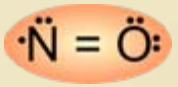
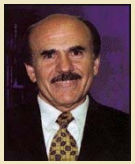
Syntázy oxidu dusnatého (NO synthases - NOSs)

Oxid dusnatý (NO)



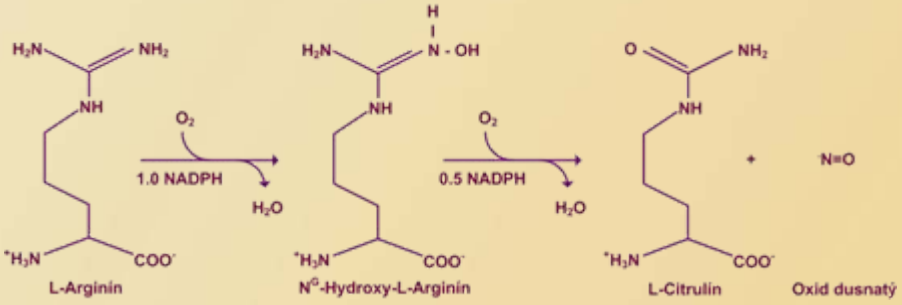
- Zloženie: atóm kyslíku a dusíku viazané dvojnou väzbou
- Atom kyslíku nesie 2 páry (nevazebných) elektónov
- Atom dusíku má 1 pár nevazebných elektrónov a jeden nepárový elektrón

Robert F Furchgott
1916
Dept. of Pharmacology,
SUNY Health Science Center
New York



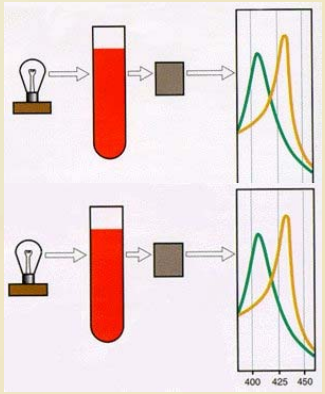
Louis J Ignarro,
1941
Dept. of Molecular and
Medical Pharmacology
UCLA School of
Medicine
Los Angeles

Ferid Murad
1936
Dept. of Integrative Biology
Pharmacology and
Physiology
University of Texas Medical
School, Houston



- V savčích buňkách je NO tvořen oxidací terminálního guanidino dusíku L-argininu molekulárním kyslíkem; kromě NO vzniká L-citrulin.
- Celou komplexní reakci katalyzuje jediný enzym, NO syntáza, která existuje 3 isoformách.

L-arg + O_2 ⟶ NO + L-cit

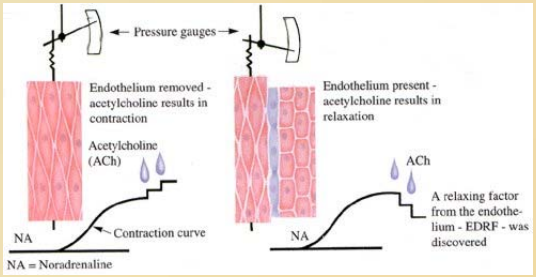


Ignarrova spektrální analýza

Ignarro pomocí spektrální analýzy prokázal, že EDRF je totožný s NO.

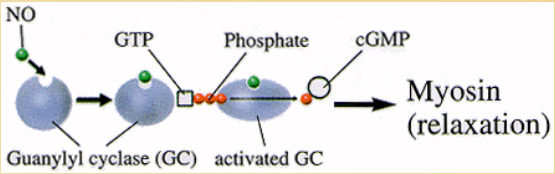
Hemoglobin (žlutý) exponovaný endoteliálním buňkám produkujícím EDRF (konverze oxyhemoglobinu na methemoglobin).

Hemoglobin (žlutý) exponovaný přímo NO. Posun v absorbční křivce je identický (EDRF = NO).



Furchgottův sandwich

Furchgott prokázal, že relaxace cév indukovaná acetylcholinem je závislá na endoteliu. Použil dva kousky aorty, u jednoho odstranil epitelium



Muradova enzymatická aktivace

Murad věděl, že nitroglycerin působí relaxaci hladké svaloviny.
Enzym guanylát cyklasa byla aktivována a indukovala zvýšení cGMP s následnou ralaxací svalu.
Působí nitroglycerin cestou uvolňování NO ???
Probublával NO přes tkáň obsahující enzym – cGMP se zvyšoval.

Něco málo chemie o NO

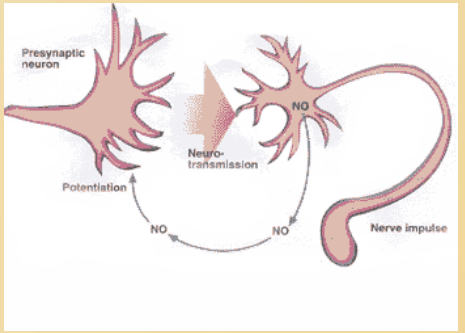
- NO je **radikál** (lichý počet valenčních elektronů, konkrétně 11 - o 1 víc než N_2, o 1 míň než O_2)
- to, že je to radikál, se někdy zdůrazňuje tečkou ($^{\cdot}NO$), to ale není nutné, "radikálovost" je implicitní v označení NO
- z N_2 a O_2 se tvoří jen za specifických podmínek při vysokých teplotách, např. při blesku; taky vzniká ve spalovacích motorech a tepelných elektrárnách
- samovolně se nerozkládá, jen za vyššího tlaku - při něm pozvolna vzniká 2-3 % toxického NO_2 za měsíc (pozor na skladování v bombách!)
- poměrně málo rozpustný ve vodě (~1.7 mmol/l při 25°C), t.j. řádově podobně jako O_2 či N_2
- v přítomnosti kyslíku podléhá **autooxidaci** za vzniku NO_2: $2\ NO + O_2 \rightarrow 2\ NO_2$
- autooxidace je asi 200x rychlejší v roztoku než v plynné fázi
- autooxidace je rychlá (několik sec), je-li NO i O_2 hodně, ale celkem pomalá, je-li NO málo - jako je tomu většinou v tkáních, kde je NO méně než 10 μM (poločas NO tam může být až 500 sec)
- ve vodném roztoku jsou produktem autooxidace nitrity (NO_2^-), pouze v přítomnosti hemoproteinů proběhne oxidace až na nitráty (NO_3^-)
- v přítomnosti **superoxidu** vzniká extrémně rychle **peroxynitrit**:
  $NO + O_2^{\cdot -} \rightarrow {}^{-}OONO$
  $^{-}OONO$ není radikál, ale je velmi reaktivní a cytotoxický
- NO je velmi rychle **inaktivován** oxidací s železem **oxyhemoglobinu** za vzniku NO_3^-

FYZIOLOGIE NO

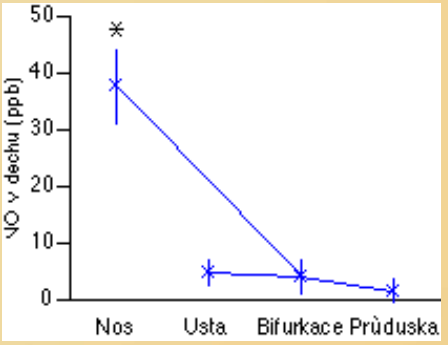
- Neurotransmitter - učení, paměť, spánek, bolest, deprese
- Zabíjí viry, baktérie, parazity, tumory (**iNOS**; indukci inhibují kortokosteroidy)
- Inhibuje mitochondriální respiraci
- Vazodilatace závislá na endotelu (ACh, **eNOS**)
- Nejvíc NO se dělá v nose a paranasálních dutinách
  - Pravděpodobně důležité pro jejich dezinfekci
  - NO lze dobře měřit ve vydechovaném vzduchu
  - Malá část vydechovaného NO pochází z dolních dýchacích cest (lze měřit odebíráním vzorků z úst) - mění se při některých nemocech, např. astma)

ÚČINKY NO NA CÍLOVOU TKÁŇ

- při velkých množstvích obvykle převládá oxidace (O_2, $O_2^{\cdot -}$) za tvorby vysoce **reaktivních, cytotoxických produktů (ONOO$^-$)** - zabíjení i jinak obtížně zlikvidovatelných baktérií (např. Mycobacterium tuberculosis), hub, parasitů a tumorů, inhibice replikace virů
- v nižších koncentracích je oxidace pomalá, většinou převládá regulační působení prostřednictvím aktivace solubilní isoformy enzymu **guanylát cyklázy** (heterodimer obsahující hem, s kterým právě NO interaguje)
- aktivace guanylát cyklázy zvyšuje v cílové buňce koncentraci cyklického guanosin-3',5'-monofosfátu (cGMP), ten zprostředkuje účinky NO



NO sensitizuje presynaptický neuron k silnějšímu signálu. To následně vede k silnější odpovědi postsynaptického neuronu.



Příklad: koncentrace NO ve vydechovaném vzduchu zdravých lidí. Hodnoty jsou nejvyšší při odebírání vzorků z nosu a podstatně nižší v ústech a distálnějších úsecích dýchacích cest. V periferní průdušce je koncentrace NO pod detekčním limitem vysoce citlivé chemiluminiscenční metody (*Chest 110: 930-938; 1996*).

Syntásy oxidu dusnatého

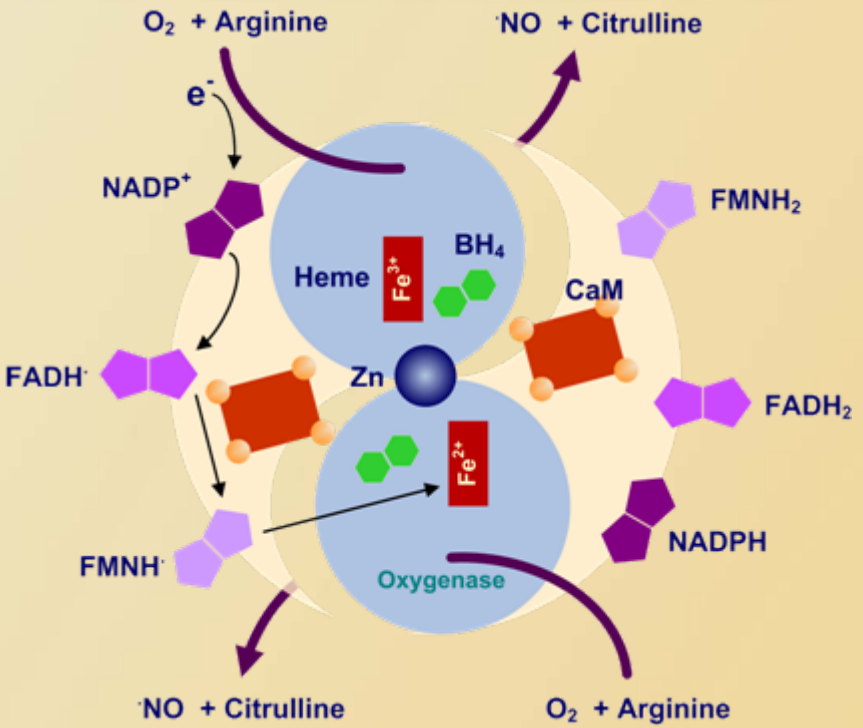
- **neuronální** syntása oxidu dusnatého (NOS1 = nNOS)
- **inducibilní** syntása oxidu dusnatého (NOS2 = iNOS)
- **endotheliální** syntása oxidu dusnatého (NOS3 = eNOS)

Každá z těchto syntáz:

- má rozdílnou tkáňovou distribuci
- lokalizovaná na různých chromozomech

Všetky 3 isoformy NO syntázy

- Sú aktívne ako homodiméry
- Obsahujú v aktívnom centre hem
- Sú stereošpecifické (D-arginín nie je substrátom)
- Jako kofaktory vyžadujú:
  - NADPH
  - 6(R)-5,6,7,8-tetrahydrobiopterin (BH_4)
  - FAD FMN
  - kalmodulin (ten se k NOS typu I a III váže po navázání Ca na kalmodulin, NOS II váže kalmodulin trvale)



Electrons (e^-) are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN redox carriers to the oxygenase domain. There they interact with the heme iron and BH_4 at the active site to catalyse the reaction of oxygen with L-Arginine, generating citrulline and NO as products. Electron flow throught the reductase domain requires the presence of bound Ca^{2+}/CaM.

Flavin adenine dinucleotide (FAD) is the precursor molecule to $FADH_2$. Upon binding to two hydrogen atoms, FAD is then changed to $FADH_2$ and is turned into an energy-carrying molecule. FAD is a coenzyme derived from riboflavin, or vitamin B_2. Many oxidoreductases, called flavoenzymes or flavoproteins, require FAD as a prosthetic group which functions in electron transfers.

Flavin mononucleotide (FMN), or **riboflavin-5'-phosphate**, is also derived from riboflavin (vitamin B_2) and serves as a cofactor of various oxidoreductases including NADH dehydrogenase.

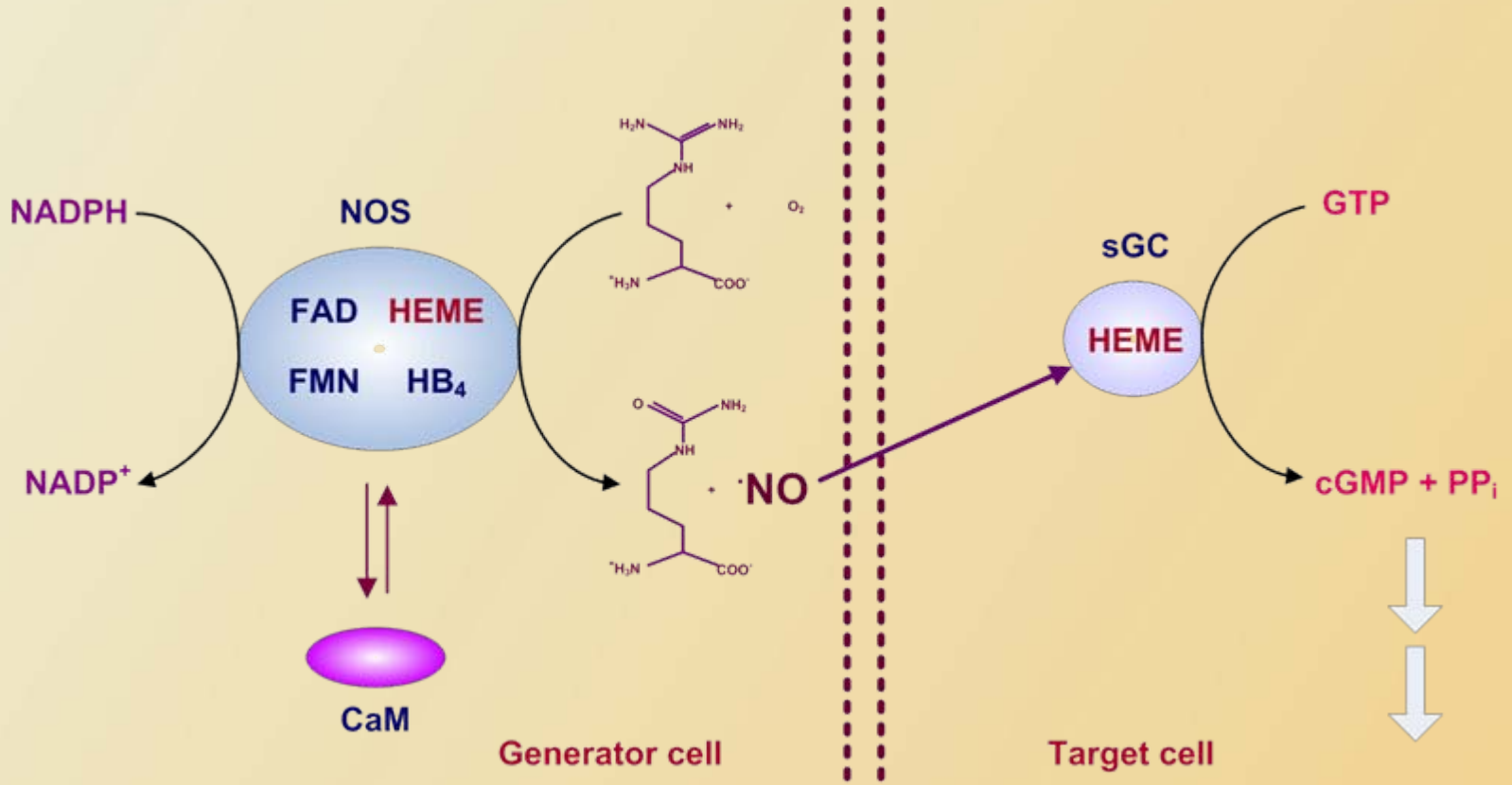
Name	Present in	Stimuli	Description
Neuronal NOS (nNOS or NOS1	Central and peripheral neurons, platelets, pancreatic β cells, epithelial cells	NMDA, insulin, thrombin	Produces NO in neuronal tissue in both the central and peripheral nervous system. Neuronal NOS also performs a role in cell communication and is associated with plasma membranes. nNOS action can be inhibited by NPA (N-propyl-L-arginine).
Inducible NOS (iNOS or NOS2)	Macrophages, endothelial cells, chondrocytes, hepatocytes, smooth muscle cells	Endotoxin, IFN-γ, IL-1, TNF-α	Can be found in the immune system but is also found in the cardiovascular system. It uses the oxidative stress of NO (a free radical) to be used by macrophages in immune defence against pathogens.
Endothelial NOS (eNOS or NOS3 or constitutive/ cNOS)	Endothelial cells, neurons, cardiac myocytes	Acetylcholine, ADP, thrombin, shear stress, VEGF	Generates NO in blood vessels and is involved with regulating vascular function. A constitutive Ca2+ dependent NOS provides a basal release of NO. eNOS is associated with plasma membranes surrounding cells and the membranes of Golgi bodies within cells.

Za fyziologických podmienok sa oxid dusnatý tvorí v organizme v nízkych koncentráciách (pM). Je rozpustný vo vode a v lipidoch, a preto veľmi rýchlo a ochotne difunduje cez cytoplazmatické aj plazmatické membrány. V takomto prípade prevláda jeho regulačné pôsobenie:

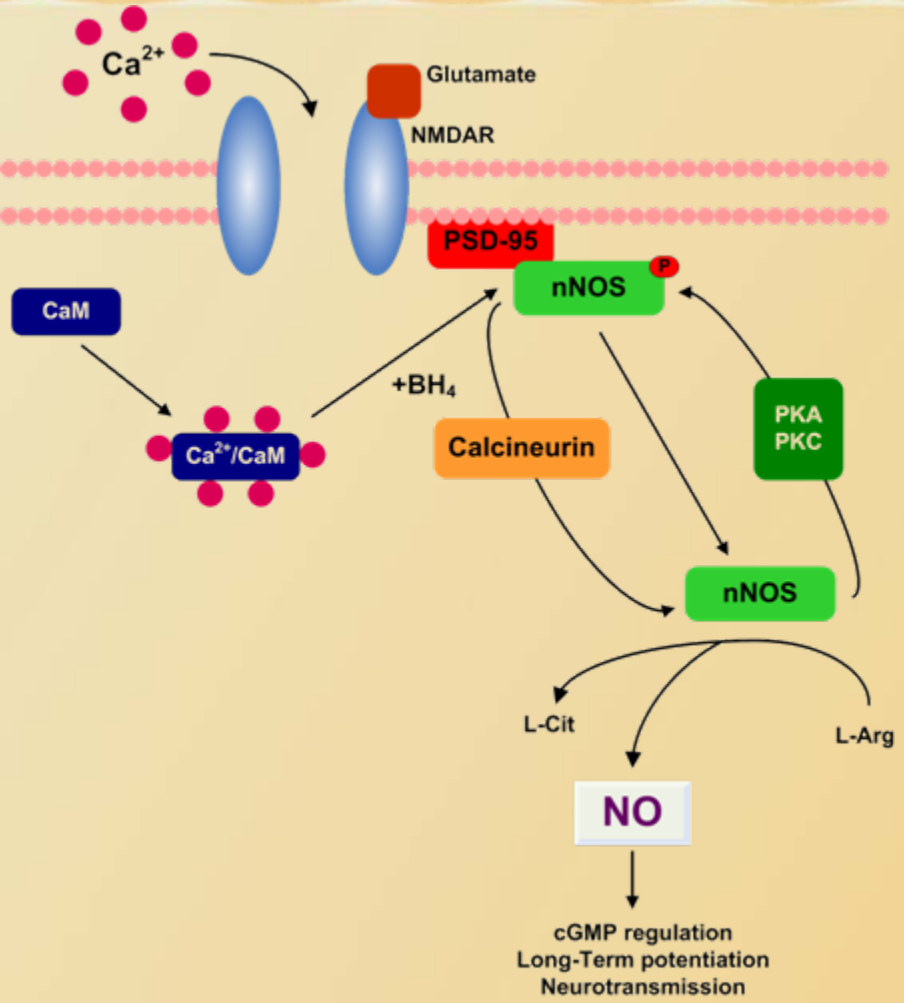
cGMP-dependentné účinky – NO aktivuje enzým guanylát cyklázu, čím sa zvyšuje koncentrácia cyklického guanozín-3',5'-monofosfátu (cGMP) v cieľových bunkách. cGMP potom priamo reguluje mnohé bunkové funkcie. Riadi niektoré bunkové kanály, znižuje intracelulárnu koncentráciu Ca^{2+} iónov a inhibuje kontraktilný aparát v hladkom svalstve. Okrem toho reguluje vazodilatáciu ciev, moduluje srdcovú kontraktilitu a znižuje zrážanlivosť krvi. Nemenej doležitý je jeho funkčný podiel na neurotransmisii a tvorbe pamäťovej stopy.

cGMP-indepedentné účinky - V tomto prípade sa oxid dusnatý uplatňuje pri inhibícii syntézy DNA a aj celkového energetického metabolizmu bunky. Reguluje metabolizmus železa.

Pri zápalových procesoch sa jeho koncentrácia v organizme mnohonásobne zvyšuje (μM). Vtedy sa NO a aj jeho reaktívne metabolity účastnia na protizápalových, antibakteriálnych, antivirálnych a antioxidačných procesoch. Cytotoxické a cytostatické účinky oxidu dusnatého sprostredkovávajú bunky imunitného systému, zúčastňujúce sa zápalových procesov. Sú to neutrofily, monocyty a makrofágy.



Neuronal Nitric Oxide Synthase (nNOS, Type I)



- Homodimers with 2 subunits (130-160 kDa)
- nNOS have binding sites for **NADPH, FAD, and FMN** near the carboxyl terminus (the reductase domain), and binding sites for tetrahydrobiopterin **(BH_4)** and heme near the amino terminus (the oxygenase domain). The reductase and oxygenase domains are linked by a calmodulin **(CaM)** binding site. Occupation of this site facilitates electron transfer from the cofactors in the reductase domain to heme during nitric oxide production.
- nNOS is associated with the **post-synaptic density protein (PSD-95)** in the neuronal membrane. In response to increased intracellular Ca^{2+}, nNOS interacts with CaM. The Ca^{2+}-CaM complex, in combination with BH_4, binds to nNOS and induces its translocation from the plasma membrane to the cytoplasm.
- The dephosphorylation of nNOS by **calcineurin** initiates the production NO. NO activates guanylyl cyclase (GC) and activates the various cGMP-regulated signaling pathways.
- nNOS is inactivated by phosphorylation by protein kinase A **(PKA)** or protein kinase C **(PKC)**.
- Neuronal nitric oxide synthase (nNOS) has been implicated in a wide variety of physiological and pathological processes. These include neurotransmission, neurotoxicity, skeletal muscle contraction, sexual function, body fluid homeostasis and atherosclerosis, among others.

- **NMDA receptor (NMDAR)** is an ionotropic receptor for glutamate
- **NMDA (N-methyl d-aspartate)** is a name of its selective specific agonist
- Activation of NMDA receptors results in the opening of an ion channel that is nonselective to cations. This allows flow of Na^+ and small amounts of Ca^{2+} ions into the cell and K^+ out of the cell.
- Calcium flux through NMDARs is thought to play a critical role in synaptic plasticity, a cellular mechanism for learning and memory. The NMDA receptor is distinct in that it is both ligand-gated and voltage-dependent.
- Activation of NMDA receptors requires binding of **glutamate** or **aspartate** (aspartate does not stimulate the receptors as strongly). In addition, NMDARs also require the binding of the co-agonist **glycine** for the efficient opening of the ion channel, which is a part of this receptor.

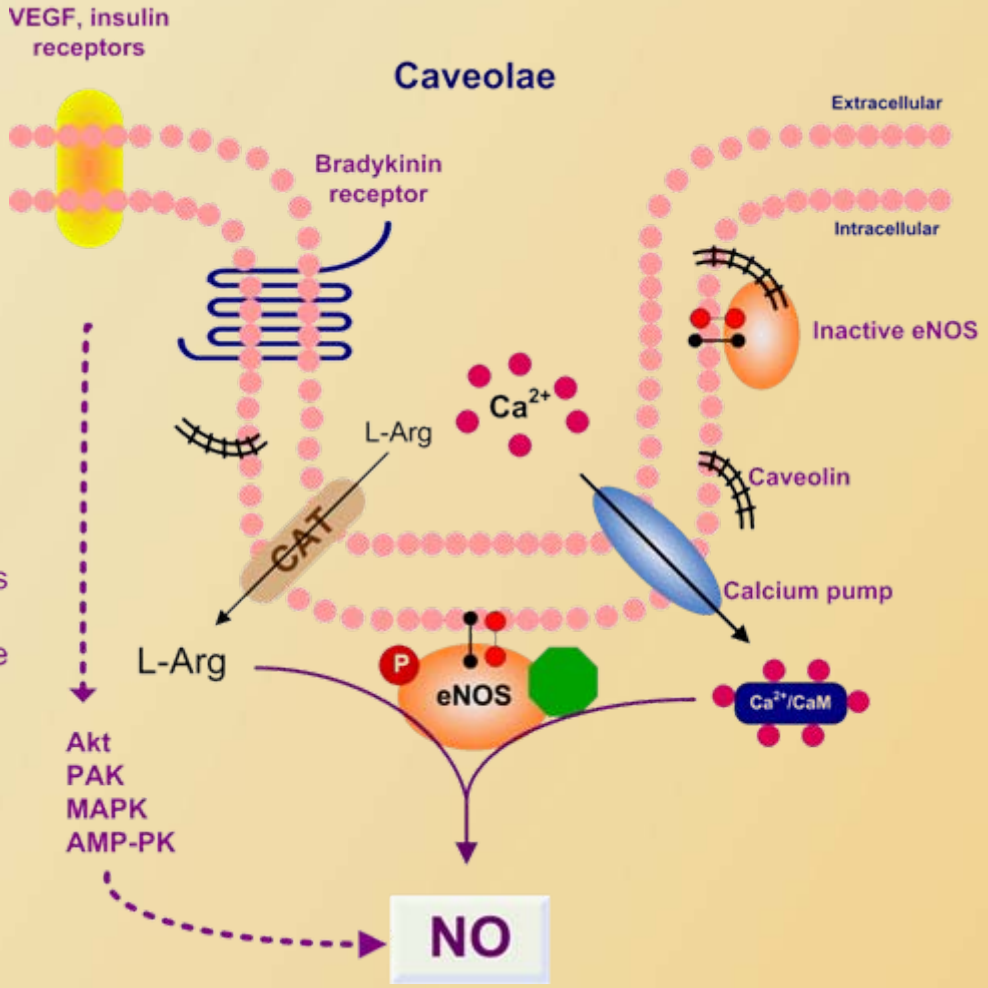
Endothelial nitric oxide synthase (eNOS or NOS3 or constitutive/ cNOS)

- localised to caveolae
- eNOS is a lipid raft/caveolar protein apparently regulated by caveolin
- Agonist stimulation induces calcium dependent association of protein cofactors and kinases ultimately resulting in generation of nitric oxide from Arginine

Regulation of Endothelial Nitric Oxide Synthase

Classical regulation by calcium

- All NO-synthases required for its activation to be bound to a calcium regulatory protein: **calmodulin**.
- iNOS tightly binds calmodulin even at resting calcium concentrations, and then it is active once it is synthetized.
- Constitutive enzymes, eNOS and nNOS, only bind calmodulin when the intracellular calcium concentration increase up to a certain value. Agents that increase intracellular calcium concentration, either by allowing calcium entrance from the outside or by stimulating calcium mobilization from intracellular stores, can activate these constitutive enzymes.
- In endothelial cells various substances increase intracellular calcium and in consequence NO synthesis: **bradykinin, histamine, serotonin**.



Calcium-independent regulation

Activity of eNOS is acutely dependent on intracellular localization and also dependent on phosphorylation at specific aminoacids.

Intracellular localization

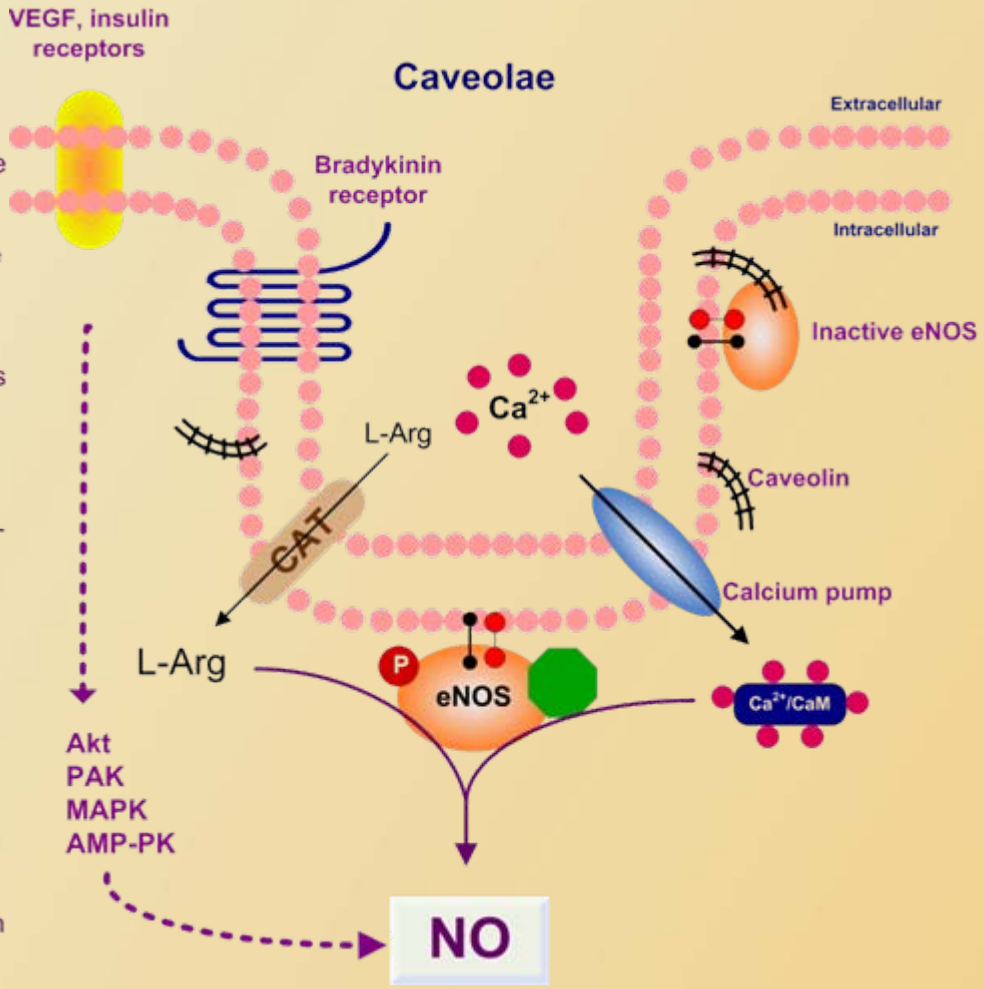
- eNOS is predominantly localized in caveolae (specialized invaginations of the plasma membrane), where it is closely regulated by interaction with caveolin-1. Modifications preventing membrane localization of eNOS also result in the absence of NO synthetic activity in the intact cells. Membrane distribution is probably needed by the presence in the same localization of other proteins important for eNOS activity: the cationic amino acid transporter CAT-1 (involved in the uptake of L-arginine, substrate for NO synthesis), calcium pump and the bradykinin receptor are also present in caveolae.
- Although membrane distribution is an essential requirement for eNOS activity, at plasma membrane the enzyme activity is closely regulated by caveolin-1. This intrinsic protein strongly reduces eNOS activity by interfering with calmodulin binding. Intracellular calcium increase or shear stress displace caveolin-1 and allow eNOS activation.
- Membrane localization of eNOS is modulated by certain post-translational modifications:
  - Myristoylation distinguish eNOS from nNOS and iNOS, that are predominantly cytosolic proteins
  - Palmitoylation is also required for a proper localization of eNOS in the membrane

Phosphorylation: Tyr-Phosphorylation, Ser/Thr-Phosphorylation

Oxygen free radicals

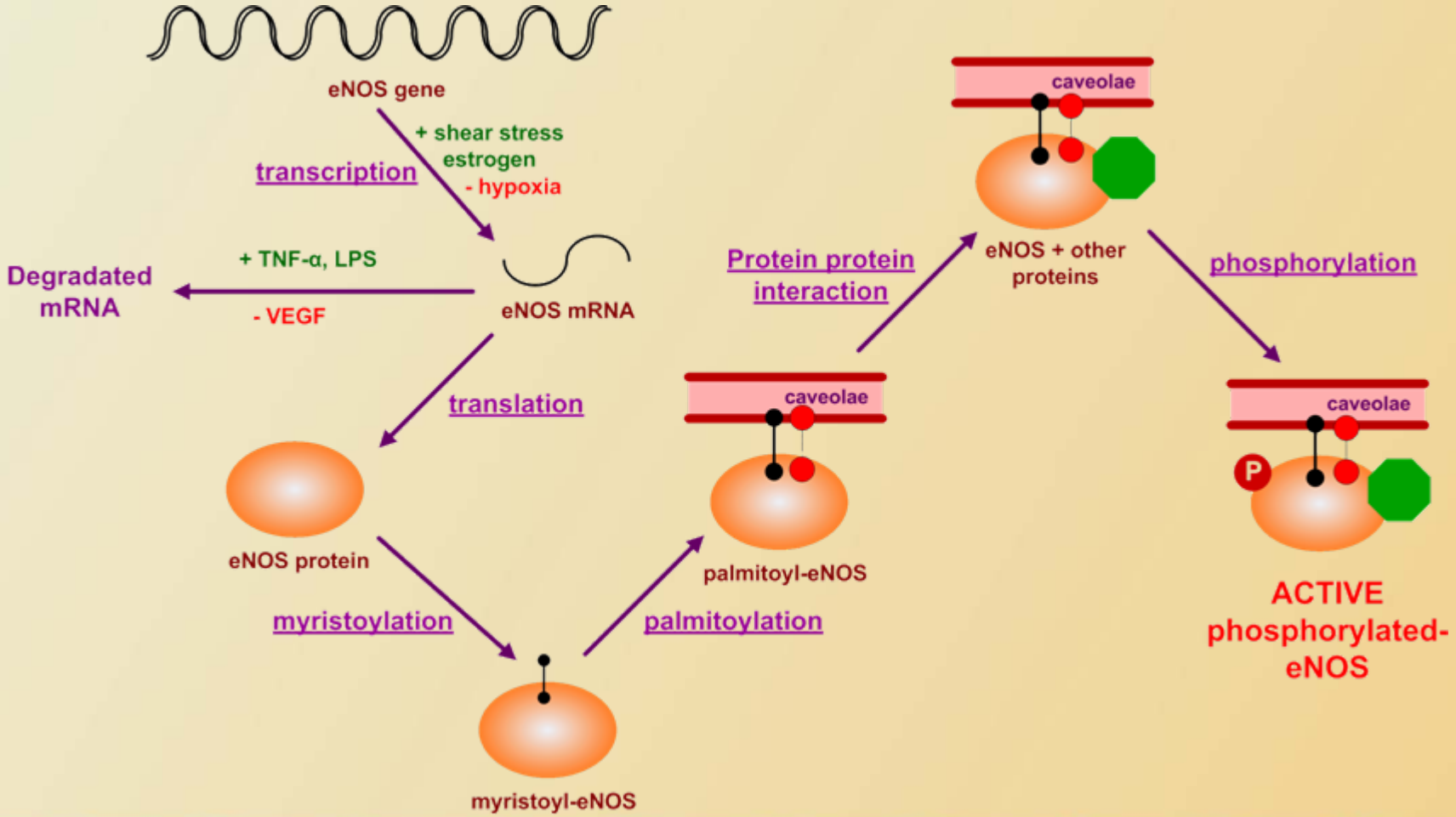
- In addition to direct regulation of NO-synthases, NO availability is also dependent on the quantity of oxygen free radicals generated by cells surrounding NO-producer cell. In fact, eNOS may generate superoxide instead of NO in certain conditions (e.g. low L-arginine levels). Whatever the origin of superoxide (eNOS, xanthine oxidase,...) this compound rapidly reacts with NO to form peroxynitrite. In certain pathological circunstances an increase in superoxide formation can be determinant in reducing NO availability.

[Based on Govers and Rabelink, Am J Physiol 2001, 280:F193]



Regulation of eNOS

- **factors that regulate the transcription** of eNOS gene (shear stress, estrogen and hypoxia)
- **factors that modulate the stability of its mRNA** (tumor necrosis factor alfa or TNF-alfa, lipopolysacharide or LPS, and vascular endothelial group factor or VEGF)
- **permanent changes of the eNOS protein e.g. myristoylation, palmitoylation**, myristoylation seems a critical factor to allow the final location of the enzyme at certain specific domains of the membrane.
- **non-permanent changes of eNOS protein e.g. phosphorylation and specific interactions with another proteins**. After those modifications the eNOS protein is active and synthetizes NO or in some cases superoxide ion (this later circunstance can take place when the substrate, L-arginine, or tetrahydrobiopterin are deficient and has pathophysiological consequences). Then, all these non-permanent modifications of eNOS revert and eNOS is deactivated. A cycle of activation-deactivation occurs in parallel with a cycle of association and dissociation from the caveoale at the plasma membrane. [Based on Govers and Rabelink, Am J Physiol 2001, 280:F193]



Caveolae

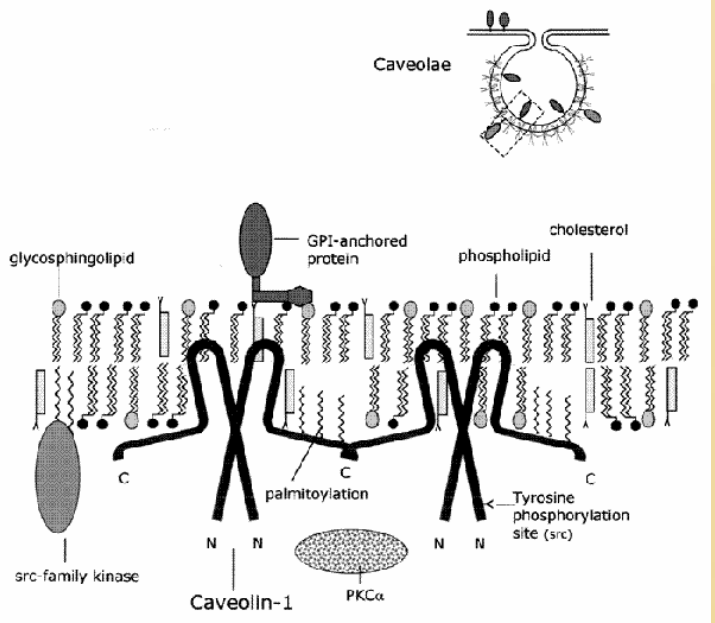
- small invaginations (vesicles) of the plasma membrane with a well-defined size (50-100 nm) and a particular lipid content
- localised in plasma membrane (**lipid rafts**-rich in glycosphingolipids and cholesterol)
- Involved in transcytosis, lipid trafficking and more recently signal transduction
- very dynamic organelles that can pinch off the plasma membrane in a process that requires the hydrolysis of GTP
- mediate trans-epithelial transport of small molecules across the cell by fusing together to form trans-cellular channels
- mediate the uptake of particular molecules and ions from the exterior and then redistribute these compounds in intracellular compartment through a process called potocytosis
- cycle between the plasma membrane and the ER for delivery of molecules inside the cell
- many receptors and cytosolic signaling proteins that do not require lipid modifications to associate with membranes, such as PKCa, are reportedly found in caveolae
- number of viruses, parasites and bacteria utilize caveolae (or caveolae-like domains) as an alternative route to enter cells.

Proteins called caveolins

- represent major components of the caveolar coat
- important for the structure of caveolae, thanks to their ability to oligomerize and bind cholesterol
- caveolin-1 and 2 have a similar tissue distribution, being mainly expressed in endothelial, epithelial and muscle cells
- caveolin-3 expression is limited to muscle cells

caveolin-1 adaptor molecule or scaffolding protein in signal transduction Caveolin-1 functions as a tumor suppressor in human colon carcinoma cells

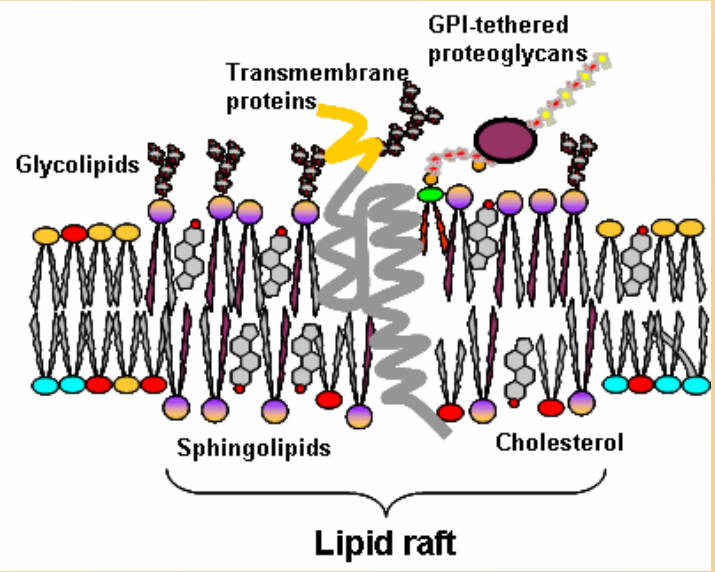
Bender et al., Biol Res 35 (2002) 139-150, Caveolae and caveolae-like membrane domains in cellular signaling and disease: Identification of downstream targets for the tumor suppressor protein caveolin-1



http://www.scielo.cl/fbpe/img/bres/v35n2/img06-01.gif

Lipid rafts

- In artificial membranes, different lipids separate from each other based on their physical properties, forming small islands called lipid rafts. These rafts have a higher concentration of certain specialized lipids, called **glycosphingolipids**, and **cholesterol** than do non-raft parts of the membrane. Rafts are also distinguished by a different assortment of proteins. Certain types of proteins cluster together in rafts, while others remain mostly outside of rafts.
- Although the existence of lipid rafts in cellular membranes remains controversial, many scientists believe they serve as communication hubs by recruiting proteins that need to come together in order to transmit a signal. They are important **signal transduction centers** in the plasma membrane, **coordinating and integrating incoming signals**, especially in tyrosine kinase signalling. Researchers are beginning to link lipid rafts with a variety of diseases, including AIDS, Alzheimer's, anthrax, and atherosclerosis.



http://www.steve.gb.com/images/science/lipid_raft.png

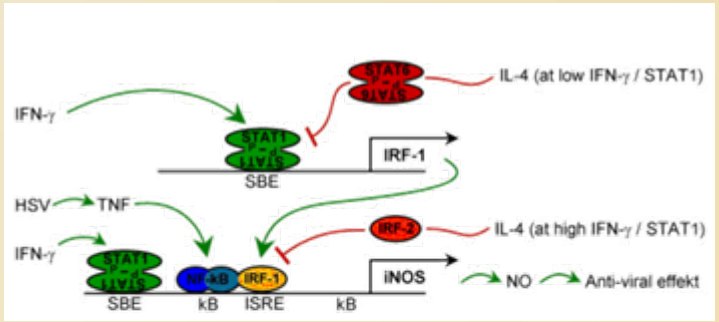
Inducible nitric oxide synthase (iNOS, NOS II)

- generates NO independently of intracellular calcium concentrations
- induced by immunostimulatory cytokines, bacterial products or infection in a number of cells e.g. endothelium, hepatocytes, monocytes, mast cells, macrophages and smooth muscle cells (function in host defense against microbial and viral pathogens)
- responsible for formation of NO radicals or S-nitrosothiols or ONOO- in the host cell or in the microbe itself
- participate in the pathology of inflammatory diseases including atherosclerosis, rheumatoid arthritis, diabetes, septic shock, transplant rejection, and multiple sclerosis, leading to cell death (F. Aktan, Life Sciences 75 (2004) 639–653)

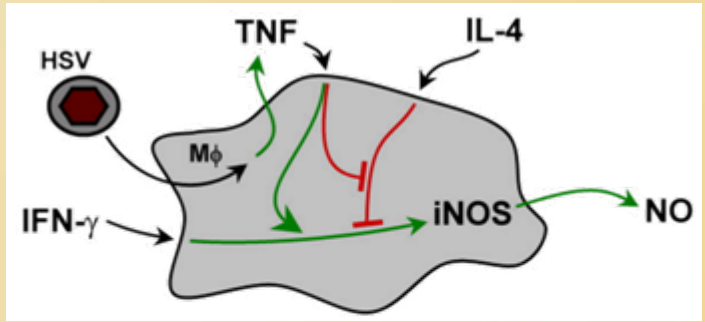
Indukcia a regulácia iNOS expresie

- Indukcia iNOS:
  - **nešpecifická** (oxidatívny stres, UV-žiarenie)
  - **Pomocou špecifických receptorov** (ligandy TNF-α, IL-1, CD-40L, LPS)
- Regulácia iNOS sa uskutočnuje na **BUNEČNEJ** a **MOLEKULÁRNEJ** úrovni
- Kľúčovým faktor pre syntézu NO je **zvýšenie expresie génu pre iNOS** a **regulácia transkripcie** tohoto génu (väzobné miesta pre transkripčné faktory, ktoré sa nachádzajú na iNOS promótore)

  →Miesta určené pre špecifické transkripčné faktory: jadrové faktory-kappaB (**NF-κB**), aktivačný proteín-1 (**AP-1**), CCAAT/enhancer-binding protein **C/EBP** a cyklický-AMP-responzívny element viažúci proteín (**CREB**)
- Makrofágy (tkanivová forma monocytov) - veľmi dôležitú úlohu v zápalových procesoch (LPS, IFN-γ, TNF...). Odpoveď na stimuláciu → produkcia prozápalových mediátorov (IL-1, IL-2,TNF-α, NO...)



Regulation of iNOS induction at the molecular level.
Transcription factors controlling induction of the iNOS gene. Activated STAT1 induces transcription of the IRF-1 and iNOS genes, an effect which is competed by activated STAT6. IRF-1 interacts physically with NF-κB, binds to the distal κB-binding site of the iNOS promoter region, and stimulates transcription. Only when NF-κB is absent, IRF-2 can bind to the ISRE site and block transcription. Stimulatory pathways are indicated by green arrows (→), and inhibitory pathways are drawn in red (Ellermann-Eriksen Virology Journal 2005).



Regulation of iNOS induction at the cellular level
Cytokines controlling the iNOS induction in macrophages during early HSV infection. IFN-γ, produced mainly by NK cells, stimulates iNOS production. This IFN-γ-induced production of iNOS can be inhibited by IL-4. Upon HSV (herpes simlex virus) infection of macrophages they produce TNF which synergizes with the IFN-γ-induced pathways and inhibits the inhibitory signals of IL-4. Thus, the virus overrules the restrictive signals and opens up for an otherwise closed pathway. Stimulatory pathways are indicated by green arrows (→), and inhibitory pathways are drawn in red (Ellermann-Eriksen Virology Journal 2005).

Toll-like receptory

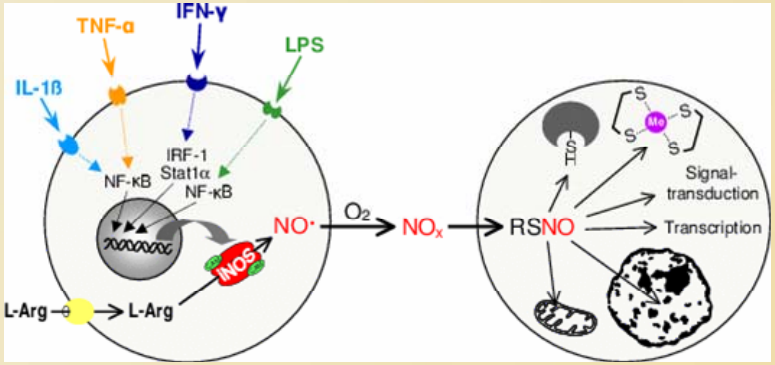
- transmembránové proteíny (monocyty , makrofágy, neutrofily, dendritické bunky, intestinálne epiteliálne a endoteliálne bunky
- 13 členov (niektoré su lokalizované intra- iné extracelulárne)
- Rozpoznávajú molekuly významné pre infekciu a zápal (LPS, Lipoproteíny, Fragmenty bakterálnych bičíkov, Bakteriálnu DNA, Dvojvláknovú (virálnu) DNA)
- Aktivácia:
  - Zahájenie rýchlych obranných reakcií
  - Dve signálne dráhy (NF-κB transkripčné faktory, Mitogén-aktivované proteín kinázy (MAPK))
- Konečné produkty TLR-4 - cytokíny (IL-1b, -6, -12, TNF) a RKM

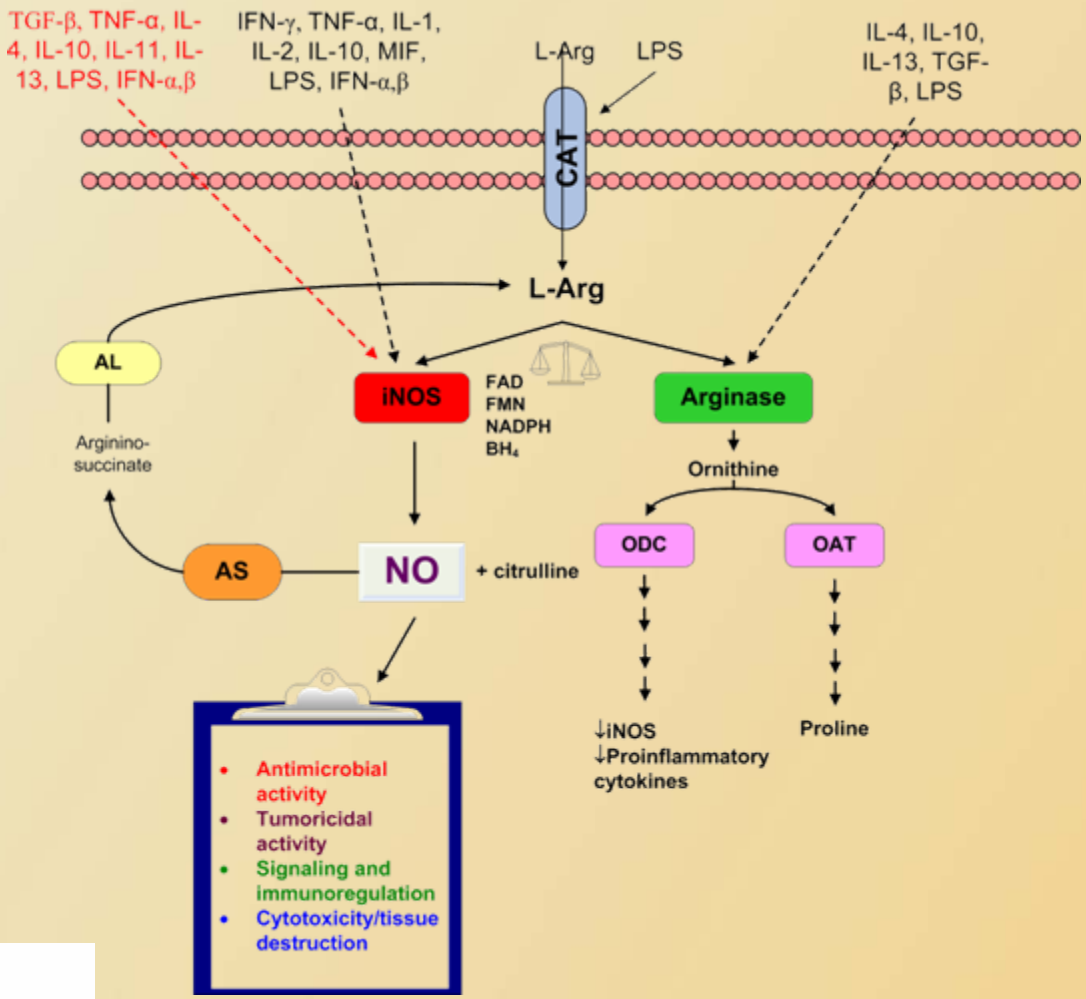
LPS

- Súčasť bakteriálnej steny Gram-neg. baktérii
- endotoxín
- vyvoláva zápalové odpovede
- interaguje s LPS-viažúcim proteínom (LPS-BP) v sére a vytvára komlex ktorý sa v prítomnosti CD14 viaže na TLR-4
- Potrebná prítomnosť sekrečného proteínu MD-2

IFN-γ, IL-1, TNF receptory

- IFN-γ indukuje aktiváciu iNOS prostredníctvom Jak-STAT signálnej dráhy





NO pathways

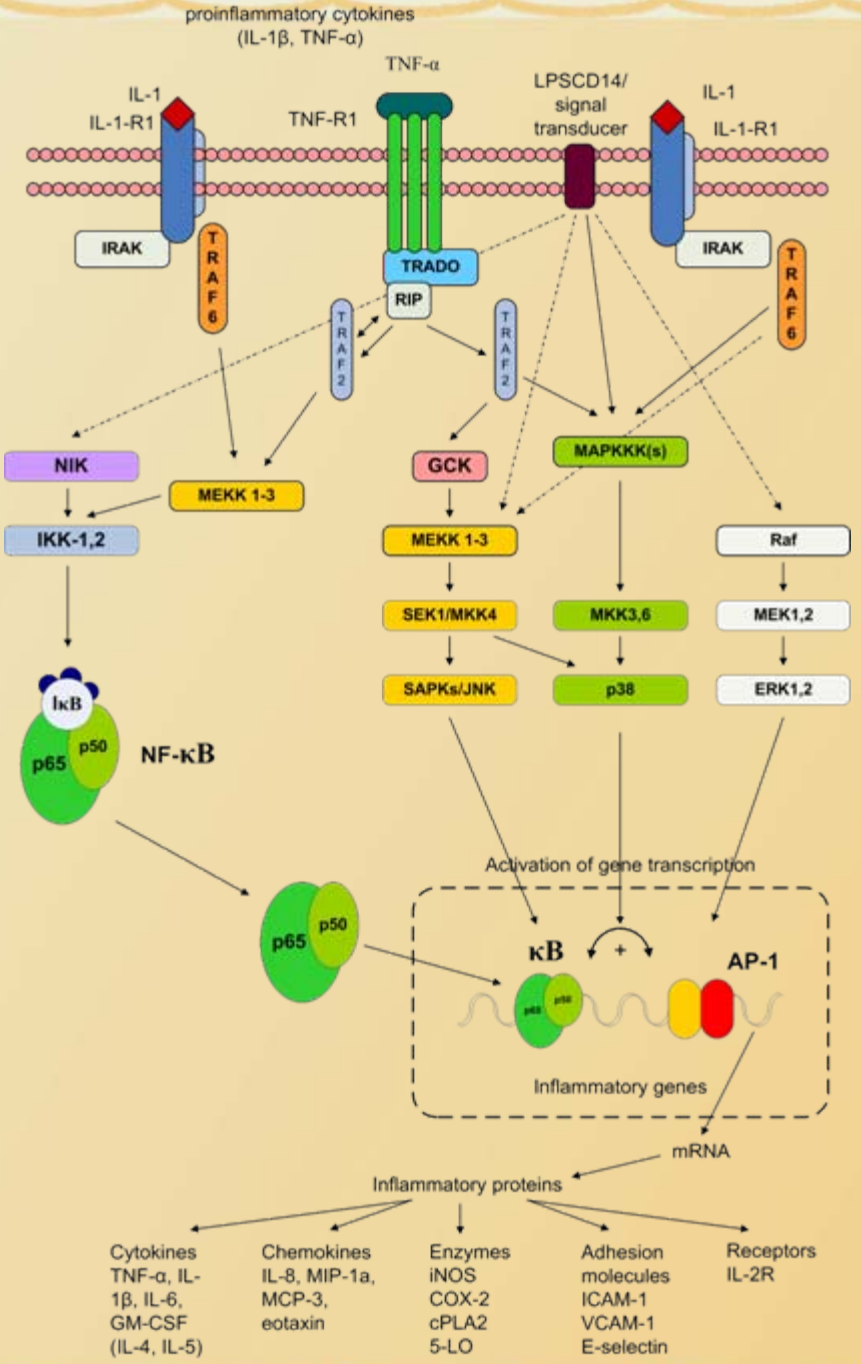
Regulation and function of inducible nitric oxide synthase, arginase and related pathways in mouse macrophages. The activity of iNOS is regulated by cytokines and microbial products (such as LPS), which affect the uptake of **L-arginine (L-Arg)** by **cationic amino acid transporters (CAT)**, the synthesis of cofactors, the expression of iNOS mRNA and protein, the enzymatic recycling of citrulline to arginine and the depletion of arginine by arginase. Polyamines- products of the arginase−ODC pathway, act as immunosuppressants and can further downregulate the production of NO. A high arginase activity in the absence of iNOS can also be associated with tissue fibrosis resulting from the increased synthesis of proline *via* the arginase−OAT pathway, which is required for collagen synthesis (for example, by fibroblasts). AL, argininosuccinate lyase; AS, argininosuccinate synthetase; MIF, macrophage migration inhibitory factor; ODC, ornithine decarboxylase; OAT, ornithine aminotransferase.

Regulácia iNOS sprostredkovaná MAP kinázami

- ERK (extracelulárnymi signálmi regulované kinázy),
- p38 MAPK
- JNK (c-Jun amino-terminálne kinázy)
- Sprostredkúvajú fosforyláciu ďalších proteínov (proteín kinázy, fosfolipázy, transkričné faktory a proteíny cytoskeletu)
- V bunkách majú rôzne funkcie
  - ERK regululujú bunkovú proliferáciu a diferenciáciu
  - p38 MAPK a JNK sprostredkovávajú apoptózu
  - p38 MAPK a ERK sú zapojené aj do regulácie expresie niektorých prozápalových génov (iNOS, IL-6)

Regulácia iNOS sprostredkovaná NF-κB

- štrukturálne a evolučne konzervovaná rodina proteínov pozostáva z piatich členov: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB a c-Rel
- Transkripčné faktory NF-κB sa v nestimulovaných bunkách nachádzajú v inaktívnej forme (diméry) a nevyznačujú sa žiadnym účinkom na transkripciu príslušných génov
- Ich aktivácia je kontrolovaná inhibičnou podjednotkou zo skupiny inhibítorov kappaB (IκB)
- K aktivácii NF-κB dochádza pod vplyvom rôznych faktorov:
  - Nešpecificky
  - Špecificky pomocou ligandov TNF-α, IL-1, CD-40L a LPS → aktivujú transkripčné faktory NF-κB prostredníctvom špecifických receptorov
  - aktivácia IκB kináz (IKK) (fosforylujú IκB zložku inaktívneho komlexu NFκB-IκB)
  - Uvoľnením inhibítoru sa NF-κB stávajú aktívnymi a sú translokované do jadra
  - Vazba na svoj responzívny element a spustenie expresie cieľových génov
- NF-κB reguluje expresiu génov, ktoré hrajú veľmi dôležitú úlohu v nešpecifickej imunite: cytokíny (IL-1, IL-2, IL-6, IL-12, TNF-α, Ltα, Ltβ a GM-CSF), adhezívne molekuly (ICAM, VCAM), proteíny akútnej fázy (SAA) a inducibilné enzýmy (iNOS a COX-2). Väzbou NF-κB na DNA dochádza zároveň k spätnej indukcii transkripcie IκB. Inhibítor sa znova viaže na aktívne proteíny NF-κB.
- **Aktivácia NF-κB je nevyhnutná pre LPS indukovanú expresiu iNOS, a používaním NF-κB inhibítorov dochádza k blokovaniu iNOS expresie a produkcie oxidu dusnatého v makrofágoch.**



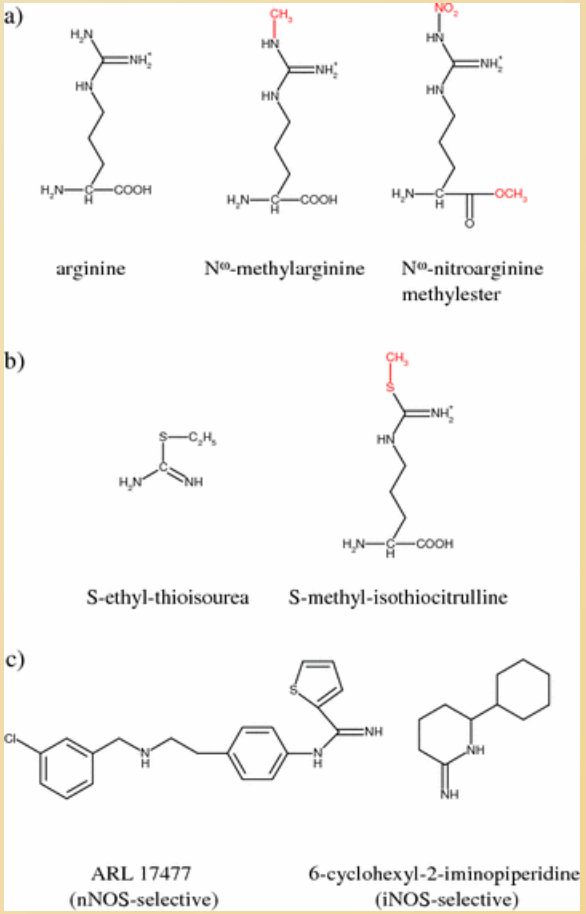
NOS inhibitor

Inhibitors of NOS have been described which interact with the NOS enzymes in a variety of ways:

- different sites (l-arginine, biopterin site, haem-binding inhibitors, flavoprotein and CaM inhibitors)
- differing time-dependence
- substrate-dependence
- mechanism of inhibition
- widely used inhibitors - l-NMMA, l-NNA and its methyl ester prodrug (*N*G-nitro-l-arginine methyl ester, `l-NAME') - effect on nNOS dimerization but did inhibit both NOS and NADPH oxidase activity of nNOS, eNOS and iNOS in a time-dependent manner

Inhibitors of NOS

- widely used in experimental research
- still in under investigation for clinical application
- Treatment with NOS inhibitors (chronic inflammatory diseases, e.g. rheumatoid arthritis)
- Some such drugs are derivatives of arginine
- alkyl derivatives of isothiourea are very potent inhibitors of NOS
- Some experimental inhibitors that indeed do show some preference for iNOS and nNOS
- selective inhibition of iNOS should be advantageous in septic shock and in chronic inflammatory diseases



http://watcut.uwaterloo.ca/webnotes/Pharmacology/noNosInhibitors_ws.png

Sildenafil1 (Viagra)

- inhibitor of the phosphodiesterase subtype 5, which is selective for cGMP
- removes cGMP and thus terminates the action of NO (if protein nitrosylation is neglected)
- Sildenafil was not developed with its now-famous application in mind; instead, the idea was to come up with another vasodilatator. Its enhancing effect on penile erection gave rise to the discovery that NO actually is the transmitter that triggers this process.
- **Thus, without NO, no one of us would even be here today!**

Detekce NO

Priame stanovenie NO:

- Gas-phase chemiluminescence assay
- Electron paramagnetic resonance (EPR)
- Electrochemical detection
- cell-permeabilní fluorescenční indikátory (4,5-diaminofluorescein diacetate (DAF-2 DA)

Nepriame stanovenie NO:

- celková koncentrace nitrátů/nitritů (Griessova metoda)
- aplikace NO donorů compounds, NO scavengerů, a guanylyl-cyklásy
- NOS aktivita v buněčných homogenátech měřením enzymatické konverze argininu na citrulin během tvorby NO
- inhibitory NOS (L-NAME)
- aplikace protilátek k isoformám NOS (imunocytochemie, imunoblotting)
- exprese genu pro iNOS

NO Detection by Gas Phase Chemiluminescence

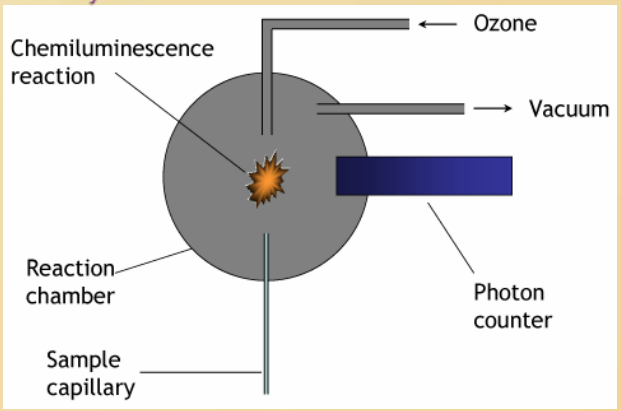
Detection Principle:

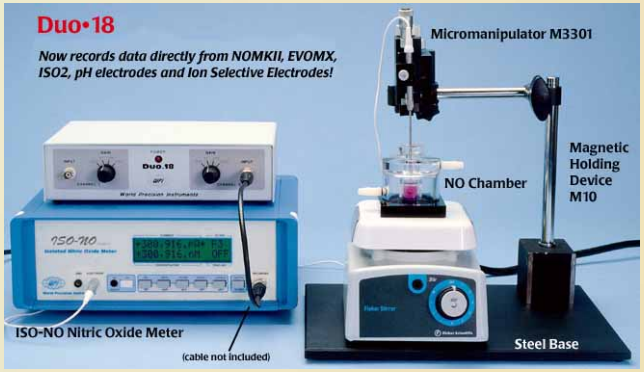
NO is purged from an aqueous solution using an inert gas such as Ar or He and transferred to a mixing chamber where it reacts with O_3 under reduced pressure.

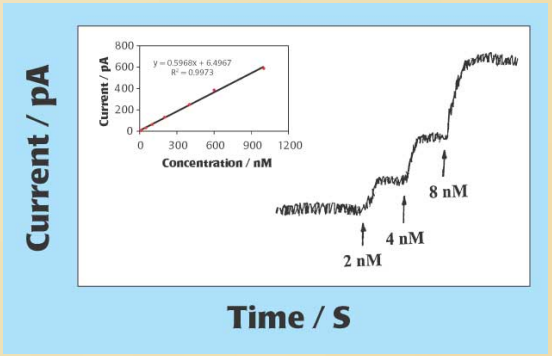
$$NO + O_3 \longrightarrow NO_2^* + O_2$$

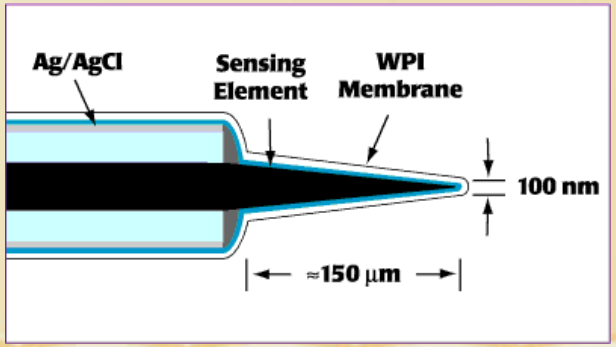
$$NO_2^* \longrightarrow NO_2 + h \cdot \upsilon$$

The light emitted by excited NO_2 upon returning to the ground state is measured by photon counting *(fmol-pmol).* Not very useful when attempting to quantify NO in physiological fluids such as serum, plasma or urine. *Why?*

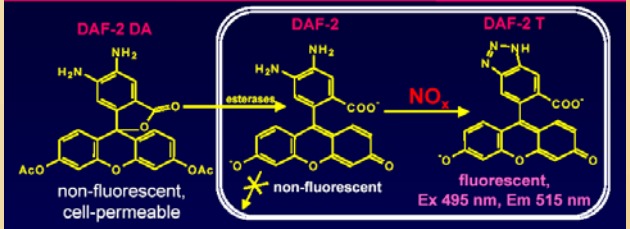








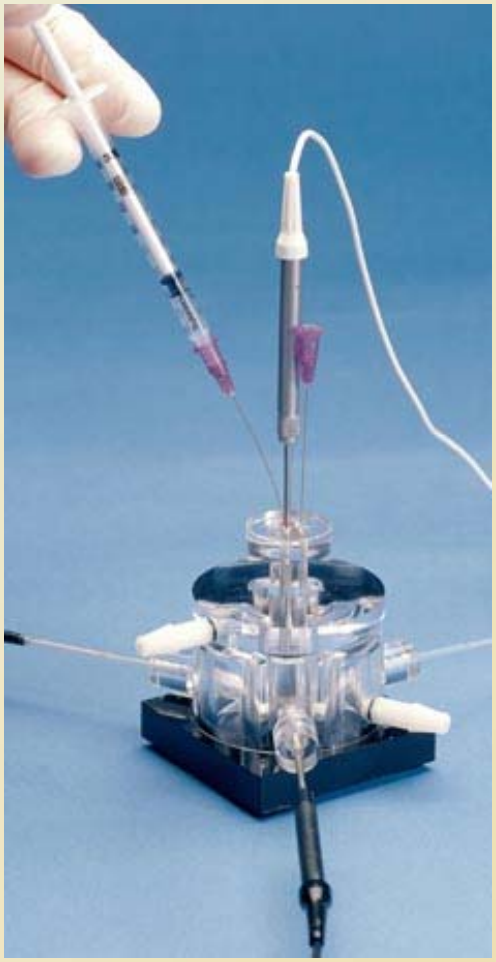
Bioimaging of Nitric Oxide Using Diaminofluoresceine-2 (DAF-2)

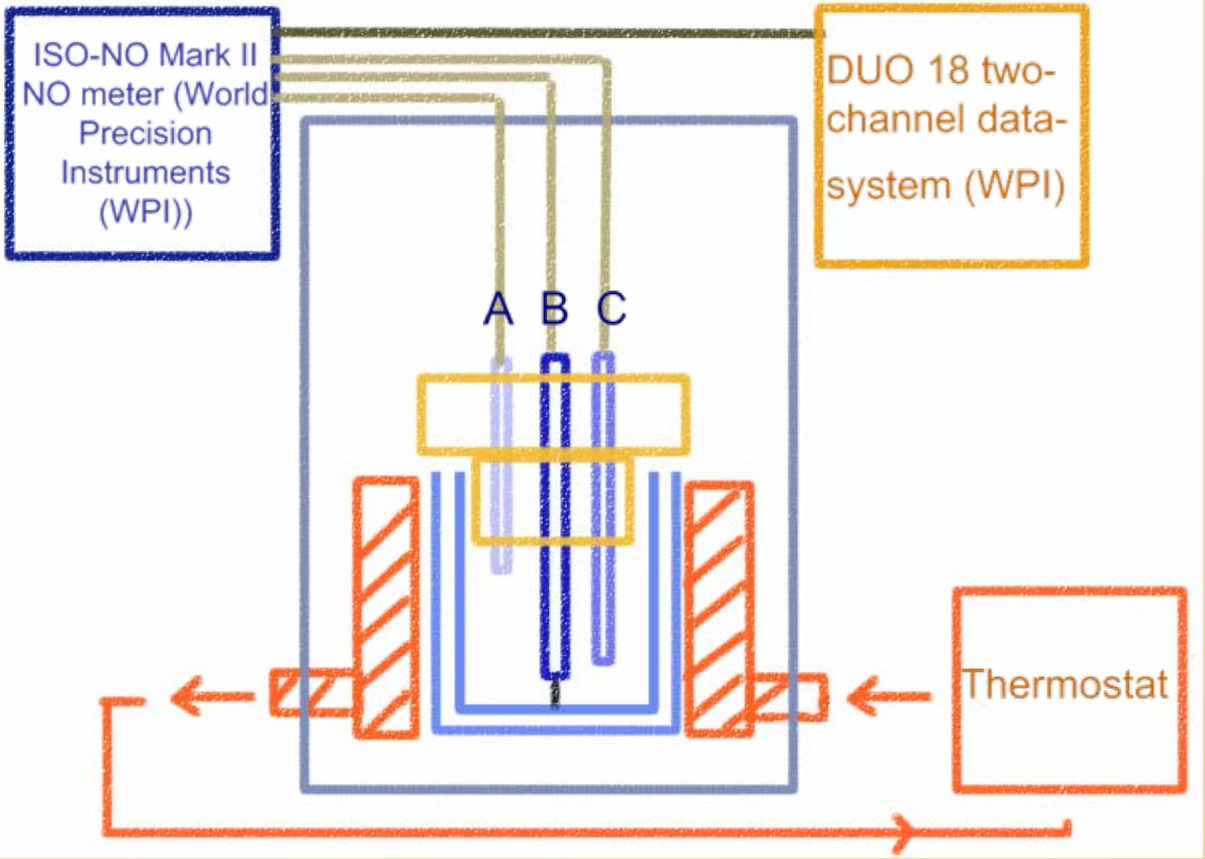


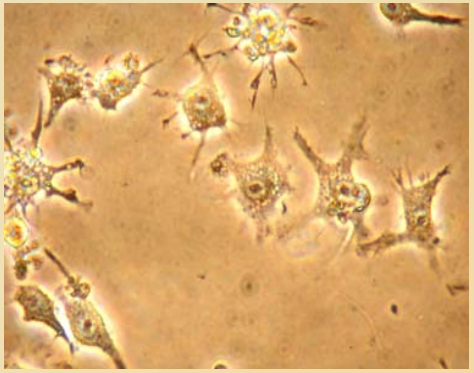
Advantages: **Sensitivity for NO (5 nM in vitro) with high temporal and spatial resolution.**
No cross-reactivity to NO_2^-/NO_3^- and $ONOO^-$
Kojima et al., Biol.Pharm. Bull. (1997)

Assay limitations: **Possible interference by reducing agents and divalent cations, requires standardized illumination conditions**

- A home-made miniature saturated silver/silver chloride reference electrode
- B porphyrinic microsensor working electrode
- C platinum wire counter electrode





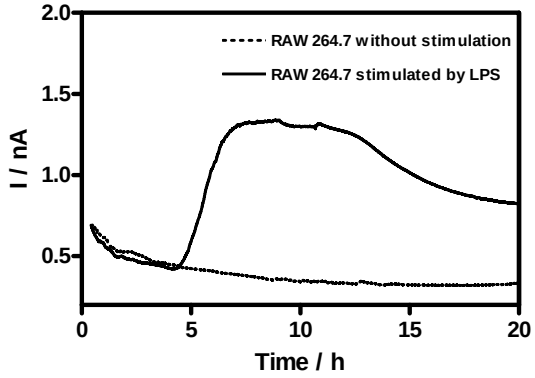


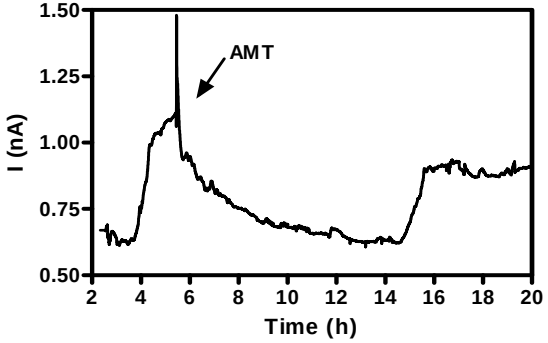
RAW 264.7 ($1x10^6$) (100x)

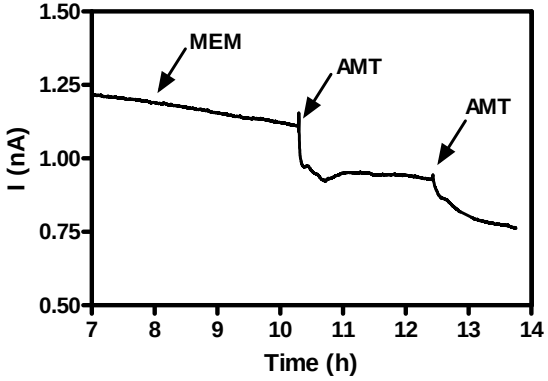
porphyrinic microsensor (7 μm in diameter, typical length 0.5-1 mm)

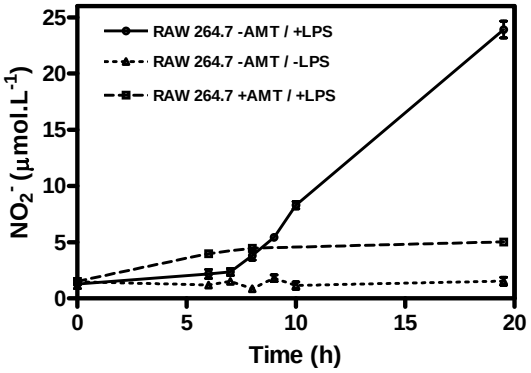
RESULTS

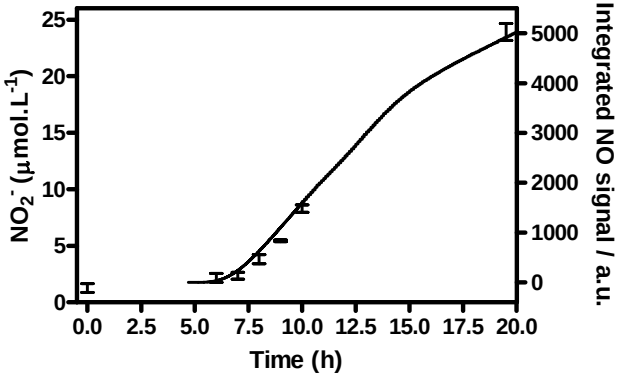
Measurement of NO release from RAW 264.7 cell line using microsensor

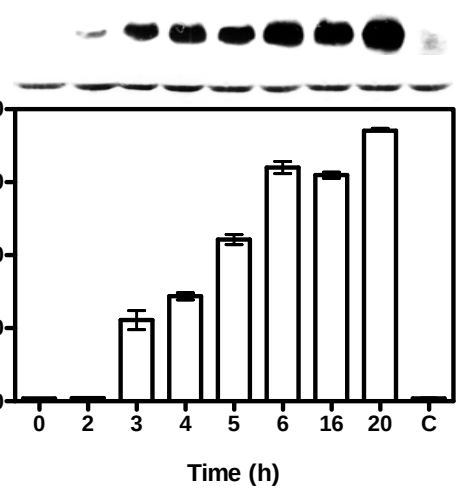


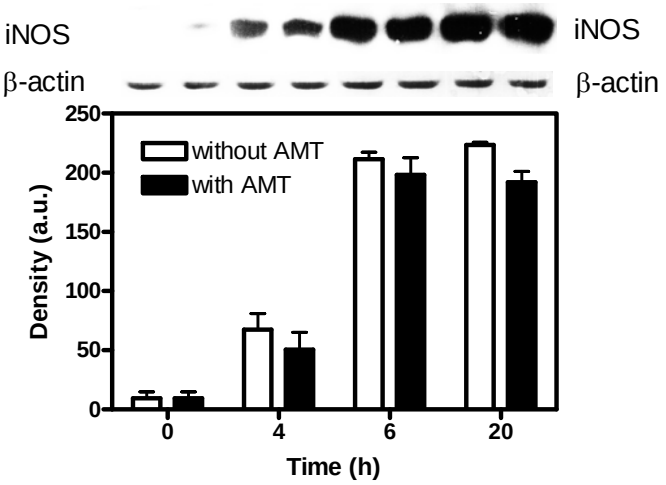


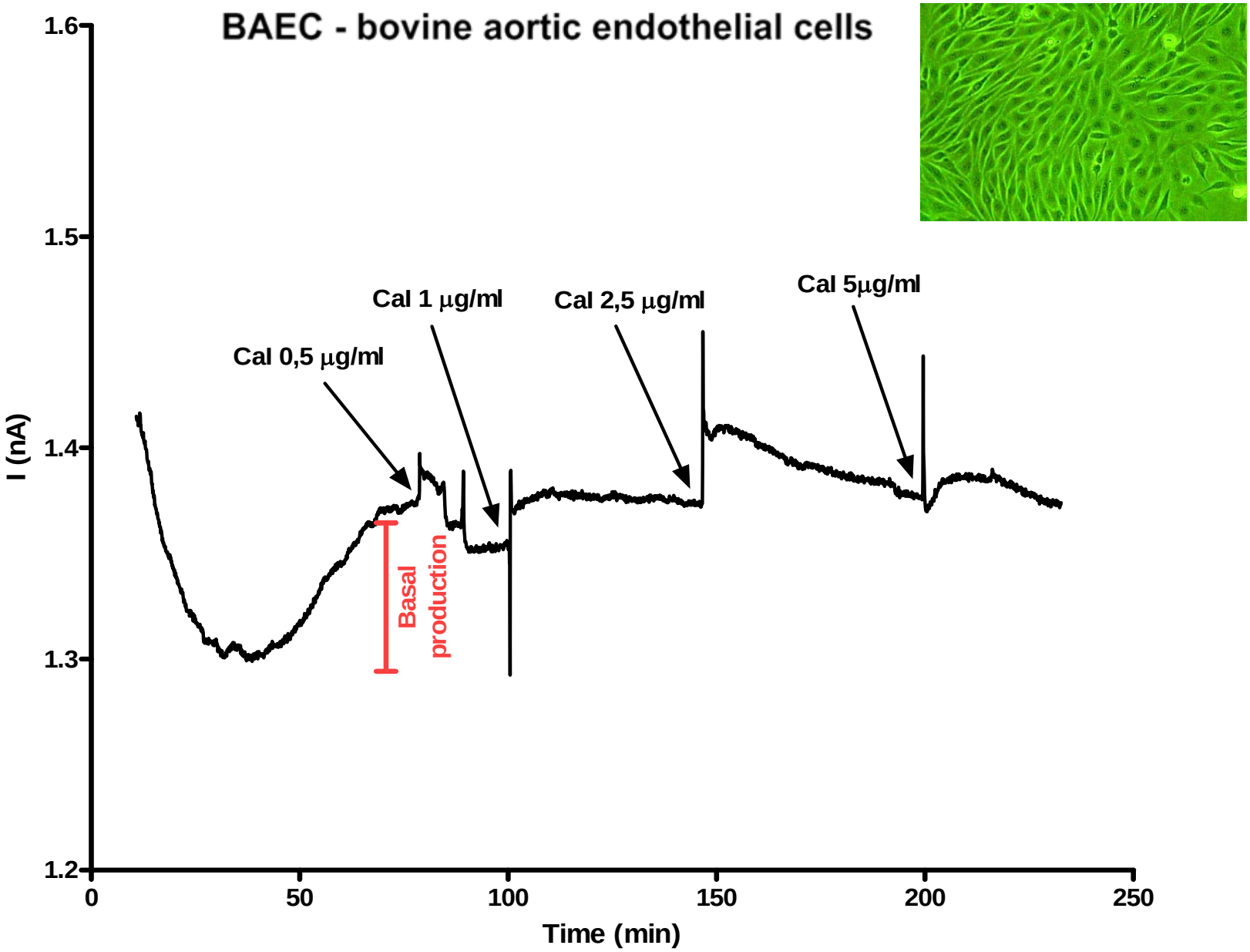


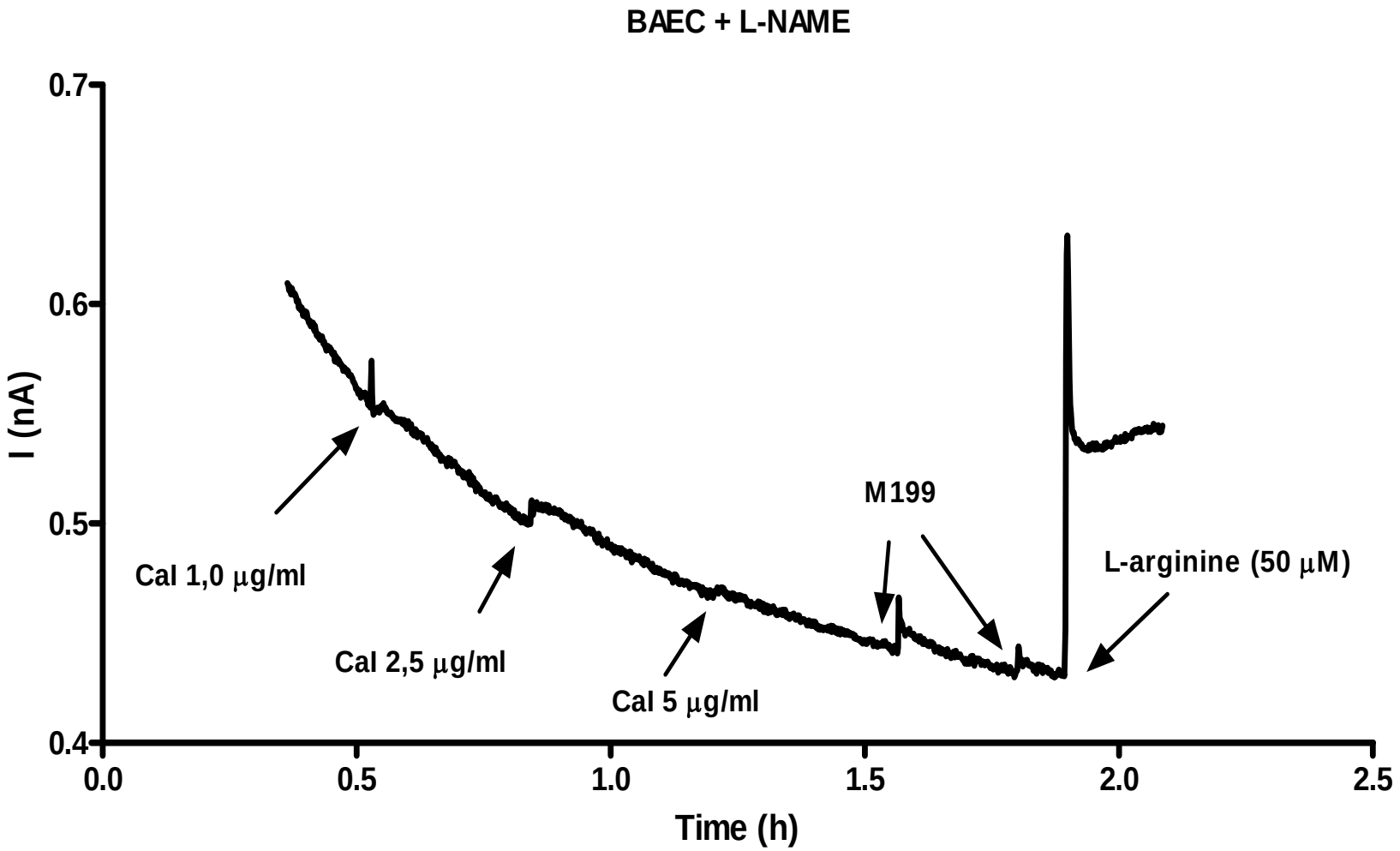


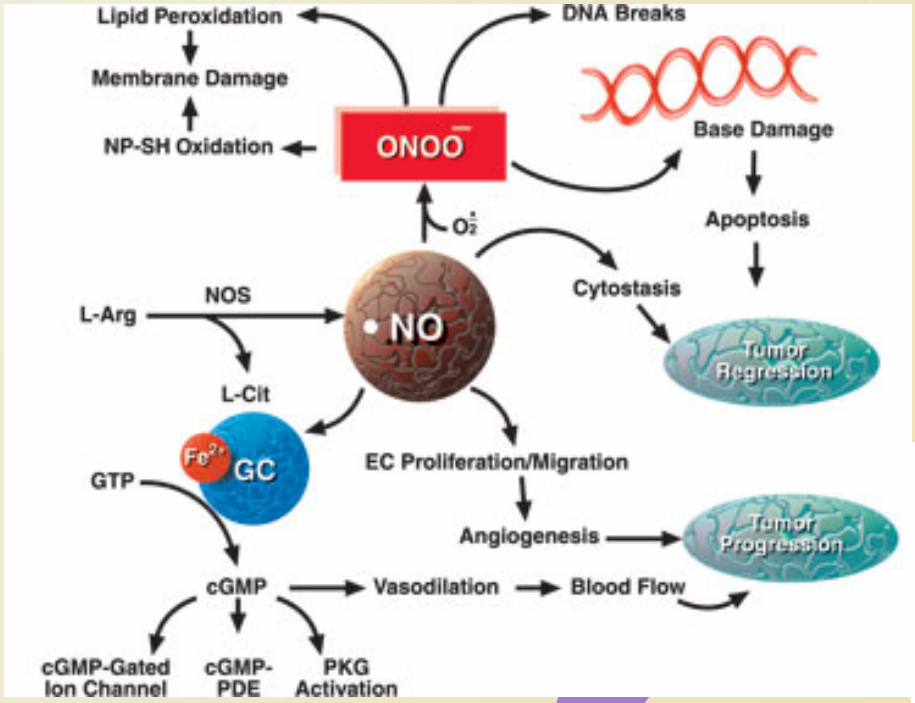


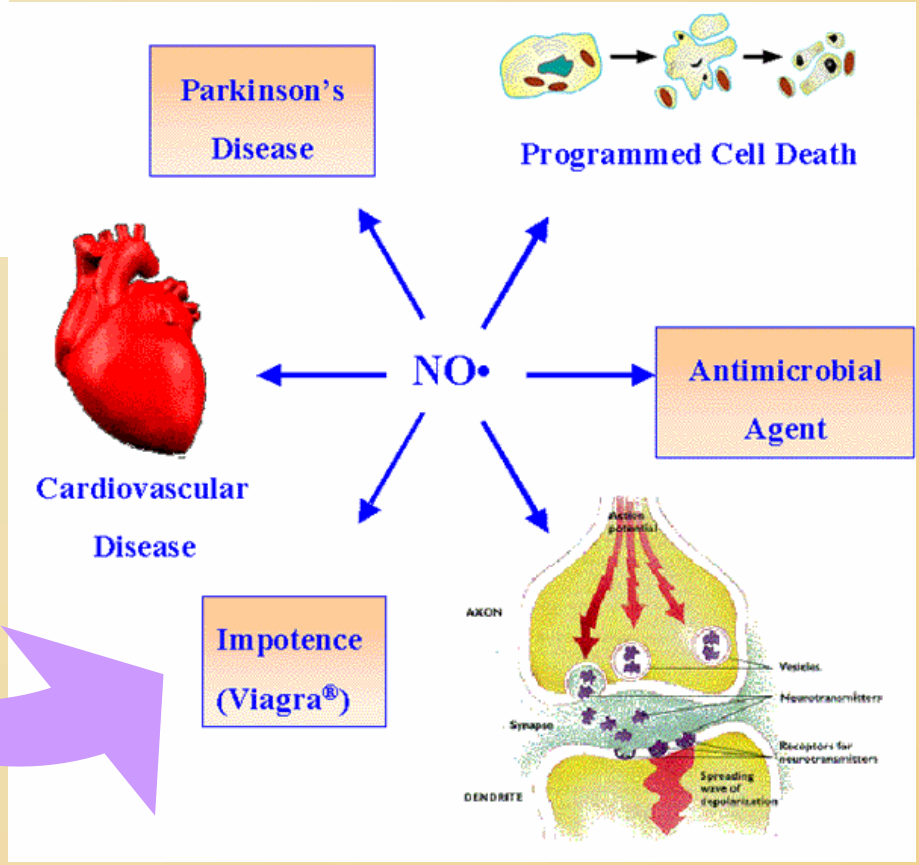





BAEC - bovine aortic endothelial cells
1.6
1.5
1.4
1.3
1.2
I (nA)
Cal 0,5 μg/ml
Cal 1 μg/ml
Cal 2,5 μg/ml
Cal 5μg/ml
Basal production
0
50
100
150
200
250
Time (min)


BAEC + L-NAME
0.7
0.6
0.5
0.4
I (nA)
0.0
0.5
1.0
1.5
2.0
2.5
Time (h)
Cal 1,0 μg/ml
Cal 2,5 μg/ml
Cal 5 μg/ml
M199
L-arginine (50 μM)


Lipid Peroxidation
DNA Breaks
Membrane Damage
NP-SH Oxidation
ONOO⁻
Base Damage
Apoptosis
O2·
Cytostasis
NOS
L-Arg
• NO
Tumor Regression
L-Cit
GTP
Fe2+
GC
EC Proliferation/Migration
Angiogenesis
Tumor Progression
cGMP
Vasodilation
Blood Flow
cGMP-Gated Ion Channel
cGMP-PDE
PKG Activation


Parkinson's Disease
Programmed Cell Death
NO•
Antimicrobial Agent
Cardiovascular Disease
Impotence (Viagra®)
AXON
Vesicles
Neurotransmitters
Synapse
Receptors for neurotransmitters
DENDRITE
Spreading wave of depolarization