

## iNOS (NOS2) at a glance

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Nitric oxide (NO) is an effector of the innate immune system. The innate immune system is a set of rapid host responses to pathogens. Cells of the innate immune system – macrophages, neutrophils and natural killer cells – use pattern recognition receptors to recognize molecular patterns associated with pathogens (Medzhitov, 2001). Activated macrophages then inhibit

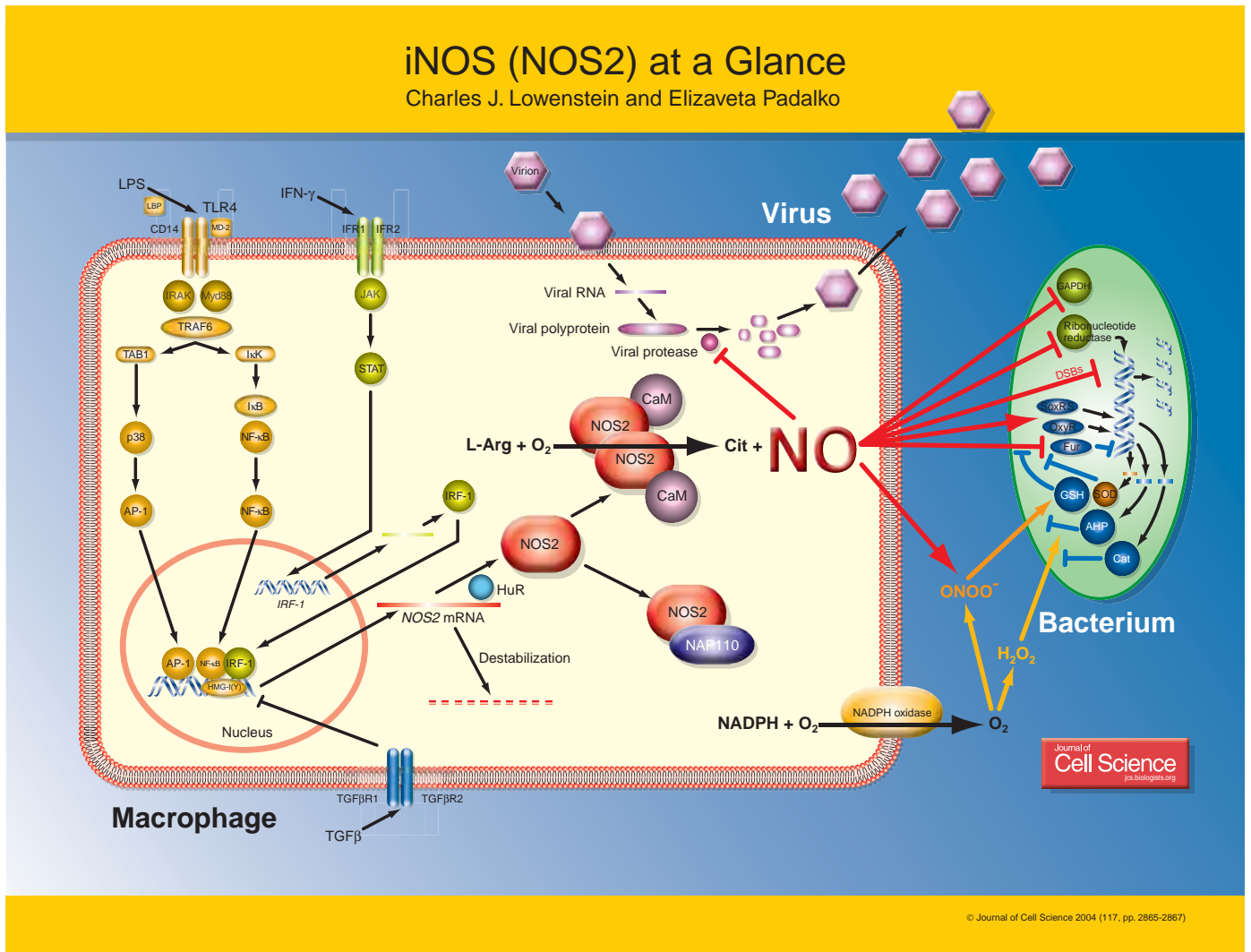
pathogen replication by releasing a variety of effector molecules, including NO.

### Extracellular signals trigger innate immunity

Resting immune cells lack the inducible NO synthase (iNOS or NOS2), the enzyme that synthesizes NO. However, a variety of extracellular stimuli can activate distinct signaling pathways that converge to initiate expression of NOS2. Cell wall components of bacteria and fungi can trigger the innate immune signaling cascade, leading to expression of NOS2. For example, lipopolysaccharide (LPS), a component of the wall of Gram-negative bacteria, can bind to LPS-binding protein (LBP), which delivers LPS to CD14, a high-affinity LPS receptor. Toll-like receptor 4 (TLR4) in conjunction with the small extracellular protein MD-2 interacts with the CD14-LPS complex, and then activates an intracellular signaling cascade via adaptors that include IRAK and MyD88, which in turn activate downstream molecules including TRAF6. The innate immune pathway then activates signaling pathways including the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathway. These pathways converge to activate NOS2 transcription.

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Cytokines released from infected host cells can also activate NO production, including tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 1 $\beta$  (IL-1 $\beta$ ). Interferon  $\gamma$  (IFN- $\gamma$ ) produced by activated immune cells can activate NOS2 expression, and can synergistically activate NOS2



expression in combination with other agents such as LPS. IFN- $\gamma$  interacts with the interferon receptor 1 (IFNR1) and IFNR2 complex, which activates kinases of the Jak family and signal transducers and activators of transcription (STAT) pathways (Darnell et al., 1994). The interferon signaling pathway can also activate NOS2 transcription.

#### **Transcriptional regulation: interacting transcription factors activate NOS2 transcription**

LPS-mediated activation of the innate immune pattern recognition receptors stimulates *NOS2* mRNA transcription within 2-4 hours, and NOS2 translation within 6 hours. LPS activation of TLR4 leads to phosphorylation of inhibitor of NF- $\kappa$ B kinase (IKK), which phosphorylates the inhibitor of NF- $\kappa$ B (I $\kappa$ B), which releases the transcription factor NF- $\kappa$ B. NF- $\kappa$ B translocates from the cytoplasm to the nucleus, where it interacts with  $\kappa$ B elements in the *NOS2* 5' flanking region, triggering *NOS2* transcription (Xie et al., 1994). IFN- $\gamma$  triggers *NOS2* transcription by activating the JAK/STAT pathway, leading to synthesis of the transcription factor interferon response factor 1 (IRF-1), which stimulates *NOS2* transcription (Kamijo et al., 1994). IFN- $\gamma$  also provides a synergistic boost to LPS induction of *NOS2* transcription because IRF-1 interacts with NF- $\kappa$ B, altering the conformation of the *NOS2* promoter (Saura et al., 1999a). Scaffolding proteins such as HMG-Y(I) and transcriptional activators such as CBP interact with IRF-1 and NF- $\kappa$ B, forming a multi-subunit complex that increases *NOS2* transcription (Perrella et al., 1999). Other transcription factors, including Stat1 $\alpha$  and hypoxia inducible factor-1 (HIF-1), can also regulate *NOS2* expression.

#### **Post-transcriptional regulation: AUUUA elements mediate NOS2 mRNA stability**

The 3' untranslated region of *NOS2* mRNA contains multiple AUUUA elements, which mediate stability of mRNAs of cytokines. The RNA-binding protein HuR interacts stabilizing *NOS2* mRNA with the AUUUA elements of the *NOS2* mRNA, (Rodriguez-Pascual

et al., 2000). Lack of HuR leads to destabilization of *NOS2* mRNA and decreased *NOS2* expression.

#### **Post-translational regulation: proteins interact with NOS2 and block NO synthesis**

*NOS2* does not synthesize NO until it forms homodimers. A set of proteins can inhibit *NOS2* activity by preventing *NOS2* homodimerization. For example, NOS-associated protein 110 kDa (NAP110) interacts with the N-terminus of *NOS2*, forming *NOS2*-NAP110 heterodimers, and blocking the formation of *NOS2*-*NOS2* dimers (Ratovitski et al., 1999). Although *NOS2* is ubiquitinated and phosphorylated, the significance of these post-translational modifications is unknown.

#### **Inhibition of NOS2 expression**

Transforming growth factor  $\beta$  (TGF- $\beta$ ) inhibits *NOS2* expression through transcriptional, post-transcriptional and post-translational mechanisms (Vodovotz et al., 1993). TGF- $\beta$  inhibits *NOS2* transcription in part by blocking expression of the scaffolding protein HMG-I(Y). A variety of other signaling molecules, including IL-4, IL-10, IL-13 and macrophage deactivation factor, inhibit *NOS2* expression by unknown mechanisms.

#### **NOS2 structure**

*NOS2* contains a C-terminal reductase domain, which binds NADPH and transfers electrons from NADPH to FAD and then to FMN, and an N-terminal oxygenase domain, which contains a heme and binding sites for arginine, tetrahydrobiopterin and calmodulin. *NOS2* utilizes oxygen and electrons from NADPH to oxidize the substrate L-arginine into the intermediate OH-L-arginine, which is then oxidized into NO and L-citrulline.

In contrast to the other NOS isoforms *NOS1* and *NOS3*, dimeric *NOS2* is always active when expressed. Although *NOS2* binds calmodulin, *NOS2* is independent of intracellular calcium levels, whereas calcium regulates *NOS1* and *NOS3* activity (Cho et al., 1992).

The  $V_{max}$  of *NOS2* is approximately 10-fold greater than the other NOS isoforms; so *NOS2* is a high-output NOS compared with the low-output isoforms *NOS1* and *NOS3*.

#### **NO and oxygen radicals**

NO and superoxide ( $O_2^-$ ) are radical effectors of the innate immune system that can directly inhibit pathogen replication (Nathan and Shiloh, 2000). Derivatives of NO can also block infections. NO can combine with  $O_2^-$  to form peroxynitrite anion (ONOO $^-$ ). NO can also form nitrosothiols, nitrogen dioxide (NO $_2$ ) and other nitrosating species. Superoxide can form hydrogen peroxide, which in turn can form hypochlorous acid and other oxidants. These reactive nitrogen intermediates and reactive oxygen intermediates can cross bacterial and fungal walls with differing facility, and react with specific pathogen targets (Fang, 1997).

#### **Anti-bacterial effects of NO**

NO is an anti-bacterial effector of the innate immune system (Fang, 1997). NO can inhibit bacterial DNA synthesis by inhibiting bacterial ribonucleotide reductase. NO can also cause double-stranded breaks (DSBs) in bacterial DNA. NO can modify cysteine residues in bacterial proteins, oxidize bacterial lipids, and interact with heme iron and iron-sulfur clusters in bacterial enzymes. However, it is unclear whether modification of these bacterial targets contributes to the anti-bacterial effects of NO. NO mobilizes zinc in bacteria, which suggests that DNA-binding proteins containing zinc are targets of NO (Schapiro et al., 2003). It can also increase the susceptibility of bacteria to oxidative DNA damage by blocking respiration (Woodmansee and Imlay, 2003). Peroxynitrite can oxidize bacterial lipids and produce nitrotyrosine of bacterial polypeptides, but the biological significance of these modifications is also unclear.

#### **Bacterial defenses against NO**

Oxidants activate bacterial defenses. The bacterial protein SoxRS serves as a

sensor for NO, and can activate transcription of a set of bacterial genes whose products defend the pathogen from oxidant damage – for example, bacterial superoxide dismutase (SOD). The bacterial protein OxyR can be modified and activated by hydrogen peroxide or NO, and directs the transcription of bacterial genes such as alkyl hydroperoxide reductase (AHP), which confers resistance to peroxynitrite, and catalase (CAT), which deactivates hydrogen peroxide (Bryk et al., 2000; Hausladen et al., 1996). The bacterial protein ferric uptake regulatory protein (Fur) also serves as an NO sensor. Fur is an iron-containing bacterial transcription factor that normally represses a set of nitrosative stress response genes. NO inactivates Fur by interacting with its iron co-factor, permitting expression of genes protective against oxidative stress (Crawford and Goldberg, 1998; D'Autreaux et al., 2002). One bacterial gene regulated by Fur encodes a flavohemoglobin that can detoxify NO, protecting pathogens from NO (Gardner et al., 1998; Hausladen et al., 2001). Thus multiple signaling pathways defend bacteria against NO.

### Anti-viral effects of NO

NO is an anti-viral effector of the innate immune system. It can inhibit replication of a variety of viruses, including Herpesviruses, Picornaviruses, Flaviviruses and Coronaviruses. Viral proteases are a target of NO. Many RNA viruses depend on viral proteases to cleave large viral polyproteins into smaller viral polypeptides. NO can nitrosylate the cysteine protease 3C<sup>pro</sup> of Picornaviruses, inhibiting viral protease cleavage of the viral polyprotein, and thus blocking viral replication (Saura et al., 1999b; Zaragoza et al., 1998).

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