response, by stimulating the release of arachidonic acid through activation of cytosolic phospholipase A2, the initial rate-limiting step of eicosanoid biosynthesis***. Most of the effects of C1P so far described seem to take place in intracellular compartments; however, the recent observation that C1P stimulates cell migration implicates a specific plasma membrane receptor that is coupled to a Gi protein. Therefore, C1P has dual regulatory capacity acting as an intracellular second messenger to regulate cell survival, or as extracellular receptor ligand to stimulate chemotaxis.

5.3. Sphingosine

Sphingosine is generated from ceramide by ***ceramidases*** (CDases). ***Sphingosine as a ceramide inhibits the Erk-cascade and activates JNK- and p38-cascades, that resultated in cell death (apoptosis).***

5.4. Sphingosine-1-phosphate

Sphingosine-1-phosphate is formed by sphingosine phosphorylation via two kinases - ***sphingosine kinase type 1*** (SK1) and ***sphingosine kinase type 2*** (SK2). Several growth factors and interleukins mediate the phosphorylation and translocation of SK1 from the cytoplasm to the plasma membrane.

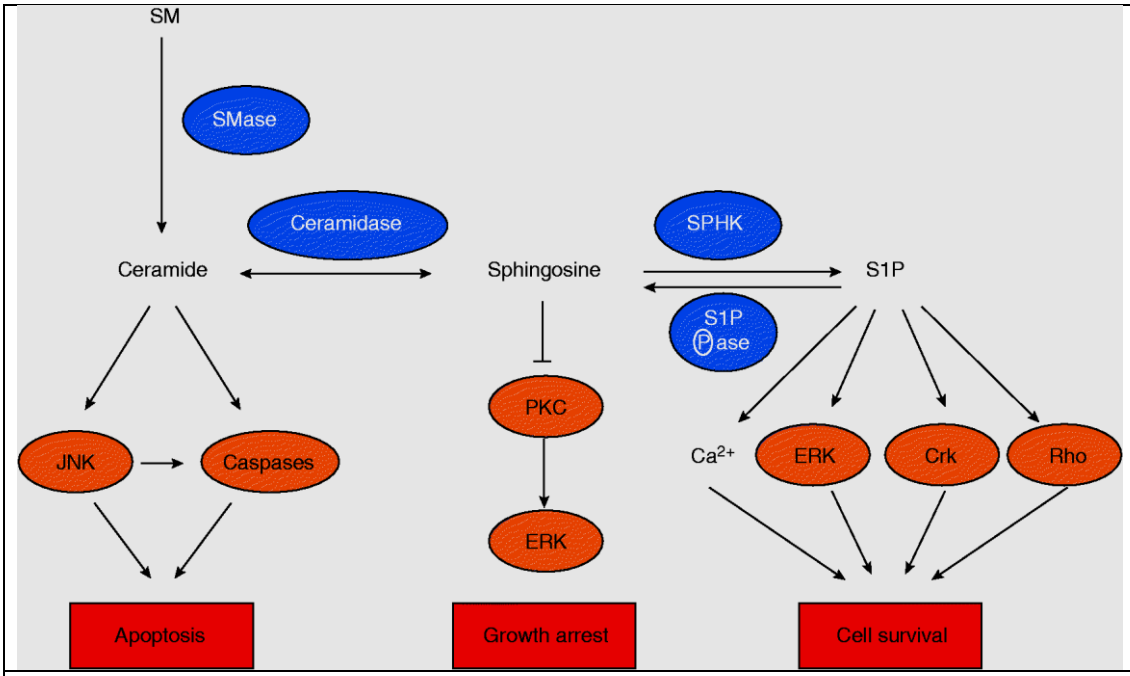
SK/S1P have been shown to be tumor-promoting molecules, because S1P signaling is implicated in cell proliferation, migration, angiogenesis, and autophagy - all processes that facilitate cancer progression, so elevated levels of these molecules have been observed in different cancer and tumor tissues. Overexpression of SKs inhibits apoptosis and blocks cell death induced by anticancer drugs, such as sphingosine and TNF-α, and downregulation of this kinases enhanced sensitivity to anticancer treatment. Sphingosine-1-phosphate also plays role in health and disease by affecting cardiac function, vascular development, immune cell function, inflammation and Alzheimer's disease.

Sphingosine-1-phosphate may also be synthesised on the inner leaflet of the plasma membrane and it is able to cross this via the action of at least two transporter proteins (from the ATP-binding cassette (ABC) family) to interact with specific receptors on the surface of the same cell or on nearby cells, or it can be transported in plasma to more distant tissues.

As extracellular receptor ligand S1P binds to a family of five G protein-coupled receptors (S1PR1-5, = EDG1-5). S1P may also function inside the cell independently of S1P receptors.

There are two types of S1P-signal termination. It can occure by the action of specific phosphatases (***spingosine-1-phosphate phosphatases, SPP1*** and ***SPP2***), located in the endoplasmic reticulum. The balance between these catabolic activities and those of the sphingosine kinases is tightly regulated. The second pathway is the cleaving of S1P irreversibly by the enzyme ***sphingosine-1-phosphate lyase***, which is also located in the endoplasmic reticulum, into fatty aldehydes and phosphoethanolamine. The reaction with sphingosine-1-phosphate lyase, which is found in many different organs, especially lymphoid tissues, but not in platelets or erythrocytes, reduces the cellular levels of sphingosine and ceramide and is ultimately the means by which all sphingolipids are removed from cells.

S1P is a potent messenger molecule with very different functions from ceramide and sphingosine. ***S1P regulates apoptosis through activating ERK- and inhibiting JNK-cascades***. ***Ceramide induces apoptosis by activating JNK-pathways and inhibiting ERK-cascade***. Due to the opposing effects of S1P and ceramide, which can be inter-convertible within cells, on the induction of apoptosis, the ***dynamic balance between S1P and ceramide/sphingosine may therefore determine the cell fate***. The balance between ceramide/sphingosine and S1P forms ***a sphingolipid rheostat model*****.** Thus, agents that regulate the inter-conversion of ceramide-sphingosine-S1P may direct the cell toward either an apoptotic or a survival program depending on the levels of ceramide/sphingosine and S1P, respectively. It leads to cell death when this balance moves towards ceramide or sphingosine, but to cell survival or proliferation when S1P levels are increased. Reduction of S1P level (by inhibition of SK or activation of S1P-phosphatase and/or S1P-lyase), that leads this rheostat towards ceramide/sphingosine, might provide a potential target for cancer therapies. So, specific SKs inhibitors, antagonists of S1P receptor subtypes, inductors and activators of S1P-lyase and S1P-phosphatase prevent tumor growth and vascularization, angiogenesis and metastasis as well as induction of apoptosis.



SM – sphingomyelinase; SPHK - sphingosine kinase; S1Pase - spingosine-1-phosphate phosphatase

5.1. Test questions

1. ***Sphingosine is synthesized from***

A)Palmitoyl CoA and Choline

B)Palmitoyl CoA and ethanolamine

C)Palmitoyl CoA and serine
D)Acetyl CoA and choline

2. ***Which of these compounds is an important mediator of the inflammatory response, by stimulating the release of arachidonic acid through activation of cytosolic phospholipase A2, the initial rate-limiting step of eicosanoid biosynthesis?***

a) Ceramide; b) Ceramide-1-phosphate; c) Sphingosine; d) Sphingosine-1-phosphate; e) Sphingomyeline

3. ***Which of these compounds of sphingolipids metabolism may function inside the cell as a second messenger and also is able to bind G-coupled membrane receptors as a extracellular ligand?***

a) Ceramide; b) Ceramide-1-phosphate; c) Sphingosine; d) Sphingosine-1-phosphate; e) Sphingomyeline

4. ***Sphingolipid rheostat***

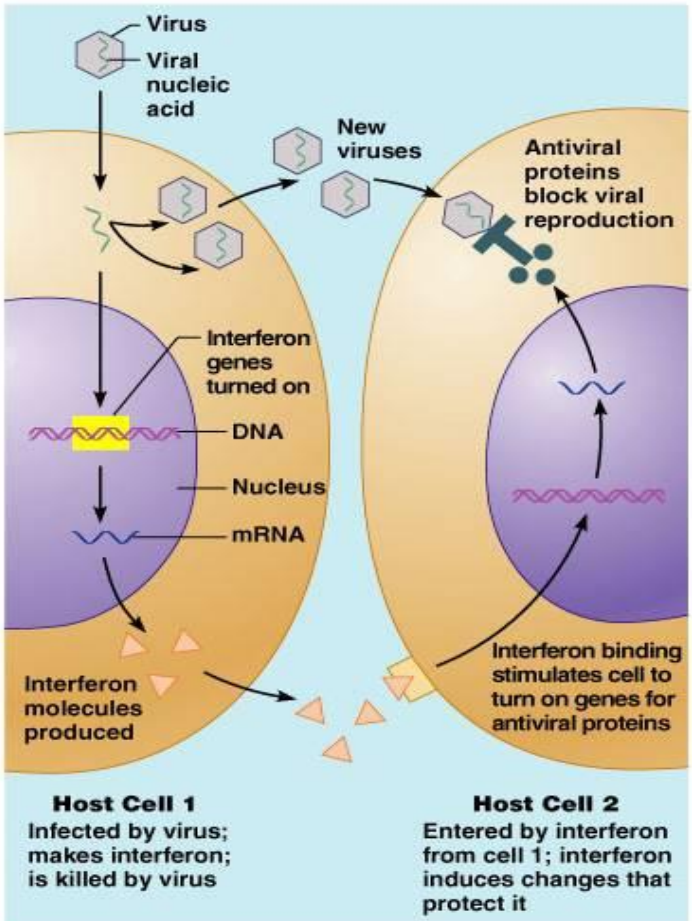
Proapoptotic sphingolipids derivatives	***Antiapoptotic sphingolipids derivatives***

5. Sphingosine-1-phosphate is cleaved irreversibly by the enzyme _____ forming phosphoethanolamine and hexadecanol

6. Interferon signaling

In 1957 Isaacs and Lindenmann did an experiment using chicken cell cultures and found a ***substance that interfered with viral replication and was therefore named interferon (IFN).*** Interferon is the first defence against viral infection as it activates the target cell antiviral defence to block the viral replication and enhances T cell recognition of the infected cell.

Interferons are small proteins released by macrophages, lymphocytes and tissue cells infected with a virus. When a tissue cell is infected by a virus, it releases interferon. Interferon will diffuse to the surrounding cells. When it binds to receptors on the surface of those adjacent cells, they begin the production of proteins that prevent the synthesis of viral proteins.



There are two main types of interferons.

<u>TYPE I interferon</u> is produced after binding viral PAMP and PRR. The main interferons of this group are ***IFN-α*** **(***leukocyte interferon***)** which is produced by virus-infected leukocytes and ***IFN-β*** **(***fibroblast interferon***)** which is produced by virus-infected fibroblasts, or virus-infected epithelial cells. They have different binding affinities but similar biological effects. The main function of IFN-α and IFN-β is antiviral activity; they also have immunomodulatory effects, for example, can increase the expression of MHC class I molecules, enhancing the cell ability to present antigen and making the cell a better target for cytotoxic T cells.

IFN-α and IFN-β bind to interferon cell receptors type 1 and both encoded on chromosome 9. There are 13 different variants of IFN-α, which are similar in amino acid sequence and differ

by a lot of variation in glycosylation and same amino acid number. IFN-β in turn encoded by one gene.

But type I of IFN is more than IFN-α and IFN-β. There are IFN- ω, - ν, -ε, –κ, -τ, -δ, and -ζ too. IFN-ω encoded by many non-primate placental mammals which express multiple IFN-ω subtypes. Only ruminants encode IFN-τ, a variant of IFN-ω. IFN-ζ is found only in mice, while a structural homolog IFN-δ is found in a diverse array of non-primate and non-rodent placental mammals. Most but not all placental mammals encode functional IFN-ε and IFN-κ genes. IFN-ν is functional in the domestic cat genome.

There is one IFN of TYPE II interferon - IFN-γ (*immune interferon****) - which is produced by certain activated T-cells and NK cells. It is made in response to antigen (including viral antigens) or mitogen stimulation of lymphocytes.*** IFN-γ binds to type 2 receptors and its genes are encoded on chromosome 12. Initially believed that ***T helper cell type 1 lymphocytes, cytotoxic lymphocytes and natural killer cells only produced IFN-γ, now evidence that B cells, natural killer T cells and professional antigen-presenting cells secrete IFN-γ also***. ***IFN-γ production follows activation with immune and inflammatory stimuli rather than viral infection. This production is controlled by cytokines secreted by interleukin 12 and 18.*** The main function of IFN-γ is immunomodulatory activity, for example, IFN-γ increases the expression of class 2 MHC molecules by macrophages to help promote antigen presentation to T cells; they also have antiviral activity.

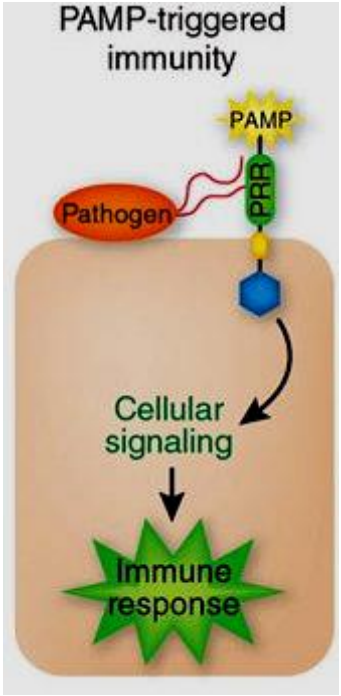
	Earlier Terms	**Principal Cell Source**	**Principal Effects**
INF α and β	**type I IFNs**	**Macrophages, neutrophils, other somatic cells**	• **Antiviral effect** • **Induction of class I MHC on all somatic cells** • **Activation of macrophages & NK cells**
INF γ	**type II IFNs**	**Activated TH1 & NK cells**	• **Induction of class I MHC on all somatic cells** • **Induction of class II MHC on APCs & somatic cells** • **Activation of macrophages, neutrophils & NK cells** • **Promotion of cell-mediated immunity** • **Induction of high endothelial venules** • **Antiviral effect**

6.1. Production of I type IFN: Recognition of antigens

Production of I type IFN predominantly occurs in response to microbes, such as viruses and bacteria, and their products. Binding of ***pathogen associated molecular patterns (PAMPs)*** - molecules uniquely found in microbes — viral glycoproteins, viral RNA, bacterial endotoxin (lipopolysaccharide), bacterial flagella, CpG motifs - by ***pattern recognition receptors (PRR)***, can trigger release of IFNs. So, PAMPs recognized by PRRs.

There are three broad classes of PRRs based on expression profile, localization, function:

- ***PRRs that signal about infection*** (***Toll Receptor Family and Cytosolic PRR*** such as RIG-I or MDA5), that are expressed externally or internally and its binding activates “pro-inflammatory” signaling pathways. This group of PRRs is needs for IFN production.



PAMP – pathogen-associated molecular patterns: groups of pathogens that share similar structures

PRR – PAMP Recognition Receptors:

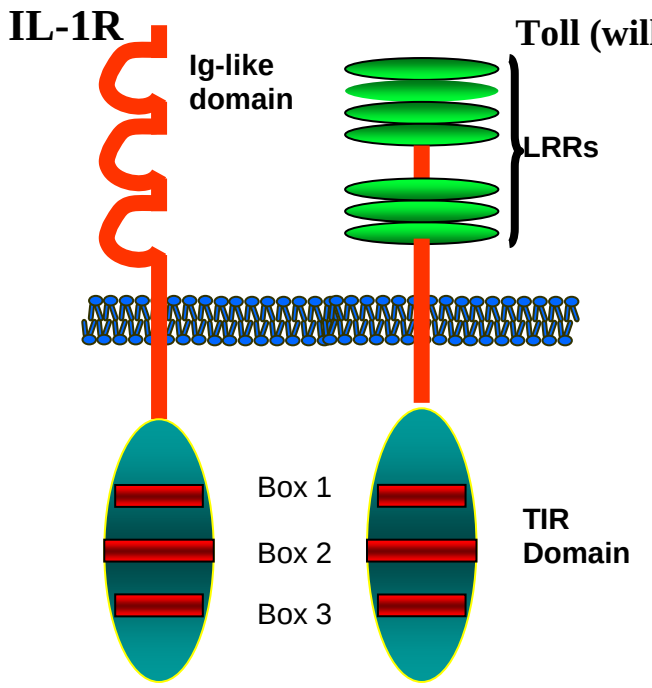
1. **Toll-like receptors (TLRs):**
   - **1,2,4,5,6,10,11 on cell surface**
   - **3,7,8,9 in cell compartments**

2. non-TLRs:

- MDA5; Protein kinase R (PkR); retinoic acid-inducible gene I (RIG-I)

- ***Phagocytic (endocytic) PRR***, that are expressed on the surface of phagocytic cells and *mediate uptake of microbe into phagocytes*;
- ***Secreted PRR***, that are secreted by macrophages, epithelial cells, hepatocytes, activate complement, opsonins and *function as accessory proteins for PAMP recognition.*

Toll-like Receptor (TLR) Family belongs to *interleukin-1 receptors (IL-1Rs) family*. These receptors were first discovered in Drosophila and these molecules are evolutionary conserved among insects and humans. ***Mammalian TLRs have homology to IL-1 receptor in cytoplasmic domain (the Toll-IL1-R or TIR domain), but extracellular domain which contains a leucine-rich repeat (LRR) differs from it because has three immunoglobulin domains***. LRR domains of TLRs may be directly involved in the recognition of PAMPs.



Toll (will become TLRs)

- **Toll receptor has an extracellular region which contains leucine rich repeats motifs (LRRs)**
- **Toll receptor has a cytoplasmic tail which contains a Toll interleukin-1 (IL-1) receptor (TIR) domain**

There is thirteen receptors (1-13) identified in mice and humans now, that are expressed differentially on immune cells (low level) and also on other cell types (e.g., endothelial cells) in respond to different stimuli. Expression of TLRs is modulated in response to stimuli – i.e., they are inducible.

TLRs are transmembrane proteins which bind to next recognized motifs (PAMPs):

-lipopolysaccharide (LPS) from Gram-negative cell walls;

-peptidoglycans from the cell walls of both Gram-negative and Gram-positive bacteria;

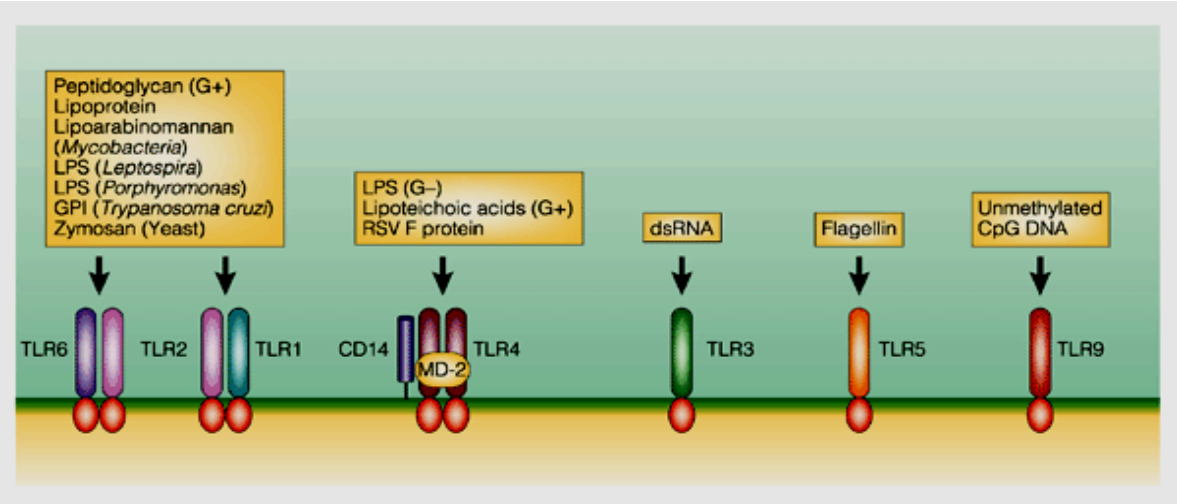
-viral double-stranded RNA;

-CpG-rich bacterial DNA;

- zymozan from yeast;

- flaggelin from flaggelated bacteria.

TLR1, TLR2, TLR4, TLR5, TLR6 localize on plasma membrane, where as TLR3, TLR7, TLR8 and TLR9 are on endosomal and lyzosomal membranes. ***In general after stimulation they functions forming homo or hetero dimers***. Individual TLR can interact with several structurally unrelated ligands of exogenous and endogenous origin.



Activation of signal transduction pathways by TLRs induces genes that function in host defense – their products are pro-inflammatory cytokines such as IFNs, IL-1, TNF-α, IL-12, chemokines, MHC and costimulatory molecules, iNOS and antimicrobial peptides that directly destroy pathogens.

TLRs have "shared" (which used by all TLRs and as essential components has adaptor proteins MyD88, TRAF6 and protein kinase IRAK *(from "interleukin-1 receptor-associated kinase")* ***and "specific" signal transduction pathways.***

TLR responses are initiated by ligand-induced dimerization. TLRs in general form homo or hetero dimers in order to induce an effective signaling cascade ***leads to the recruitment of primary adaptor molecules – depending on the TLR involved.***

As points above, the ***majority of the adaptor proteins identified to date contain a TIR domain. So the TRL-adaptor interactions are mediated by homotypic associations between TIR domains of the receptor and the adaptor.***

The core TLR signaling pathways use MyD88 as the primary adaptor protein and results and NF-kB and MAPK activation and the secretion of a core panel of cytokines. So, TLR2 (in combination with TLR1 or TLR6) and TLR4 utilize ***MyD88*** (*Myeloid differentiation factor 88*) as primary adaptor to activate NF-kB and pro-inflammatory cytokine secretion such as IFNs (main α), IL-1, TNF-α, IL-12.

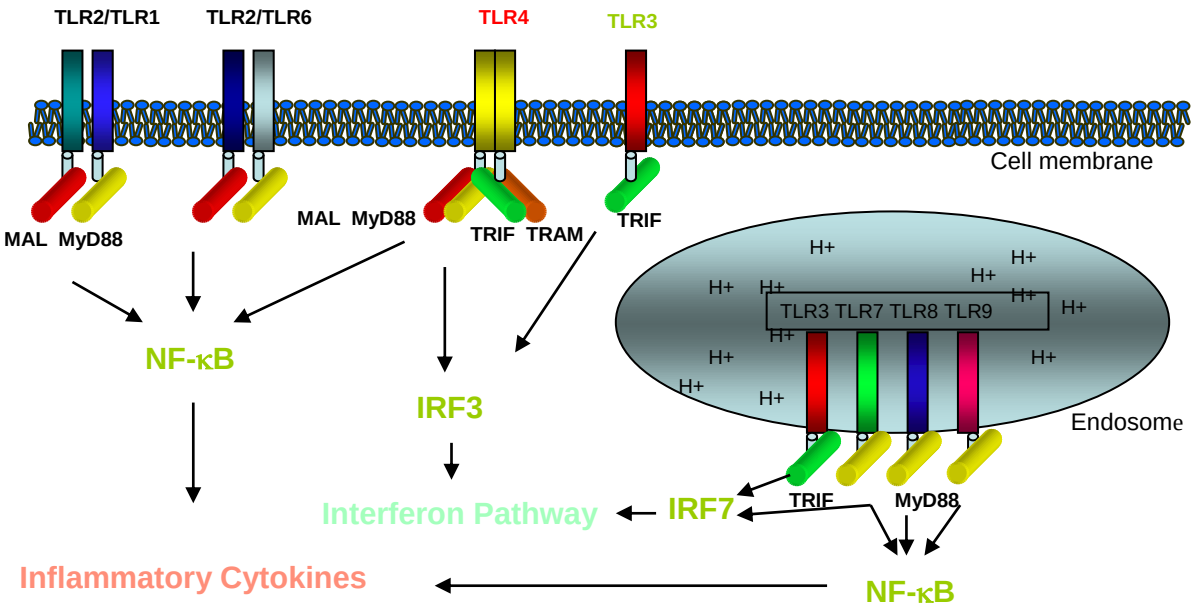
Although most TLRs utilize the MyD88-dependent pathway, a subset of TLR responses remains MyD88 independent. These pathways use a variety of different signaling networks that employ a variety of different adaptors.

For example, ***TLR4*** uses ***TRIF*** (*Toll/IL-1 receptor domain-containing adaptor inducing IFNβ*) and the ***TRAM*** (*TRIF-related adaptor molecule*) to activate IRF3 and the IFN pathway. TLRs 3, 7, 8 and 9, which typically localize to endocytic compartments, detect a variety of nucleic acids and activate the IFN pathway. ***TLR3*** is important for inducing interferon in response to the presence of double-stranded RNA viruses; the ligand for this receptor is double-stranded RNA (dsRNA). It utilizes TRIF, but not TRAM, to activate IRF3; ***TLRs 7, 8 and 9*** trigger inflammatory cytokine secretion and the IFN pathway (main α) through MyD88 and involve IRF-7.

Interferon regulatory factors (IRFs) are proteins which regulate transcription of interferons. These molecules contain a conserved N-terminal region of about 120 amino acids, which folds into a structure that binds specifically to the interferon consensus sequence (ICS), which is located upstream of the interferon genes. The remaining parts of the interferon regulatory factor sequence vary depending on the precise function of the protein.

IRF3 has been characterized and shown to contain several functional domains including a nuclear export signal, a DNA-binding domain, a C-terminal IRF association domain and several regulatory phosphorylation sites. IRF3 is found in an inactive cytoplasmic form that upon serine/threonine phosphorylation forms a complex with CREBBP. This complex translocates to the nucleus and activates the transcription of interferons alpha and beta (main β), as well as other interferon-stimulated genes**.**

IRF7 has been shown to play a role in the transcriptional activation of virus-inducible cellular genes, including the type I interferon genes (main α). Constitutive expression of IRF7 is largely restricted to lymphoid tissue, largely plasmacytoid dendritic cells, whereas IRF7 is inducible in many tissues. The IRF7 pathway was shown to be silenced in some metastatic breast cancer cell lines, which may help the cells avoid the host immune response. Restoring IRF7 to these cell lines reduced metastases and increased host survival time in animal models. IRF7 has been shown to interact with IRF3.

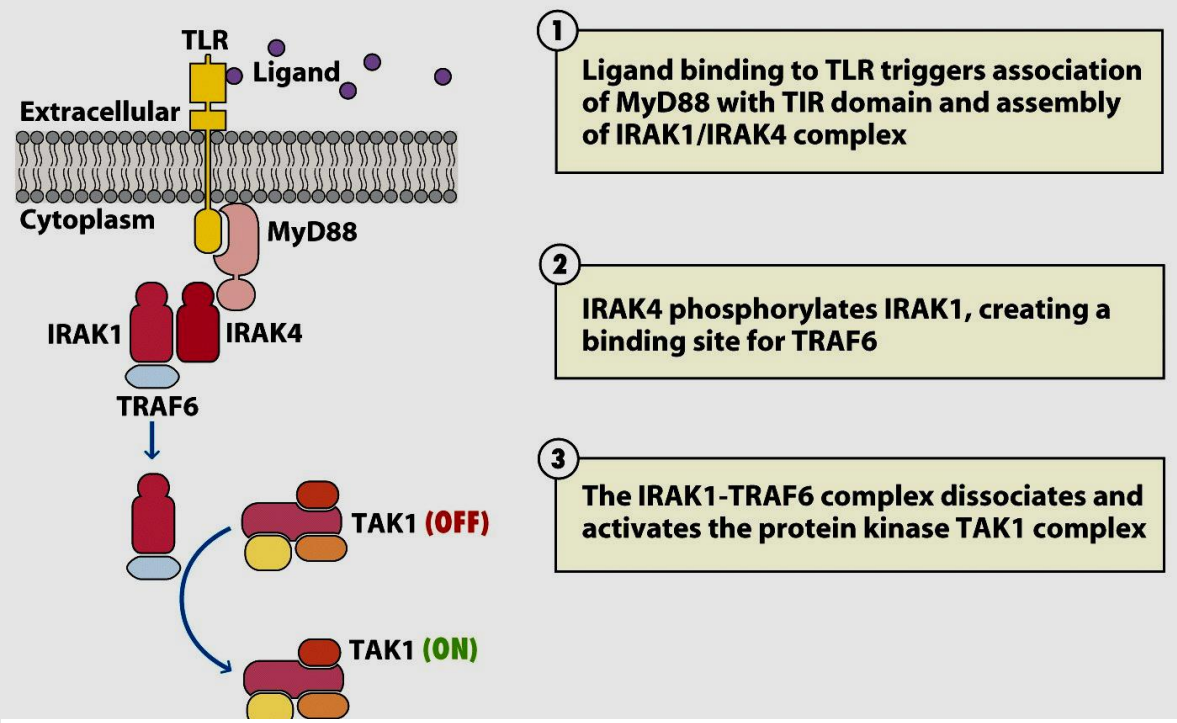


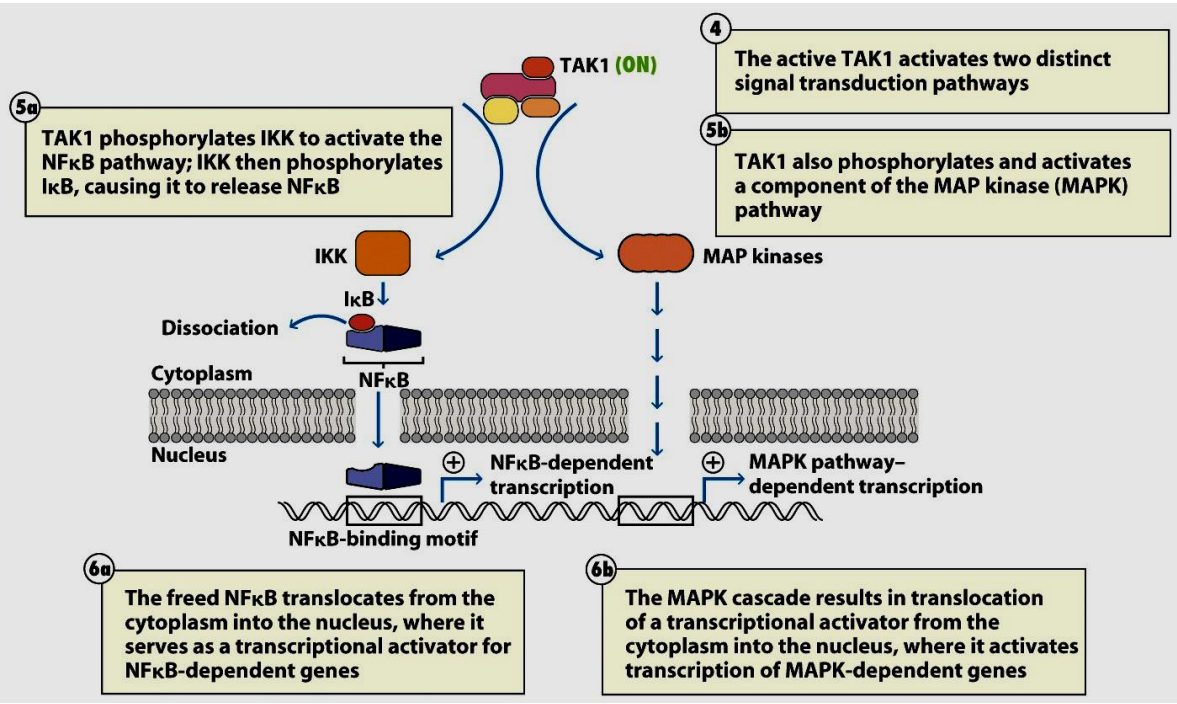
Example: TLR-4 signaling

TLR4 predominently present on immune cells and is the receptor for LPS (lipopolysaccharides) which are major components of outer membrane of Gram-negative bacteria. So, binding these compounds to TLR4 leads to production of a variety of proinflammatory cytokines (TNF, IFN-β, NO).

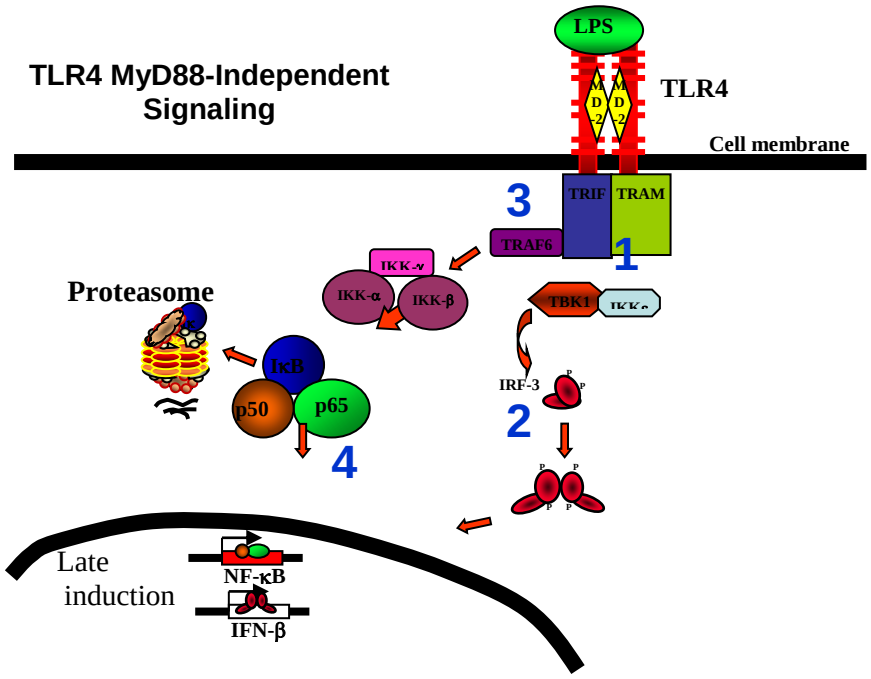
Upon LPS binding TLR4 homodimerizes and initiates the ensuing signaling cascade that bifurcates into two signaling pathways: MyD88 (Myeloid differentiation primary-response protein 88) dependent and independent pathways.

In first, ligand binding to TLR triggers association of TLR TIR domain with MyD88 TIR domain and assembly of IRAK1/IRAK4 complex to MyD88. IRAK4 phosphorylates IRAK1, creating a binding site for TRAF6. The IRAK1-TRAF6-complex then dissociates and activates the protein kinase TAK1 oligomeric complex. The active TAK1 activates two distinct signal transduction pathways. So, it phosphorylates inducible inhibitor NF-kB kinase (IKKi) to activate the NF-kB pathway; IKKi then phosphorylates IkB (inhibitor NF-kB kinase) causing it to release NF-kB which activates transcription of NF-kB-dependent genes (for example, IFN-β). Or TAK1 phosphorylates and activates MAPK (JNK-cascade) that resulting in activation of transcriptional factor AP-1 and MAPK-dependent genes transcription (for example, IFN-β and –γ).





In the MyD88-independent pathway (TRIF & TRAM-dependent pathway) TRIF and Toll-receptor-associated molecule (TRAM) recruit the kinase TBK-1/IKKi heterodimer (1). This complex phosphorylates IRF-3 (interferon-regulation factor 3), which dimerizes and translocates to the nucleus leading to the activation of IFN-β genes (2). TRIF binds to TRAF-6 via the N-terminal TRAF6-binding domain leading to the activation of the signalosome – followed by the ubiquitination and the degradation of IkB, culminating in the late phase NF-kB activation (3, 4). TRAM can also activate IRF-7 which in turn induces the production of IFN-alpha.



Cytosolic PRRs: RIG-1, MDA5 & PkR

RIG-I (retinoic acid-inducible gene 1) and ***MDA5 (Melanoma Differentiation-Associated protein 5) are the RIG-I-like receptors with dsRNA helicase activity, that function as a pattern recognition receptor that is a sensor for viruses.***

RIG-I typically recognizes short (< 4000nt) 5′ triphosphate dsRNA where as MDA5 typically recognizes dsRNA that is over 2000 nts in length. These PRRs signaling needs in IPS1 (IFN-β-promoter stimulator 1, = VISA) as adaptor protein.

Protein kinase receptor (PkR) is activated upon binding to dsRNA (viruses), can blocks viral and cellular protein synthesis (through phosphorylation of eIF2a) and activates NF-kB, MAP kinases, STATs and IRF signaling pathways which resulting in inducing of infected cells apoptosis and IFNα/β production.

6.2. Interferons signaling

6.2.1. JAKs/STATs signalling

IFN-α and IFN-β can be induced and released within hours of infection. IFN molecules bind to specific receptors on the neighbouring cells and induce the production of antiviral proteins which are then activated when they bind to dsRNA. So ***IFN-α and IFN-β are inductors of antiviral proteins production, dsRNA is these antiviral proteins activator***.

Receptor for IFN type I (IFN-α and IFN-β) is known as the ***IFN-α receptor*** (***IFNAR***) that consists of IFNAR1 and IFNAR2 chains.

Interferon type II binds to ***IFNGR*** that consists of IFNGR1 and IFNGR2 chains.

Interferon type III transmits signal through a ***receptor complex consisting of IL10R2 (also called CRF2-4) and IFNLR1 (also called CRF2-12)***.

By interacting with their specific receptors, IFNs through cytosolic tyrosine protein kinases JAKs family activate signal transducer and activator of transcription (STAT) complexes. STATs are a family of transcription factors that regulate the expression of certain immune system genes. Some STATs are activated by both type I and type II IFNs. However each IFN type can also activate unique STATs.

STAT activation is initiated by the most well-defined cell signaling pathway for all IFNs, the classical Janus kinase-STAT (JAK-STAT) signaling pathway. JAKs associate with IFN receptors and, following receptor engagement with IFN, phosphorylate both STAT1 and STAT2, which after that can form dimer. As a result, an IFN-stimulated gene factor 3 (ISGF3) complex forms and moves into the cell nucleus. This complex contains STAT1, STAT2 and a third transcription factor called IRF9. Inside the nucleus, the ISGF3 complex binds to specific nucleotide sequences called IFN-stimulated response elements (ISREs) in the promoters of certain genes, known as IFN stimulated genes (ISGs). Binding of ISGF3 and other transcriptional complexes activated by IFN signaling to ISRE induces transcription of ISGs.

Additionally, ***STAT homodimers or heterodimers form from different combinations of STAT-1, -3, -4, -5, or -6 during IFN signaling; these dimers initiate gene transcription by binding to IFN-activated site (GAS) elements in gene promoters***.

Type I IFNs act through JAK1 and TYK Janus kinases, formation of STAT homodimers or heterodimers and can induce expression of genes with either ISRE or GAS elements. Type II IFN acts through JAK1 and JAK2 Janus kinases, STAT1 homodimers formation and causes gene induction only in the presence of a GAS element.

6.2.2. Other signaling cascades

Both type I and type II IFNs activate a member of the CRK family of adaptor proteins called CRKL, a nuclear adaptor for STAT5 that also regulates signaling through the C3G/Rap1 pathway.

Type I IFNs further activate *p38 mitogen-activated protein kinase* (MAP kinase) to induce gene transcription. Antiviral and antiproliferative effects specific to type I IFNs are resulted from p38 MAP kinase signaling.

The *phosphatidylinositol 3-kinase* (PI3K) signaling pathway is also regulated by both type I and type II IFNs.

6.2.3. Virus defense from IFNs effects

Viruses have mechanisms to resist interferon activity. ***These mechanisms can occur by preventing IFN production, blocking downstream signaling events that occur after the IFN binds to its receptor, inhibiting the functions of proteins that are induced by IFN***. Viruses that inhibit IFN-signaling include Japanese Encephalitis Virus (JEV), dengue type 2 virus (DEN-2) and viruses of the herpesvirus family, such as human cytomegalovirus (HCMV) and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8).

Reducing IFN-α activity may prevent signaling via STAT1, STAT2, or IRF9 (as with JEV infection) or through the JAK-STAT pathway (as with DEN-2 infection).

Several poxviruses encode soluble IFN receptor homologs — like the B18R protein of the vaccinia virus that binds to and prevents IFN interacting with its cellular receptor, impeding communication between this cytokine and its target cells.

Some viruses can encode proteins that bind to double-stranded RNA (dsRNA) to prevent activation of IFN-induced antiviral proteins or minimize the ability of interferon to induce antiviral protein production from interferon stimulated genes (ISGs).

6.3. Effects of IFN type I

6.3.1. Antiviral effects IFN Type I

Type I IFN mediates a wide range of biological effects, including direct antiviral, antiproliferative, antiangiogenic and antitumour activities.

Interferons increase expression of interferon-stimulated genes (ISGs) that induce production of hundreds of other proteins that have roles in combating viruses.

So, cells in response to interferon produce large amounts of an enzyme known as ***protein kinase R (PKR).*** PKR is induced by interferon in a latent state. Binding to dsRNA is believed to activate PKR by inducing dimerization and subsequent auto-phosphorylation reactions. ***This enzyme phosphorylates a protein known as eIF-2 in response to new viral infections; eIF-2 is a eukaryotic translation initiation factor that forms an inactive complex with another protein, called eIF2B, to reduce protein synthesis within the cell.*** This inhibits further cellular mRNA translation, thereby preventing viral protein synthesis. Both cellular and viral translation is inhibited, so PKR also causes apoptosis.

Active PKR is also able to mediate the activation of the transcription factor NF-kB by phosphorylating its inhibitory subunit, IkB. Activated NF-kB upregulates the expression of Interferon cytokines, which work to spread the antiviral signal locally **(***II state INF I production*).

Viruses have developed many mechanisms to outfox the PKR mechanism. It may be done by decoy dsRNA, degradation, hiding of virus dsRNA, dimerization block, dephosphorylation of substrate or by a pseudosubstrate. For instance, Epstein - Barr virus (EBV) uses the gene EBER-1 to produce decoy dsRNA. This leads to cancers such as Burkitt's lymphoma, Hodgkin's Disease, nasopharyngeal carcinoma and various leukemias

Cells in response to interferon also produce large amounts of an enzyme known ***2'-5' oligo(A) synthetase*** which produces strands of ***2'-5' adenylic acid*** (versus the normal 3'-5'; ATP-dependent); these strands induce activation of ***RNase L*** which then forms a dimer and proceeds to degrade all RNA within the cell.

RNAse L destroys RNA within the cells to further reduce protein synthesis of both viral and host genes. Inhibited protein synthesis destroys both the virus and infected host cells. Mutations in this gene have been associated with predisposition to prostate cancer and this gene is a candidate for the hereditary prostate cancer 1 (HPC1) allele. In 2002, the "hereditary prostate cancer 1" locus (HPC1) was mapped to the RNase L gene, implicating it in the development of prostate cancer. Impairments of the 2'-5' oligoadenylate (2-5 A) synthetase/RNase L pathway in chronic fatigue syndrome (CFS) also have been investigated.

Interferon-inducible Mx proteins (myxovirus proteins) belong to the family of large GTPases and are highly homologous with dynamins within their GTP-binding domain. Interferon-induced GTP-binding cytoplasmic protein MxA is a protein that in humans is encoded by the *MXA* gene. ***MxA tightly interacts with viral nucleocapsids by binding to the nucleoprotein component and also blocks viral RNA polymerase***. It has effects on Influenza virus, Thogoto virus, Vesicular stomatitis virus, Measles virus, Hantaan virus, Coxsackievirus, Semliki Forest virus.

Human MxB protein is found both in the cytoplasm and in the nucleus and has not been found to have any antiviral activity.

Double-stranded RNA-specific adenosine deaminase (ADAR) is an enzyme that in humans is encoded by the *ADAR* gene. ***This enzyme destabilizes double stranded RNA through conversion of adenosine to inosine. Inosine has structural similarity to guanosine (G), so Inosine selectively base pairs with cytidine (C) and therefore functions in translation and replication as guanosine (G). In addition, conversion of adenosine present in A:U pairs in duplex structures leads to I–U mismatches, that destabilizes the duplex structure and leads to more stable I:C pairs***.

6.3.2. Immunomodulatory activity of IFNs I type

Type I IFN also has a broad spectrum of immunomodulatory activities on both innate and adaptive immune responses by upregulation of major histocompatibity complex (MHC) class-I, increasing natural killer cell activity, enhancement of dendritic cell (DC) activation. But the main biological activity of Type I IFN appears to be antiviral.

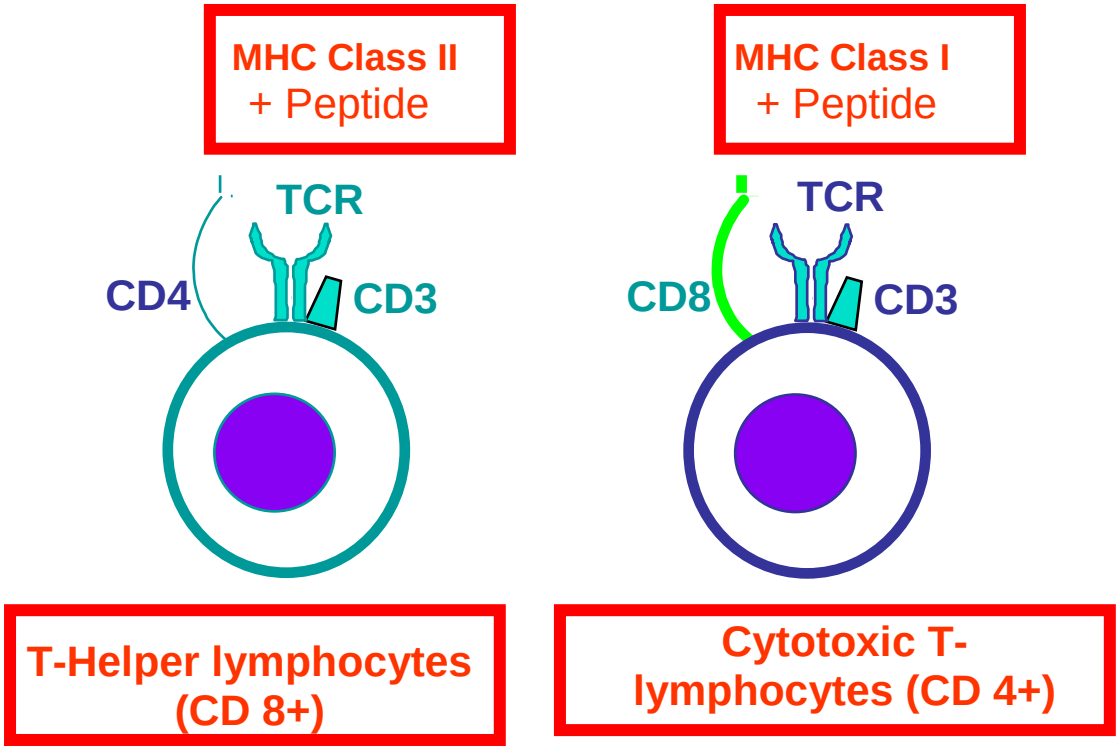
Induction of expression of major histocompatibility complex molecules - MHC I – on all somatic cells causes presentation of viral peptides to cytotoxic T cells and natural killer cells, thereby increasing the recognition and killing of infected cells.

6.4. Type II IFN effects

In contrast to IFN-α and IFN-β, which can be expressed by all cells, IFN-γ is secreted by T helper cells (specifically, Th1 cells), cytotoxic T cells (TC cells) and NK cells. ***IFN-gamma has antiviral and antiparasitic activities and also inhibits the proliferation of a number of normal and transformed cells. The main biological activity of IFN-gamma appears to be immunomodulatory in contrast to the other interferons that are mainly antiviral.***

Among the effects are:

1. Promotion of NK cell activity;
2. Increasing of antigen presentation and lysosome activity of macrophages;
3. Activation of inducible Nitric Oxide Synthase iNOS;
4. Induction of the production of IgG2a and IgG3 (antibodies) from activated plasma B cells;
5. Promotion of Th1 differentiation, ultimately leading to cellular immunity: cytotoxic CD8+ T-cells and macrophage activity - while suppressing Th2 differentiation which would cause a humoral (antibody) response;
6. Causing of all somatic cells to increase expression of class I MHC molecules as well as class II MHC on antigen presenting cells - specifically through induction of antigen processing genes in addition possibly to the direct upregulation of MHC heavy chains and B2-microglobulin itself. ***Higher MHC II expression on antigen presenting cells increases presentation of viral peptides to helper T cells; these cells release cytokines (such as more interferons and interleukins, among others) that signal to and co-ordinate the activity of other immune cells;*** For example, the helper T cells then help to trigger an appropriate immune response which may include localized inflammation and swelling due to recruitment of phagocytes or may lead to a full-force antibody immune response due to activation of B cells.



7. Promotion of adhesion and binding required for leukocyte migration;
8. Induction of the expression of intrinsic defense factors, representing directly antiviral effects;

9. IFN-γ is the primary cytokine which defines Th1 cells: Th1 cells secrete IFN-γ, which in turn causes more undifferentiated CD4+cells (Th0 cells) to differentiate into Th1 cells, representing a positive feedback loop — while suppressing Th2 cell differentiation.

6.5. Test questions

1. In some insects, such as Drosophila, fungal cell wall elements can activate the protein Toll. What is Toll's function?

a) acts as a receptor that, when activated, signals synthesis of antimicrobial peptides
b) functions directly to attack the fungi presented to it
c) produces antimicrobial peptides by interaction with chitin
d) secretes special recognition signal molecules that identifies specific pathogens
e) causes some hemocytes to phagocytize the pathogens

2.

	Interferon type (I or II)
α	
β	
γ	
Generates by macrophages, neutrophils, other somatic cells	
Generates by activated TH1 & NK cells	
Is produced as a result of activation immune cells with immune and inflammatory stimuli	
Is produced after binding viral PAMP and PRR	
The main effect is antiviral action	
The main effect is immunomodulatory action	
Induction of class I MHC on all somatic cells	
Induction of class II MHC on APCs	

3. Inductor of 2,5-oligoadenylate synthase and protein kinase R is _____, and their activator is _____.

4.Which of these interferon-induced proteins destabilizes double stranded RNA through conversion of adenosine to inosine?
a) interferon; b) dsRNA; c) 2,5-oligoadenylates; d) 2,5-oligoadenylate synthase; e) protein kinase R; f) PAMP; g) ADAR; h) MxA; i) RNAse L

5. **Cells, specific for the MHC class _____ complex will recognize and kill the presenting cell; cells, specific for the MHC class _____ complex will recognize and help to trigger an appropriate immune response by macrophage activation or B cells activation and B cell antibody secretion.**
(I; II)

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