Review

S-nitrosylation/Denitrosylation and Apoptosis of Immune Cells

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Nitric oxide (NO) as an immunoregulatory molecule, predominantly depending on S-nitrosylation, acts as a versatile player that executes its regulation and signal transduction for exerting its multi-functions and pleiotropy. Apoptosis of immune cells is an intricate process coupled with positive/negative selection depending on integrated diverse endogenous and exogenous signals and functions to sustain homeostasis in the immune system. Here, the dual roles of NO depending on its concentration in apoptosis are reviewed, breeding up a switch mode in the apoptotic process. Following comments of different switches from apoptosis-death, a new finding of checkpoint (early fluorescence point) of GSNO-initiated thymocyte apoptosis and NOS-GSNOR double control are highlighted. Moreover, S-nitrosylation/denitrosylation, being as a redox switch, logically approaches to networks of metabolism itself and further accesses the neuroendicrine-immune-free radical network as a whole. Moreover, the host defense mediated by NO on pathogens, *via* protein S-nitrosylation are also discussed. *Cellular & Molecular Immunology*. 2007;4(5):353-358.

Key Words: immune cell, nitric oxide, apoptosis, S-nitrosylation/denitrosylation, switch, host defense

Introduction

Nitric oxide (NO) as an immunoregulatory molecule, ubiquitously produced in almost all immune cells, is recognized as one of the most versatile players in the immune system (1). NO executes its regulation and signal transduction exerting its multi-function and pleiotropy, predominantly depended on S-nitrosylation, the covalent modification of cysteine sulfurs of proteins by NO or its derivatives to form S-nitrosothiols (SNOs). S-nitrosylation serves as NO-related functions involved in the proliferation, differentiation and apoptosis of macrophages, thymocytes, lymphocytes, endothelial cells and the cross talks among immune cells and diverse cells both in the nitric oxide synthase (NOS)-containing cells and in the intercellular signaling network (2, 3).

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It is well recognized that the model of NO-induced macrophage apoptosis is used to investigate macrophage function *in vitro* (4). In cultured macrophages, changes in SNO levels depend upon cytokine induction of inducible nitric oxide synthase (iNOS).

Thymocyte apoptosis is an intricate process depending on integrated diverse endogenous and exogenous signals and functions, coupled with positive/negative selection to sustain homeostasis in the immune system (5-7). NO and its donors S-nitrosoglutathione (GSNO) are able to induce immune cell apoptosis, such as macrophages, thymocytes, lymphocytes and endothelial cells *via* various signal pathways, especially *via* S-nitrosylation/denitrosylation as a reversible redox switch (5, 8). Negative selection in thymocyte development is a process, in which immature thymocytes expressing T cell receptors (TCRs) with high affinity for self-peptide: major histocompatibility complex (MHC), are induced to undergo apoptosis (9). Thus, S-nitrosylation/denitrosylaton proteins in post-translated modification have multifunction, especially, thymocyte apoptosis and its negative selection (5, 10).

Many papers contribute to study post-translational modification of S-nitrosylation/denitrosyaltion and its biological signification, like those of phosphorylation/ dephosphorylation. The specificity of S-nitrosylation is characterized to reaction of NO^+ derived from NO with sulphydryl of cysteine residue on the proteins in high

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; GSNO, S-nitrosoglutathione; GSNOR, S-nitrosoglutathione reductase; SNO, S-nitrosothiol; TCR, T cellreceptor; DP thymocytes, double positive thymocytes; GAPDH, glyderaldehyde-3-phosphate dehydrogenase; MHC, major histocompatibility complex.

reaction constant, which either covers recognition function of nitric oxide or reveal S-nitrosyl reactivity in the optimal micro-environments. However, the major specificity of protein S-nitrosylation is determined by nitric oxide-dependent protein-protein interactions (2, 9, 11). The further research findings demonstrate that specificity of S-nitrosylation should be conferred by structural motifs of corresponding target protein. The Cys residue between Asp(acid)-His(base) motif becomes a valid Cys for S-nitrosylation in many proteins (including Hb) (12).

This article focused on the advancement in the investigation of protein S-nitrosylation/denitrosylation in apoptosis of immune cells to open up a new approach of exploring switch mechanism on thymocyte apoptosis coupled with the positive/negative selection during thymocyte development.

GSNO-induced thymocyte apoptosis and S-nitrosylation/denitrosylation

Dual role of nitric oxide in immune cell apoptosis

NO concentration within immune cells plays a key role in determining whether NO stimulates or inhibits apoptosis coupled with the negative selection of thymocytes. GSNO as an NO donor is able to up- or down-regulate thymocyte apoptosis. GSNO lower than 0.6 mmol/L would trigger thymocyte apoptosis, while higher than 2 mmol/L would suppress apoptosis. Inhibition of apoptosis by NO is analogous to the action of the thiol-blocking compound N-ethylmaleimide (NEM), which implies that the inhibition of thymocyte apoptosis results from thiol modification of critical proteins in response to NO treatment (13). Our experimental findings also indicated GSNO with different concentration (0.3, 0.6, 1.2, 2.4 mmol/L) has dual roles during thymocyte apoptosis. Indeed, the above findings would imply that the concentration of GSNO controls thymocyte apoptosis switch mode.

As mentioned above, experimental results seem to only describe protein S-nitrosylation; in fact, alternation (or alternated) between S-nitrosylation/denitrosylation should be proceed non-enzymatically or enzymatically in cells. Meanwhile, both S-nitrosylation and denitrosylation cooperate to coordinate the cells' apoptosis (7, 12). All mammalian cells including untreated mouse thymocytes, contain low levels of protein S-nitrosylation (14), which has been supported by the very weak bands of protein S-nitrosylation detected using Biotin switch coupled with Western blot. But both glutathione and thioredoxin are able to make S-nitrosylated proteins denitrosylated *via* transnitrosation (15).

Mannick reported that it is predominantly the subpopulation of caspases, which is S-nitrosylated, residing in mitochondria of human lymphocyte cell lines. A subset of caspase-3 zymogens are inhibited by the catalytic site cysteine S-nitrosylation in untreated human lymphocyte cell lines (16). However, Fas, as an apoptotic stimuli, would activate caspase-3 *via* denitrosylation and promote the release of caspase-3 from mitochondria in the presence of Apaf1 (14). Consequently, increase of caspase-3 activity results in cytosol caspase-3 denitrosylation, eventually, which leads to cell apoptosis. Therefore, in this case Fas activates caspase-3 not only *via* promoting the cleavage of the caspase zymogen to its active subunits, but also through stimulating the denitrosylation of its active-site thiol, in essence, thiolation (17). Thus protein S-nitrosylation/denitrosylation can cooperate to coordinate in the intricate signal transduction pathways.

Lipopolysaccharide could induce the up-regulation of the 120 kDa iNOS in dendritic cell (DC) resulting in increase of NO, which is able to inhibit caspases *via S*-nitrosylation of the valid cysteine residues at the active sites. Thereby, nitric oxide-inhibited caspases could become an essential mechanism in controlling the activity of caspases during DC maturation (18). S-nitrosylation of Bcl-2 inhibits its ubiquitin-proteasomal degradation, therefore suppresses apoptosis (19).

In general, non-enzymatic reaction is characterized by S-nitrosylation, but recently the growing evidence of enzymatically mediated S-nitrosylation has been recognized, such as formaldehyde dehydrogenase selectively denitrosylates the S-nitrosylated peptide GSNO. GSNO serves as a reservoir of NO in cells. Therefore, both deletion and knockout of formaldehyde dehydrogenases would result in the increases of GSNO and protein S-nitrosylation, which would increase death rate due to endotoxic or/and bacterial challenge. But NOS inhibitor can decline the accumulation of proteins' S-nitrosylation (20).

Thymocyte apoptosis switch mode and S-nitrosylation/denitrosylation

Regulation, especially dual roles of GSNO during thymocyte apoptosis, and signaling transduction *via* S-nitrosylation and denitrosylation in cells imply an implicit switch which is characterized in living system. Recently, growing reports contribute to be visually integrated many life phenomena by switch mode. In regards to apoptotic switch mode, many scientists only focus on studying the mechanisms on apoptosis-necrosis switch mode. Fujita R only provided from apoptosis to necrosis switch mode depended on ATP level coupled with energy metabolism (21-23), whereas Gramaglia found it wasn't shut off apoptotic switch as ATP was exhausted (24). Those contradictory viewpoints result from the malpractice of methodology and limitation of measured apparatus (such as resolution, sensitivity, laser damage to cells, photobleaching, and so on) besides the different cell types and the working conditions. To avoid the disadvantages caused by the micro-imaging apparatus, we fully exert superiority of high sensitivity and high resolution of advanced ICCD-based real-time fluorescence micro-imaging, and also minimized the laser-caused damage on cells by autosynchronizing the exciting shutter and the exposure shutter. Thereby, the acquired checkpoint (early apoptotic fluorescence signals) initiating thymocyte apoptosis has been found about 2 h after exposure to 0.3 mmol/L GSNO in the study of thymocyte apoptotic switch mode by fluorescence microscopy, two photon laser confocal microscopy and near-field scanning optical microscopy (NSOM) (25, 26).

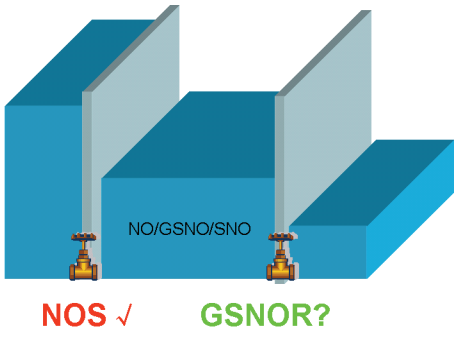

Figure 1. NOS-GSNOR double gate-control of the homeostasis of endogenous NO/GSNO/SNO. NO is produced by NOS and stored or metabolized as GSNO or SNOs, which are controlled by GSNOR directly/indirectly. This diagram is a hypothesis that in immune response, there is a homeostasis of endogenous NO/GSNO/SNO which is under NOS-GSNOR double gate-control. Compared to NOS, the roles of GSNOR are much less understood.

This pioneer checkpoint of initiated apoptosis just matches a moment of appearing peak value of fluorescence, which denotes pH value decrease (acidification of lysosome). The event that turned the apoptotic switch on, inducing thymocyte apoptosis, in 0.3 mmol/L GSNO-treated thymocyte lysosome, was detected by two photon laser confocal microscopy with LysoSensor 7545 in our laboratory. This not only revealed the relation of a variety of protons in lysosome, marking an alternation between acidification/alkalification in cell microenvironment that initiated thymocyte apoptosis, but also indirectly proved the checkpoint of GSNO-initiated thymocyte apoptosis is consistent with a sharp raise of fluorescence intensity (acidification) in thymocyte lysosome resulting in apoptosis (data not shown).

Meanwhile, our findings have been supported by real time imaging in plasma membrane of apoptotic monocytes and macrophages (27). As Mannick has legibly provided S-nitrosylation/denitrosylation of critical cysteine residues on proteins serves as a redox switch that regulates the function of a wide array of proteins (7, 9), this proposition has further demonstrated our findings are reasonable and corresponding scientific rule.

Moreover, more active findings also showed that the occurrence and regulation of protein S-nitrosylation is the checkpoint of the susceptibility of neuronal cells to NO (28). In addition, it has been reported that NO has multiple effects on immune cells. For instance, NO alters the Th1-Th2 balance (29-31) and NO is involved in the apoptotic deletion of thymocytes, selection of TCR-stimulated DP thymocytes (32). S-nitrosylated mechanism on NO-induced/inhibited cell apoptosis has been moved to further study in wide area by means of advanced multi-techniques. It remains to be applied in immunology.

The diverse stressors as apoptotic stimulators, in essence, different concentration of GSNO-treated thymocytes denote an S-nitrosative stress, produce coordinated S-nitrosylation and denitrosylation events in intricate and delicate pattern, which cooperate in concert to control the apoptotic pathway (17).

In summry, we further develop the dual roles of GSNO during thymocyte apoptosis underlying NO-triggered cell apoptosis which implies a switch function in the apoptotic process, breeding up a switch mode with our active experiments. Since the different apoptosis-death switches have been studied, a new finding--checkpoint (early fluorescence point) of GSNO-initiated thymocyte apoptosis has been highlighted in GSNO-induced thymocyte apoptosis coupled the negative selection during process of thymocyte development. Integration of our findings with propositions of Mannick JB indicates S-nitrosylation/denitrosylation serves as a redox switch which logically approaches to networks of metabolism and further accesses neuroendocrine-immune-free radical network as a whole.

NOS-GSNOR double gate-controlled S-nitrosylation and the immune response

The reductants, such as thiols and ascorbate, can enhance metal-ion-dependent decomposition of S-nitrosothiols by copper and iron *in vitro* (33). The endogenous SNO levels are modulated not only by three classes of NOS, but also by specific S-nitrosoglutathione reductase (GSNOR). It belongs to the alcohol dehydrogenase (ADH) class III family of enzymes, also known as glutathione-dependent formaldehyde dehydrogenase (FDH), and is capable of catalyzing the NADH/NADPH-dependent degradation of GSNO to glutathione sulphinamide, oxidized glutathione (GSSG) and ammonia (34). Most importantly, although this enzyme does not act on SNO-protein substrates, GSNOR deficiency leads to increases in basal levels of SNOs. While S-nitrosothiols play an essential role in biological processes such as vascular homeostasis and contribute to the pathogenesis of endotoxic/septic shock (20), the current biology of NO centered on NOS is being changed.

Evidence strongly proves that increase of NOS in the exhaled breath denotes signature of asthma, but asthmatic syndrome is almost unchanged in mice knockout or inhibiting NOS. Whereas mice with knockout GSNOR gene present increases in lung SNOs and are protected from airway hyperresponsivity (35). GSNOR controls levels of GSNO directly and protein SNO indirectly, and is essential for SNO metabolism. Therefore, we propose that in immune response, there is a homeostasis of endogenous NO/GSNO/SNO which is under NOS-GSNOR double gate-controlled (Figure 1). However, compared to NOS, the effects of GSNOR are much less understood.

Host defense and S-nitrosylation

NO possesses not only immunoregulatory function but also direct effects on microbes. In general, it is able to either killing pathogens or inhibiting their replication. It is important to inhibit the growth of bacteria, parasites, and fungi (36). Pulmonary tuberculosis is cured with inhaled NO

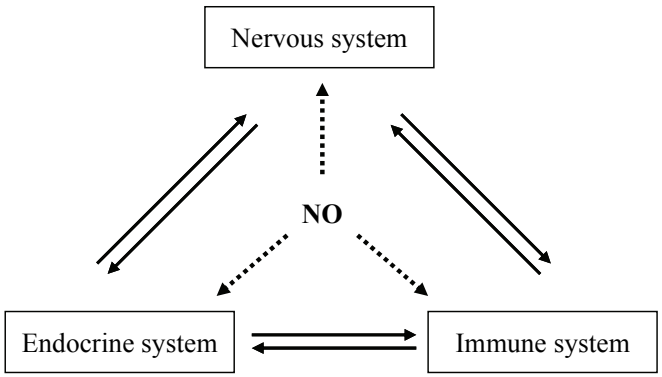

Figure 2. Regulation and signaling transduction of nitric oxide in the intricate network among nervous system-endocrine system-immune system through S-nitrosylation/denitrosylation of proteins. Nitric oxide, as a common modulator and messenger coupled with reactive oxygen species, may serve as a bridge for the regulation among these three systems. NO-regulating process implies its signaling transduction. The NO regulation would be carried out in the signaling transduction which should be mediated by S-nitrosylation/denitrosylation. S-nitrosylation/denitrosylation may be the novel molecular mechanism.

which directly touches *M. tuberculosis* in the lung and *M. tuberculosis* could be killed when cultured in 90 ppm NO (37).

NO also inhibits virus replication *via* two approaches: (1) oxidation modification by a potential oxidant peroxynitrite ($ONOO^-$), produced by reactions of NO with superoxide in the high reaction constant, is able to oxidative modification of capsid and coxsackie viruses resulting in the inhibition of viral entry into cells; (2) virus proteins S-nitrosylation coupled with inhibition of transcription factors (38, 39). For example, NO-mediated S-nitrosylation of viral and host (macro) proteins, e.g., proteases, reverse transcriptase, and ribonucleotide reductase, would become an intriguing general mechanism for inhibition of HIV-1 replication, antiviral effects and the control of the virus life cycle (40, 41). Viral proteinase S-nitrosylation, which required for viral replication, was carried out in post-translational modification manner leading to viral variety (42, 43).

NO is an anti-viral effector of the innate immune system, but few of NO-targeted viral proteins have been defined by further valid experiments, especially, regarding the mechanism of S-nitrosylation.

It has been reported that spermine-NONOate as NO donor inhibited over 67% adenovirus titer and proved that NO was able to inhibit adenovirus infection *in vitro* by preventing from the processing of the virion precursor proteins and the synthesis of infection virus. Furthermore, evidence indicated that NO is able to inhibit adenovirus proteinase (AVP)-pVIc (an 11-amino acid peptide from the precursor to adenovirus precursor protein pVI) by S-nitrosylation of Cys122 (42).

It is important to keep in pathogen latency for the active host immune response. NO delivery from host cells may be critical for maintaining latency because inhibition of NO synthesis either by NOS inhibitors or by targeted disruption of the iNOS leads to reactivation of *M. tuberculosis*, *L. major*, *T. gondii*, and Epstein-Barr virus (38, 44, 45). R410C, as a new class of S-nitrosylated proteins, human serum albumin with two free thiols at Cys-34 and Cys-410, is an NO carrier which possesses antibacterial activity against *Salmonella typhimurium* and cytoprotective properties both *in vivo* and *in vitro* (46). Moreover the preceded findings are proved by the results that mice with a targeted deletion of iNOS or treated with NOS inhibitors had less severe disease than control mice (47).

Perspective

Present new findings in the proteins S-nitrosylation/denitrosylation in post-translational modification have been driving the advance of theory and practice of life sciences. We face challenges from new concepts, new proposition, and new theory in booming life sciences underlying development of mathematics, physics, chemistry and current biology as a whole. So we need to gain perspective in the future as follows:

1) It is going to the further study of the relation between the structure of a key protein S-nitrosylation and their functions which regulate immune proteins in the processes of signal transduction, underlying advance in S-nitrosylated mechanism on NO-induced immune cell apoptosis, especially, proteins S-nitrosylaton in post-translational modification;

2) S-nitrosylation/denitrosylation of proteins is able to take part in the network of metabolism itself, and further approach to the neuroendocrine-immne-free radical network (Figure 2). It tries to create a platform of cytokine network and network of the key enzymes, transcription factors, receptors for developing immunology, immunopharmacology under theories of the traditional Chinese medicine and system biology.

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