The L-Arginine-Nitric Oxide-Peroxynitrite pathway (LANOP pathway): Does it protect or worsen the course of Chagas disease?

MARÍA EUGENIA CÁRDENAS, MSc1, DIEGO TORRES, MD, MSc, PhD1,2, ANA MARÍA MUJICA1, JASSON SEBASTIÁN SANABRIA1

SUMMARY

Many different immunological processes have been described in the Chagas infection, some of them associated with the Chagas disease. In this scenario, the L-Arginine-Nitric oxide (NO) - Peroxynitrite (NOOO-) pathway (LANOP pathway) appears as an essential component of that process. The relationship is well known between cytokines that can induce Oxide Nitric Synthase (iNOS) genes, such as TNF-α and IFN-γ, and other molecules that can inhibit their expression (TGF-β, IL-10 and others), which are involved in both acute and chronic stages of the disease pathogenesis. However the participation of the LANOP pathway seems complex, given that evidence shows different roles for it during the course of the infection. In this article, the authors review the immunological and inflammatory response leading to the activation of the LANOP pathway during the Chagas infection, and the role this via plays, including different effects, protector or deleterious, observed in parallel during the development of the infection.

Colomb Med. 2010; 41: 388-95

Keywords: iNOS; Nitric Oxide; Trypanosoma cruzi; Chagas disease; Chagasic cardiomiopathy; Peroxynitrite.

Vía L-Arginina-Óxido Nítrico-Peroxinitrito (Vía-LAONP): ¿Protege o empeora el curso de la enfermedad de Chagas?

RESUMEN

En la infección chagásica se han descrito diversos procesos inmunológicos, algunos de los cuales se han asociado con la enfermedad. En este escenario, aparece la vía L-Ariginina-Óxido Nítrico (NO) - Peroxinitritó (ONOO-) (Vía-LAONP) como un componente esencial de estos procesos. Se conoce la asociación entre citocinas inductoras de los genes de la Sintasa del Óxido Nítrico (iNOS) tales como TNF-α e IFN-γ, así como moléculas que inhiben su expresión (TGF-β e IL-10 entre otras), involucradas en la patogénesis tanto de la fase aguda como crónica de la enfermedad. No obstante, la participación de la vía-LAONP parece ser compleja, una vez que las evidencias señalan papeles diferentes de ésta durante el curso de la infección. Por tanto, los autores revisan la respuesta inmunológica e inflamatoria que da lugar a la activación de la vía-LAONP durante la infección chagásica, y el papel que ésta desempeña, incluyendo efectos diversos, tanto protectores como deletéreos, que han sido observados en paralelo durante el curso de la misma.

Colomb Med. 2010; 41: 388-95

Palabras clave: iNOS; Óxido nítrico; Peroxinitrito; Trypanosoma cruzi; Enfermedad de Chagas; Cardiomiopatía chagásica.

The hemoflagellate Trypanosoma cruzi protozoan is the etiological agent of Chagas disease in Latin America, infecting close to 15-million people, even when there are nearly 28-million individuals exposed1. Its manifestations during the acute phase are non-specific. Two to three decades after primary infection, between 30 and 40% of the seropositive individuals in undetermined phase, present signs and symptoms of dilated cardiomyopathy2. Herein, we reviewed physio-pathological aspects of the Chagasic infection, with emphasis on the L-Arginine-Nitric oxide (NO) – Peroxynitrite (NOOO-) pathway (LANOP pathway), along with the most notable evidence of the dual...
actions (beneficial and deleterious) of these free radicals on the affected tissue. It is worth knowing and understanding the dimension acquired by the LANOP pathway in the physiopathology of the disease for the basis of future studies.

INVASION OF THE HOST BY TRYPSANOSOMA CRUZI AND INTERACTION WITH THE IMMUNE SYSTEM

The interaction between cells from different hosts and the parasite, a process of fundamental importance in understanding its pathogenicity, has been extensively reviewed by different authors3,4, and although it is beyond the objective of this review, we will address some relevant aspects given its close relationship with LANOP pathway.

*Trypanosoma cruzi*, an obligate intracellular protozoan with high genetic variability (lineage) within its sole species5, manifests a diversity of biological behaviors and cellular interactions, which require it to put into action its virulence factors, in response to the defense mechanisms of the host to be able to perpetuate its replication6-8.

The parasite has two phases in its development cycle: one in the vertebrate host (which includes humans), and another in the insect vector. When the *T. cruzi* penetrates the vertebrate host, it multiplies locally as a amastigote, and appears in peripheral blood as a blood trypomastigote, responsible for parasitemia. From there, it is ingested by the triatomine, goes through the epimastigote stage, and reaches the hindgut of the vector, where it is multiplied as a metacyclic trypomastigote and is eliminated in the feces; this way, it becomes infectious to mammals. Here, the parasite penetrates via continuity solutions in skin or mucous membranes, thus closing its life cycle9.

Many types of mammalian cells, including humans, have been studied as a model to understand the initial membrane-membrane interactions between host and parasite, through cell culture systems, cell lines, and primary cultures10,11. Some of the models mostly used by researchers have been mammalian cells, usually macrophages, fibroblasts, epithelial and muscle cells of murine origin, or cells from the phagocytic mononuclear system, proceeding from human peripheral blood12-14.

It is known that in vertebrates the first contact of the *T. cruzi* occurs with the mononuclear fagocytes15,16, via Toll-like receptors (TLRs), and the recently described NOD-like receptor (NLRs), which recognize multiple molecular patterns from different pathogens17,18. There are other interactions between both cell surfaces, via lipid and protein sialoglyco-conjugate molecules, present in the mononuclear phagocyte and in the parasite, permitting cell activation through specific signals19,20. Among these molecules some lectins and integrins are described, as well as other molecules of cellular and vascular adhesion: fibronectin and sialic acid. The parasite manages to transfer itself through the phagocyte membrane, given that it expresses trans-sialidase-neuraminidase enzyme activity, and cysteine protease (Cruzipain)21,22. Other cells implied in the initial control, via induction of the iNOS expression, are the NK23.

As of this moment, a series of complex and critical processes are generated to mediate the production of inflammatory mediators, initially at the expense of innate mechanisms of the immune system and, thereafter, these go on to guiding the actions of the antigen-presenting cells (APCs), which would be key for inducing the adaptive immune mechanisms24,25; two moments that could correspond *in vivo* to the acute and chronic forms of the infection.

Within this context, NO, as a parasiticide molecule, is one of the main initial mediators relevant in parasite control; it is produced in considerable levels a few hours after activation of via TLR and NLR phagocytes, which induce the expression of the iNOS gene14,17,26. Given that some inflammatory mediators (Tumoral Necrosis Factor alpha, TNF-α, Interleukin 1, IL-1, and Interferon gamma, IFN-γ), also strongly induce the gene in various cell types, among them the mononuclear phagocyte; others like the transforming growth factor beta (TGF-β) can repress it11,27. NO is considered indispensable for infection control, at least in mice11,27-29. Hence, it is probable that its level of production, as well the equilibrium in the level of inflammatory mediators controlling said production, are key for the outcome of acute infection, as has been described30,31.

The adaptive immune system is activated once the APCs process and introduce parasite antigens into T cells within the context of the HLA-II molecules. As in other intracellular infections, the Th1 profile would be responsible for greater IFN-γ production, which stimulates the production of NO from L-Arginine, by the activated macrophages, for the purpose of destroying...
parasites\textsuperscript{13,32,33}; however, for several reasons, this response may be modified in favor of the \textit{T. cruzi}, permitting perpetuation of the infection.

In this sense, there is experimental description of various mechanisms of evasion or modification by the \textit{T. cruzi} to defense mechanisms of the host, both in the innate as in the adaptive immunity. For example, the glycoprotein gp160, which has the trypomastigote, bonds to the C3b and inhibits recruitment of subsequent members of complement cascade, preventing the formation of the convertase and parasite lysis\textsuperscript{34}, while modulating functions during the antigen presentation, like inhibiting the expression of HLA-II\textsuperscript{35}. It has also been described to induce cell apoptosis of T cells (more Th1 than Th2), and diminishes the expression of IL-2 and IL-12\textsuperscript{31}, which generates disequilibrium at the Th1/Th2 level, favoring parasitic persistence\textsuperscript{32,36}.

It may be said, then, that NO plays an important role in infection persistence and that in the parasite-host relationship there are specific aspects of the pathogen (sustained expression of surface glycoproteins that maintain the parasite-cell interaction, and capacity to evade the defense of the host), and specific aspects of the host (proper recognition, presentation and activation of immune mechanisms associated to parasite control), which determine the outcome of said infection\textsuperscript{37}.

**L-ARGININE-NITRIC OXIDE-PEROXYNITRITE PATHWAY**

Chemically, NO is a low-molecular weight radical gas (30 daltons), soluble in water and lipids. It reacts in water with the oxygen and its intermediary reactives\textsuperscript{14,38} to produce other radicals and anions of diverse stability. Given its characteristics, it easily spreads the eukaryote and prokaryote cell membranes. Once it is produced by any cell type, the human body performs its actions, physiological or physio-pathological, according to its synthesis triggering process. Because NO is produced in constitutive or inducible manner, according to the expression of the gene of the enzyme synthesizing it, this gas will have a different level and synthesis duration, but always with a short mean biological lifespan of a few seconds\textsuperscript{39} (6-10 s).

The biochemical basis of the toxicity induced by the nitric oxide on phagocytized organisms, apparently depends on the combination of such with molecules containing iron in their structure, e.g., key enzymes in the cell respiration and replication cycle\textsuperscript{14} like the hapta-protein enzyme complex of the NADPH oxidase, critical in multiple cell processes. Not only does the phagocytic mononuclear system produce large and sustained amounts of NO under induction stimulus from iNOS, but the activity of this enzyme has also been detected in other cells like: hepatocytes, vascular smooth muscle cells, polymorphonuclear neutrophils and endothelium, among others. Hence, NO is involved in acute and chronic inflammation, as well as in the innate and acquired immunity\textsuperscript{39,40}. Specifically, for the case of American trypanosomiasis NO has been described as a participating molecule in the pathogenesis of the disease, but with different effects in the diverse phases of the disease\textsuperscript{37}.

Also, NO has been described as key in the synthesis of several agents considerably important in the physiopathology of acute and chronic inflammatory processes among which there is peroxynitrite (ONOO-), which results from the reaction between NO and the superoxide ion (O$_2^\cdot$). O$_2^\cdot$, also described as a direct microbicide agent, is released from the granulocyte and macrophage respiratory chain, through a redox reaction in which the NADPH oxidase transfers an electron from the equivalent NADPH reducer to the molecular oxygen\textsuperscript{41} (Graphic 1).

Although, initially NADPH oxidase was only described in phagocytic cells, currently it has also been identified in non-phagocytic cells like fibroblasts, endothelial cells, vascular smooth muscle cells, renal mesangial cells, and renal tubular cells. O$_2^\cdot$ is also produced during autoxidation of hemoglobin, myoglobin, and cytochrome c; as well as enzymes like xanthine oxidase, aldehyde oxidase, and a variety of flavin dehydrogenases\textsuperscript{41}. The resulting superoxide is subsequently converted to various intermediate reactive species with microbicide functions consisting of structural damage on the microorganisms\textsuperscript{41}.

ONOO$^-$ is recognized as an oxidizing agent of highly reactive nitrination, with a half-life of approximately 1 second at 37°C, pH=7.4, and stable in alkaline solutions\textsuperscript{41}. It reacts with a variety of biomolecules, including lipids, proteins, carbohydrates, and deoxyribonucleic acid. From the point of view of physio-pathological effects, ONOO$^-$ has been identified as an important microbicide agent, triggering lipid pero-
oxidation, sulphydryl oxidation, inactivation of sodium transport, and nitration of tyrosine residues in a significant variety of proteins that include the inactivation of enzymes and/or receptors; associated to oxidative stress and consequential endothelial.

NITRIC OXIDE (NO) SYNTHESIZING ENZYMES

The L-arginine amino acid was described in 1988 as the precursor in biosynthesis of nitric acid in endothelial cells. Said synthesis is catalyzed by a perfectly studied, classified, and characterized enzyme complex called nitric oxide synthase (NOS) of which there are three described isoforms, whose expression differs from one tissue to another, regarding the control of the gene transcription and the enzymatic activity. One is neural (nNOS), another is inducible (iNOS), and the other is endothelial (eNOS), or types I, II, and III, respectively. The NO synthases have relevant differences in relation to inducer mechanisms of their expression, amounts, and duration time of the NO produced; thus, eNOS and nNOS, being of constitutive expression, depend on calcium/calmodulin for their activation and synthesize NO in picomolar amounts and for short periods (seconds to minutes), while iNOS is independent of calcium and is activated by another series of mediators that induce the gene expression to produce sustained nanomolar amounts of NO.

The catalytic reaction of molecular oxygen with L-arginine to form its two end products: nitric oxide and L-citrulline, is a reaction common to the three isoforms. Structurally, these enzymes are hemoproteins 40% similar to cytochrome P-450. They exist as dimers to be functional, have a reducer end and the other is oxidizing in each monomer, and in their active form bind molecules of NADPH, FAD, FMN, calmodulin, heme, and tetrahydrobiopterin.

Graphic 1. LANOP Pathway. The oxidation of the reducing agent, NADPH by the NADPH Oxidase is necessary for superoxide (O$_2^-$) formation. Superoxide ion and Nitric Oxide (NO), synthesized from the reaction between L-arginine and molecular Oxigen O$_2$, are both critical for the synthesis of the final product: Peroxynitrite (ONOO·).
FISIOPATOLOGÍA DE LA ENFERMEDAD DE CHAGAS: PARTICIPACIÓN DE LA VÍA LANOP EN LA RESPUESTA IMMUNO-INFLAMATORIA

El parásito inicia la infección penetrando como trypomastigote metacíclico en el hospedador vertebrado, y una vez que logra invadir una amplia variedad de células, la inmunidad se activa en el hospedador con participación activa de mecanismos efector de múltiples tipos que mediante la control del ataque infeccioso, permitiendo en algunos casos el progresso a la etapa crónica y predominantemente afectando a la musculatura 45. El óxido nítrico (NO) es uno de estos mediadores, derivado del gen iNOS, que durante más de una década ha sido conocido por su gran potencia microbicida en "vitro" 29, incluyendo Trypanosoma cruzi 29. Este efecto parásitico en la tripanosomiasis se logra a través de mecanismos oxidantes en respuesta a la estimulación del gen inducible, presente en una gran cantidad de células del cuerpo. A pesar del efecto parásitico del NO sobre T. cruzi 29, algunas investigaciones han mostrado que la participación del NO no es totalmente esencial para este control 46 y, en algunos casos, a un cierto grado -dependiendo del estado de la infección- puede desempeñar un papel predominante deletério en la patogénesis de la enfermedad crónica 47.

iNOS está principalmente implicado en la respuesta inmunológica a T. cruzi. Su gen está localizado en el cromosoma humano 17 y fue inicialmente descrito en las macrófagos, aunque actualmente se ha encontrado en diferentes tejidos, incluyendo el músculo cardíaco. Polimorfismos genéticos han sido descritos en varios loci del gen codificador de iNOS; sin embargo, no hay evidencia de asociación entre estos y la susceptibilidad para T. cruzi 48. En macrófagos no estimulados, la actividad de iNOS no está normalmente presente; tras un tiempo fijado de activación celular a través de productos bacterianos (lipopolisacárido bacteriano o LPS) o citocinas inflamatorias pro-inflamatorias como IL-1, IFN-γ, y FNT-α, hay expresión para la síntesis de iNOS en "novo". Una vez que se expresa, NO es producido durante un largo periodo (48-72 horas) y en grandes cantidades, lo que permite la acción de NO en la respuesta inmunológica. No obstante, esto puede resultar en manifestaciones tóxicas en el tejido adyacente y, en algunos casos, contribuye al desarrollo de la enfermedad, como en el caso de la Chagas experimental 12.

El mismo iNOS puede ser estimulado, y también se puede bloquear por diferentes sustancias inhibidoras, como L-N-MMA, L-NAME, y aminoguanidina 43, que han demostrado, a través de estudios experimentales, el efecto protector de NO contra la infección con T. cruzi, dado que la parasitosis no puede ser controlada en la ausencia de NO, o cuando éste está inhibido 50. Vale la pena mencionar el existencia de un mecanismo de regulación que puede contrarrestar la expresión de iNOS, por ejemplo, NO producido en grandes cantidades debido a la estimulación de TNF-α sobre iNOS se puede contrarrestar ya que inhibe a un cierto grado la constituyente del enzima.

Una vez que el parásito es interiorizado, su muerte depende del NO producido. La producción de NO por macrófagos activados es estimulada por TNF-α y IFN-γ, y por moléculas quimiotácticas, como el factor activador plaquetario (PAF), las C-C quimokinas, y el leucotrieno B4, que también inducen la expresión de iNOS 51. Moléculas como el factor estimulante de colon macrófago (GM-CSF) pueden indirectamente amplificar el efecto parásitico del NO, induciendo mayor producción de TNF-α y IL-12; lo que favorece la síntesis de IFN-γ. Por el contrario, células como células NK (células de tipo natural) que están presentes en el proceso fisiopatológico de la infección, pueden inducir amplificación directa del NO a través de la INF-γ 2. Otros agentes inmunosupresores, como TGF-β y IL-10, también producidos durante la infección de Chagas, son reguladores negativos de la producción de NO, revisado en 27, sugiriendo que la patogénesis de la enfermedad (al menos en la cardiomiopatía Chagásica observada en ratones) depende de un equilibrio entre las citocinas inflamatorias y anti-inflamatorias, es decir, las citocinas de clones de CD4+ Th1 linfocitos, pero no las producidas por clones de CD4+Th2 linfocitos, activan a los macrófagos para eliminar el parásito con mediación de NO 13,32.

Un aspecto interesante de la respuesta tripanocida mediada por NO, confirmado experimentalmente a través de la administración de iNOS-/-, altamente susceptibles a la infección, se debe a que la participación de la reducción de radical libre depende del tiempo de infección. Así, como se mencionó antes, NO es esencial durante las primeras etapas del ataque infeccioso para disminuir la parásitemia y disminuir en mortalidad, pero no es igualmente
relevant during the late period of the acute phase, or during the chronic phase. This reveals fundamental differences in the infection’s control mechanisms, which probably behave differently against other related microorganisms.

It was mentioned previously that one of the ways NO exerts its parasiticidal action is by binding to iron-containing structures, nevertheless, it is worth noting that when NO reacts with $O_2$ (equally synthesized by the macrophage), to produce ONOO- (more cytotoxic), it may be considered a positive modulator of the organism’s defense system. In other words, NO confers resistance to the macrophage activated with IFN-α in the presence or absence of the metabolic burst, which was previously described. Also, it has been shown that it could be involved in processes suppressing the immunological or immune-modulation system, to the extent that it induces in vivo and in vitro apoptosis of the cells affected during the acute phase of the experimental infection. The apoptosis induced by NO involves the Fas-FasL interaction, whose inhibition (by using Fas expression deficient mice), generates a decrease in NO production as a consequence of increased synthesis of Th2 cytokines; which would attribute NO a regulating or modulating effect of the response through this mechanism, as in the case of immune suppression against T. cruzi, via the apoptosis induction in T cells T54.

Regarding the immunoregulatory effect mediated by NO, there is knowledge of a protein released by the T. cruzi parasite (Tc52), which synergizes with IFN-γ to produce NO through iNOS induction and, indirectly, stimulates synthesis of IL-12 and IL-1. The experimental immunization with this antigen partially improves the immune-suppressing state described during acute Chagas infection. However, given that immune response has only been detected against Tc52 in T. cruzi infection, it is probable that this parasitic molecule plays a role in modulating the biological functions of NO7.

Until now, discussion has centered mainly on the trypanocidal effect of NO. However, studies of experimental Chagas in iNOS-deficient mice reveal parasitemia and mortality equal to the wild controls, suggesting that iNOS is not essential to control the infection. Interestingly, studies conducted with mice immunized with T. rangeli and then infected with T. cruzi revealed protection and increased survival associated with the rise of the Th2 pattern and diminished Th1 pattern (including NO), which particularly questions the protective role of NO during the acute phase of the infection with T. cruzi56. Furthermore, it has been observed that nitric oxide could be responsible for the reactivity decrease of the Th1 immune profile, which generates Th1/Th2 disequilibrium. Nevertheless, it has also been noted that in animals infected with T. cruzi, there is a decrease of the Th1 pattern via NO-induced apoptosis, favoring the persistence of the parasite58.

It must be noted that the increased levels of NO and TNF-α in chronic Chagasic individuals, correlated to low levels of antioxidants like glutathione peroxidase and superoxide dismutase, favor the evolution of the disease in chronically infected patients. Additionally, other studies have shown that NO from iNOS plays an important role in the development and progression of ventricular dilatation and systolic dysfunction in acute Chagasic myocarditis observed in mice infected with T. cruzi60.

**CONCLUSIONS**

In summary, this review permits concluding that the LANOP pathway is involved in the pathogenesis of acute and chronic Chagas disease studied in experimental models and in infected patients. Nonetheless, the role of this pathway is not unidirectional, given that the actions of such are dependent on time, because depending on the acute or chronic stage of the disease process, the pathway may have different actions or even contrary, protective or deleterious. This situation challenges researchers into broadening and delving deeper into these aspects and clearly defining the true dimensions of the role of the LANOP pathway in the physio-pathological process of the trypanosomiasis and, consequently, its plausible pharmacological manipulation.

Also, macrophages are definitely key cells in all aspects of host-parasite interaction occurring during infection; and when observing the diversity of responses these cells have against the very parasite and/or against cytokines produced during the course of the infection, it is easy to understand that a greater number of studies is rendered to understand the complexity of cell interactions taking place during different phases of the disease and, thus, be able to obtain greater clarity on the
protective or deleterious role of NO against *Trypanosoma cruzi*, given that frequently both effects have been observed during the infection.

ACKNOWLEDGMENTS

This review was conducted as part of the research project executed at Universidad Autónoma de Bucaramanga through financial support from Instituto Colombiano de Ciencia y Tecnología (COLCIENCIAS), contract N° 133-2000.

REFERENCES


31. Abrahamsohn IA, Coffman RL. Trypanosoma cruzi: IL-10, TNF, IFN-γ, and IL-12 regulate innate and acquired immunity to infection. Exp Parasitol. 1996; 84: 231-44.