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The origins of nitric oxide and peroxynitrite research in Uruguay: 25 years of contributions to the biochemical and biomedical sciences

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A B S T R A C T

In this review, I provide historical background on how nitric oxide (•NO) and peroxynitrite research was originated in Uruguay in the early 90′s and how the investigations evolved through over more than two decades within a context related to human biology. This process involved the participation of multiple local investigators, in conjunction with collaborations at the regional (Latin American) and international levels. The discoveries have been integrated with parallel investigations from other research groups worldwide and, have provided a body of knowledge to unravel how the free radical nitric oxide (•NO) can shift its signal transduction action towards oxidative processes *via* its interactions with superoxide radical ($O_2^{\bullet-}$) to yield peroxynitrite, a strong biological oxidant. The oxidative biochemistry of peroxynitrite involves both direct reactions and the formation of secondary oxidizing species (*i.e.* hydroxyl radicals (•OH), carbonate radicals ($CO_3^{\bullet-}$) and nitrogen dioxide ($^{\bullet}NO_2$)) that cause oxidative modifications of biomolecules, including thiol oxidation and tyrosine nitration. Due to the intrinsic instability of peroxynitrite in biological systems, its half-life and fate are largely dictated by its reaction kinetics with biotargets. The direct actions of •NO and peroxynitrite in the modulation of intracellular redox processes are disparate, with peroxynitrite typically causing permanent modifications of cellular components and resulting in severe alterations of cell and mitochondrial homeostasis. Herein, I highlight the evolution and progression of •NO and peroxynitrite research in Uruguay during over 25 years of work, emphasizing hypothesis- and mechanistic-oriented biochemical studies and their translation to medically-relevant conditions.

1. Introduction

This review, part of a special issue dedicated on science in Latin America, provides historical background on how nitric oxide (•NO) and peroxynitrite research was originated in Uruguay in the early 90′s and how the investigations evolved through over more than two decades with a strong biochemical basis and translated to the biomedical sciences. Due to the nature of this article, the reader will be referred to significant number of works from our group and integrated with other parallel contributions. I will particularly quote collaborative research activities that we have performed with other Latin American groups mainly based in Argentina, Chile and Brazil and that have been essential to keep a strong research program. Overall, the article highlights a discovery process based at our laboratories at the Universidad de la República in Montevideo, Uruguay, that contributed to create a solid body of knowledge to unravel at the molecular level how the free radical •NO shifts its signal transduction action towards oxidative processes *via* its interactions with superoxide radical ($O_2^{\bullet-}$) to yield peroxynitrite, a strong biological oxidant. In parallel to the main specific discoveries described herein, the research program implicated the simultaneous training of various generations of local and regional scientists and the development of research infrastructures.

2. The tradition of free radical and redox research in Uruguay

In the mid 80's, I started to perform work on xanthine oxidase enzymology characterizing $O_2^{\bullet-}$ formation by chemiluminescence probes [1,2]. This type of studies in our Department were, in fact, initiated in the late 50's during a sabbatical visit of Dr. John R. Totter [3,4], an (north) American biochemist, and continued in the 60's and early 70's by one of his disciples, Dr. Eugenio Prodanov [5,6]. This advanced research program was halted in the early 70's due to the irruption of a military government in the country that dismantled the research capabilities at the University and produced the exile of Prodanov in France. Upon the regain of democracy and University autonomy in 1985, Prodanov returned from France and together with Homero Rubbo (at that time the two of us medical students) a new research group originated. In the period 1986–1988, we did a large amount of work characterizing the kinetics of production of $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2) by xanthine oxidase and also initiated early work on the peroxidatic activity of cytochrome *c* [1,2]. The research training obtained in redox enzymology and kinetics during those years with Prodanov was really solid, and upon the completion of my medical degree, I left for postdoctoral studies at the University of Alabama at Birmingham (UAB), under the mentorship of Bruce A. Freeman. When I arrived to UAB in 1989, I met Joe S. Beckman who was Bruce's former postdoctoral fellow

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and then an Assistant Professor, who was really interested on the reaction of $^{\bullet}NO$ with $O_2^{\bullet-}$, the generation of peroxynitrite and the biological effects of that reaction. Early after my arrival to UAB, Joe handed to me the draft of a paper that he wished me to analyze; the manuscript basically dealt with the possible generation of $^{\bullet}OH$ from the proton- catalyzed homolysis of peroxynitrite and proposed this pathway as a novel mechanism of tissue oxidative injury. This paper, published in PNAS, represents a hallmark contribution to the field [7]. I must admit that at that time, I only had a faint knowledge of "nitric oxide". Indeed, the first papers characterizing $^{\bullet}NO$ as the endothelial-derived relaxation factor [8,9] and its role in neurotransmission [10] and cellular immune responses [11] had been just published in the previous years. After I read Joe's paper, I became fascinated with $^{\bullet}NO$ and peroxynitrite. Indeed, at this time I fully realized the radical character of $^{\bullet}NO$ and that then it should be a central player in free radical and redox biology and medicine. I do not think that in those early days of $^{\bullet}NO$ research in biology, the physiology field appreciated the radical nature of $^{\bullet}NO$ and, in fact, it took some time for the biochemists to convince physiologists that $^{\bullet}NO$, in addition of being a signal transduction agent through reversible reactions with heme protein targets such as guanylate cyclase, could also participate in a complete different set of reactions involving reactive intermediates, free radical processes and oxidative events.

I became really intrigued about the oxidative chemistry of peroxynitrite and started characterizing it. One aspect that was particularly interesting to me was its p*Ka* near neutrality, resulting in the coexistence of the anionic ($ONOO^-$, peroxynitrite anion) and the protonated form (ONOOH, peroxynitrous acid) under biologically-relevant conditions. Since at the time I arrived to UAB Bruce A. Freeman was really interested on comparing the sensitivity of thiol oxidation *vs* lipid peroxidation as oxidative stress biomarkers, we designed experiments to assess the reactivity of peroxynitrite with protein and non-protein thiols [12] and phosphatidyl choline liposomes [13]. These studies became hallmarks papers on the field: the overall conclusions of these two works were that 1) peroxynitrite can directly react with thiols promoting their two-electron oxidation in a process typically faster than that by H_2O_2 and 2) peroxynitrite can promote lipid peroxidation in a transition-metal independent fashion, by the secondary formation of oxidizing radicals after the homolysis of ONOOH. Later works clearly confirmed that ONOOH homolysis leads to $^{\bullet}OH$ and $^{\bullet}NO_2$ in about 30% yields (recently reviewed in Ref. [14]). The original works by Beckman et al. [7], together with those of Radi et al. [12,13] and Ischiropoulos et al. [15,16], all based at UAB, paved the way for the expanding the paradigm of the molecular mechanisms of oxidative stress, incorporating a central role of $^{\bullet}NO$ and merging it with the $O_2^{\bullet-}$ pathway (recently reexamined in Ref. [17]).

3. The incorporation of nitric oxide and peroxynitrite research in Uruguay

Upon my return back to Uruguay in 1992, I was committed to continue with $^{\bullet}NO$ and peroxynitrite research, as during my three years at UAB, we made a number of observations that clearly showed us that this was potentially a groundbreaking area, and that the only way to consolidate the "peroxynitrite theory" was to keep up with contributions and experiments for the next several years (Fig. 1). The initial years of "peroxynitrite in biology" were seen with some skepticism by many researchers, in particular those coming form physiology and there was a need to accumulate much more data and information directly to assess questions such as the biological formation and quantitation of peroxynitrite, its radical and non-radical oxidative pathways and its role in pathology *in vivo*, among other several questions.

To this end, in early 1992 I set up in Montevideo the "quenched flow reactor" for the synthesis of peroxynitrite [12,18] and with my colleague and friend Ana Denicola we generated peroxynitrite for the first time in Uruguay. We were really pleased to see the instant generation

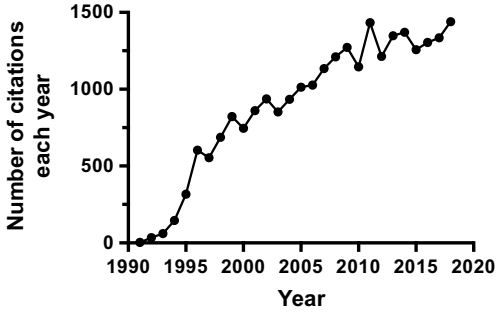

Fig. 1. Evolution of the number of citations each year on "peroxynitrite" from work performed at Universidad la República, Uruguay. Data for the citation report was obtained from the "Web of Science" with the search terms "peroxynitrite" (Topic) AND "University of the Republic Uruguay" (Organization) over the period 1990–2018. The total number of citations for the period is 25.278, with an *h*-index of 79.

of a "yellow" solution containing peroxynitrite (*i.e.* $ONOO^-$, $\lambda_{302} = 1700\ M^{-1}cm^{-1}$, reviewed in Ref. [14]); the reaction consists in the rapid mixing of solutions of sodium nitrite with H_2O_2 in acidified media which readily yields the unstable ONOOH, which in turn is immediately "quenched" in the form of $ONOO^-$ by concentrated NaOH. The yield and purity of that peroxynitrite solution was high and we were able to aliquot and freeze it for storage and later use. The successful synthesis was a critical step to initiate a vigorous program on peroxynitrite research in Uruguay. A little later, we also started to generate saturated solutions of $^{\bullet}NO$, first through a chemical synthetic procedure (*i.e.* with a "Kipp generator¨ after mixing solutions of sodium nitrite with ferrous sulfate in acid, [19]) and later using a pure $^{\bullet}NO$ gas tank. As we already had experience in purifying and working with xanthine oxidase (the $O_2^{\bullet-}$ -generating enzyme) and also purchased a series of $^{\bullet}NO$ donors, all the necessary components to study the biochemistry of the $^{\bullet}NO/O_2^{\bullet-}$ interactions and of peroxynitrite were ready to go (reviewed in Ref. [20]).

In this initial period we started to explore four research questions, two related to the reaction chemistry and two on the biology of peroxynitrite. The chemical aspects related to the free radical chemistry arising from peroxynitrite reactions, namely, 1) the homolysis of ONOOH to hydroxyl radicals ($^{\bullet}OH$) and nitrogen dioxide ($^{\bullet}NO_2$) and 2) the modulatory role of CO_2/HCO_3^- on peroxynitrite-dependent reactions. The formation of $^{\bullet}OH$ from ONOOH, originally proposed by Beckman et al. [7], was unambiguously identified by electron paramagnetic resonance (EPR)-based experiments performed together with Ohara Augusto and collaborators at the University of Sao Paulo (in the mid 90's there was no EPR instrumentation in Uruguay, and therefore we intensely collaborated with this expert group in Brazil) [21–23]. The modulatory role of the CO_2/HCO_3^- pair on peroxynitrite biochemistry was suggested by experiments on luminol chemiluminescence that I initiated during my last months at UAB and completed in Montevideo [24]. Later different groups and ours made progress on this area, clearly showing a fast reaction between $ONOO^-$ and CO_2 that yields carbonate and nitrogen dioxide in about 33% yields (recently reviewed in Ref. [14]). The direct detection of $CO_3^{\bullet-}$ by the peroxynitrite/CO_2 reaction was also unambiguously shown by EPR, again, in a collaborative project [25] (in the late 90's an EPR instrument was installed in our laboratory and the first *in-house* EPR data published on the formation of protein-derived radicals during the reaction of oxyhemoglobin with peroxynitrite, [26]). Due to the ubiquity of CO_2 in biological systems and its rapid reaction with peroxynitrite, this process turned to be biologically-relevant, influencing the biological fate and actions of peroxynitrite *in vitro* and *in vivo* (for a detailed analysis see Refs. [14,27]). The biological aspects studied in this first research period related to 1) the role peroxynitrite as a cytotoxic effector molecule against unicellular invading pathogens in the phagosome of

immunostimulated macrophages (which involve the simultaneous release of $O_2^{\bullet-}$ and $^{\bullet}NO$); in this context we have studied the infection mediated by the unicellular parasite *Trypanosoma cruzi*, the causative agent of Chagas Disease [28,29], and 2) the action $^{\bullet}NO$ and peroxynitrite in the mitochondrial electron transport chain and mitochondrial aconitase [19,30,31]; the initial observations confirmed that peroxynitrite caused far more oxidative damage to mitochondrial components than $^{\bullet}NO$ both to mammalian mitochondria, observations that were later extended to *T. cruzi* mitochondria and its impact on the disruption of parasite calcium homeostasis in works led by Leonor Thomson in collaboration with Anibal Vercesi's laboratory [32,33] (Universidad Federal de Campinas, Brazil). Our early observations on chemical and biological aspects of $^{\bullet}NO$ and peroxynitrite were also studied, complemented and extended by various groups worldwide, in a very seminal period of free radical and redox biochemical research [34–36]. A special note deserves our long lasting relationship with two mitochondrial biology research groups at the Universidad de Buenos Aires, led by Alberto Boveris and Juan José Poderoso, respectively, with whom we had many interactions in the 90′s, including a shared publication on the reactions of peroxynitrite with mitochondrial ubiquinone [37].

4. Modulatory action of nitric oxide on lipid peroxidation and the formation of nitrolipids

In 1994, Bruce A. Freeman visited our laboratories in Montevideo under the auspices of the Fulbright Foundation. After publishing together at UAB the observation that peroxynitrite could promote lipid peroxidation [13] and an independent observation indicating that simultaneous fluxes of $^{\bullet}NO$ and $O_2^{\bullet-}$ (the precursors of peroxynitrite) led to human low density lipoprotein lipid peroxidation [38], Bruce and us became interested to investigate whether lipid peroxidation yields and product distribution were similar with “authentic” peroxynitrite (added either as bolus or infusion) to those with $^{\bullet}NO$ and $O_2^{\bullet-}$ fluxes in a model liposomal system. As free radicals processes in membranes are highly dependent on the propagation period and termination reactions are favored at high concentrations of radical intermediates, it was likely that the mode of oxidant addition (bolus *vs.* infusion) and the coexistence of $O_2^{\bullet-}$ and $^{\bullet}NO$ would influence the overall process. Thus, experiments were planned in which fluxes of $^{\bullet}NO$ and $O_2^{\bullet-}$ were simultaneously generated by the xanthine oxidase system and S-nitrosothiols or by an infusion of a pure $^{\bullet}NO$ solution, respectively. We calibrated the radical fluxes through a large range of $^{\bullet}NO/O_2^{\bullet-}$ ratios spanning values from 0.3 to 3. For example, a 1/1 ratio could be obtained with equal $^{\bullet}NO$ and $O_2^{\bullet-}$ fluxes of 1.5 μM/min [39]. Upon radical exposure to a suspension of egg yolk phosphatidyl choline liposomes, lipid peroxidation yields and product distribution was assessed [39]. The data obtained were striking and readily opened new avenues of research. The main observation was that under a constant $O_2^{\bullet-}$ flux. lipid peroxidation yields increased as a function of the $^{\bullet}NO$ flux to reach the highest value at a $^{\bullet}NO/O_2^{\bullet-}$ flux ratio *ca.* 1. At ratios > 1, a consistent $^{\bullet}NO$ flux-dependent inhibition of lipid peroxidation was observed. The “biphasic” data was rationalized considering that the first phase of increase of lipid peroxidation reflected the formation and actions of peroxynitrite (with the highest peroxynitrite level formed at ratios near 1) and the second, inhibitory phase, corresponded to termination reactions of $^{\bullet}NO$ with lipid-derived radicals and the formation of novel nitrogen-containing lipid-derivatives including nitroso- and nitrolipids (these latter determined by MS-based measurements). Three outcomes of these studies arose immediately: 1) the biochemical data obtained with the utilization of authentic peroxynitrite can not be linearly assimilated to that obtained with $^{\bullet}NO$ and $O_2^{\bullet-}$ fluxes, 2) $^{\bullet}NO$ could exert potent antioxidant actions in biomembranes and lipoproteins through radical-radical termination reactions and 3) a new family of lipid-derived products emerged that turned to be important biological mediators, the nitroalkenes [40].

In the next years, and in collaborative works with the Argentinian/Chilean scientist Eduardo Lissi, we studied the diffusion properties and permeation characteristics of $^{\bullet}NO$ in model lipid systems, biomembranes and lipoproteins [41,42].

5. Peroxynitrite in neurodegeneration

In the mid 90′s Luis Barbeito, a fine Uruguayan neurobiologist (at the time located at the Instituto de Investigaciones Biológicas Clemente Estable and currently at the Pasteur Institute of Montevideo) became very enthusiastic to initiate work together on the role of $^{\bullet}NO$, oxidative stress and peroxynitrite in neurodegenerative processes. At the time I contacted Joe S. Beckman (that had moved from UAB to Oregon State University, OSU) to join our research plans as he was already interested on the role of peroxynitrite in Lou Gehrig Disease (*i.e.* amyotrophic lateral sclerosis, ALS) [43]; thus, we initiated a long and prolific collaborative research project in the area of redox neurobiology. Indeed, Barbeito, Beckman and myself together with Alvaro Estevez first and Patricia Cassina and associates later made progress to understand conditions that promote peroxynitrite generation by central nervous system cells, in particular motor neurons and astrocytes, and the possible alterations of cell and tissue homeostasis that could result in neuronal cell death and neurological disease. Actually, other and us showed at about the same time that peroxynitrite was capable to induce apoptosis in neurons [44,45], in a process that could be triggered by the deprivation of growth factors [46] and involved alterations in mitochondrial redox homeostasis and caused mitochondrial dysfunction [47]. The early cellular studies were complemented through collaborative efforts in the next two decades with studies showing the relevance of these pathogenic mechanisms *in vivo* in the mouse and rat animal models of ALS [48]; we also assessed the potential of mitochondrial-targeted pharmacology for alleviating disease progression [49,50]. A particular hallmark observation and methodological advance during these years was made when protein-derived radicals were identified immunochemically using the anti-DPMO-nitrone antibodies developed by Ron Mason and associates (NIEHS, USA), being the first successful application of the “immunospintrapping” technique *in vivo* [48](recently reviewed in Ref. [51]).

6. Studies on protein tyrosine nitration

The capacity of peroxynitrite to cause the nitration of protein tyrosine residues was observed in the early 90′s at UAB by Ischiropoulos, Beckman et al. [15,16]. Tyrosine nitration was initially conceived as an oxidative posttranslational modification and a “footprint” of the reactions of peroxynitrite, a concept that was later extended to other $^{\bullet}NO$-derived oxidants (reviewed in Refs. [14,52]). A large number of contributions have shown the presence of tyrosine nitrated proteins in human disease conditions such as atherosclerosis [53], neurodegeneration [54] and inflammation [55]. In regards to the biochemistry of peroxynitrite-dependent nitration reactions in 1996 we made two contributions to the field, one indicating that the presence of CO_2 could enhance the yields of peroxynitrite-mediated tyrosine nitration [56] and another showing that tryptophan was a also a molecular target for nitration from peroxynitrite [57]. Important later contributions on protein tyrosine nitration from our group include: 1) it was observed that peroxynitrite and other $^{\bullet}NO$-derived oxidants promote the nitration of cytochrome *c*, and the specific modification of solvent-exposed Tyr74 triggers a conformational change under physiological pH, resembling the so-called alkaline transition [58–61]. The conformational change has profound consequences of cytochrome *c* biochemical activity and subcellular location [62] and continues to be matter of active investigation in terms of its detection and biological significance *in vivo* (recently reviewed in Refs. [63,64]). Structural biology work on nitrated forms of cytochrome *c* have benefitted by nuclear magnetic resonance [60] and Raman resonance spectroscopy studies [61]

performed in collaboration with Argentinian groups lead by Alejandro Vila (Universidad de Rosario) and Daniel Murgida (Universidad de Buenos Aires), respectively; 2) we have helped to rationalize the reported nitration and inactivation of MnSOD *in vitro* and *in vivo* [65,66] (recently reviewed in Ref. [67]); indeed, nitration of critical Tyr34 requires the fast reaction of peroxynitrite with the enzyme Mn center followed by free radical chemistry at the active site microenvironment [68,69]. These reactions have relevant consequences in the context of mitochondrial dysfunction during inflammatory and oxidative stress conditions; 3) it was found that tyrosine nitration processes associated to biomembranes and lipoproteins require the participation of lipid-derived radicals, including lipid peroxyl and alkoxyl radicals, which are capable to promote the one-electron oxidation of tyrosine to tyrosyl radical and promote nitration reactions: these observations have provided the concept of a "connecting reaction" between lipid peroxidation and tyrosine nitration in hydrophobic biostructures [70–72] and the possibility of lipid- and tyrosyl-radical combination reactions leading to the formation of Diels-Alder adducts [73].

7. Biological half-life of peroxynitrite and permeation across biomembranes

In the 90's a large amount of kinetic data was gathered (including a growing list of second order rate constants of peroxynitrite with biomolecules) with the use of both direct stopped flow spectrophotometry following peroxynitrite decay at 302 nm and by competition kinetics [72]. Then, it became evident that peroxynitrite would be readily consumed in biological systems and with an estimated half-life in the ms range. Considering a diffusion constant for peroxynitrite similar to that of nitrate (*ca.* 1500 $\mu m^2 s^{-1}$) and competing reactions for peroxynitrite, a mean travel distance of 5–20 μm was predicted, corresponding to roughly 1–2 cell diameters (for a recent analysis see Ref. [14]). Thus, peroxynitrite should be considered a local mediator. However, at that time it was unknown whether its predominant form under physiological pH of 7,4, $ONOO^-$, was capable to cross biological membranes. This latter property was important if peroxynitrite represented a cytotoxic mediator that could be diffuse from one cell type to another cell type, such macrophage-mediated toxicity to invading pathogens or cancer cells. Reports in the late 90's using model phospholipid vesicular systems [74] and red blood cells [75] found that peroxynitrite could permeate membranes. In our work [75], we exposed red blood cells to external peroxynitrite and found that the anionic form was able to traverse the biomembranes *via* anion channels (*i.e.* in a similar way to what it had been previously reported for $O_2^{\bullet-}$ [76]), as revealed by intracellular oxyhemoblogin oxidation and protein tyrosine nitration. This was a particularly important manuscript for our group [75], since it was the first time we were able to publish an article in PNAS (direct submission) and upon acceptance found that the handling editor had been the NAS Member, Irwin Fridovich, the co-discoverer of SOD and an admirable and highly respected senior scientist. The observations supporting anion channels as the main entry sites of peroxynitrite to erythrocytes [75], was early after elegantly confirmed using an erythrocyte ghost model with an entrapped mutated variant of CuZnSOD, which served as a peroxynitrite "probe "upon nitration [77]. In later works we studied in deeper detail the competition between extracellular peroxynitrite consumption by biotargets such as CO_2 and penetration to target cells, as a function of target(s) concentration and intercellular distances [78,79].

8. Peroxynitrite detoxification systems in pathogens in virulence

The cytotoxic action of peroxynitrite against invading microorganisms is modulated by the endogenous antioxidant armamentarium of the pathogens [80]. In this regard, the antioxidant enzyme networks of pathogenic microorganisms are now considered to participate in their virulence to host cells, including macrophages. Notably, a family of thiol-containing enzymes, the peroxiredoxins have been shown to participate in the catalytic two-electron reduction of peroxynitrite to nitrite and attenuate its cytotoxic effects [81,82]. Second order of the reactions of peroxynitrite with a variety of peroxiredoxins are in the order of 10^5-10^7 $M^{-1}s^{-1}$ (recently reviewed in Ref. [83]), a process three to four order of magnitude faster than the reaction of peroxynitrite with free cysteine. Through a combination of experimental and computational approaches (with the key participation of Dario Estrín and associates at the Universidad de Buenos Aires), we have embarked ourselves to define structural and physico-chemical aspects in the active site of these peroxiredoxins that can rationalize the enhanced reactivity, and therefore target selectivity, of these so-called, fast reacting thiols [84–86]. The enhanced reactivity many times is seen not only for peroxynitrite but also for H_2O_2, although distinctive reactivities have been clearly disclosed ((77).

In the case of *Trypanosoma cruzi* the cytosolic and mitochondrial variants of peroxiredoxin play roles in infectivity [29,87]. More recently, these studies have been expanded to other microorganisms [86,88] and also include another type of thiol-peroxidase that generates resistance against peroxynitrite and lipid hydroperoxides in *Salmonella* [89](this latter work in collaboration with Luis Netto's group, at the University of Sao Paulo, Brazil).

9. Peroxynitrite in the vasculature and human research studies

The interactions of endothelial-derived $^{\bullet}NO$ with vascular derived $O_2^{\bullet-}$ were appreciated early on [90,91] and excess $O_2^{\bullet-}$ leads to decreased bioavailability of $^{\bullet}NO$ and endothelial dysfunction. In addition, there is a consequent formation of peroxynitrite that can participate in vascular damage during degenerative processes related to, for example, atherosclerosis, hyperglycemia and hypertension and aging [17,92]. Our group has contributed to understand the molecular basis of enhanced $O_2^{\bullet-}$ formation by vascular endothelial cells [93–95] and also performed a research study in humans [95]. This latter work, led by our esteemed friend and colleague Dr. Gonzalo Peluffo,[1] was performed in "healthy "smokers and found that these subjects already had vascular endothelial dysfunction, likely due to enhanced $O_2^{\bullet-}$/$^{\bullet}NO$ reactions in the arteries. *In vitro* experiments with vascular endothelial cells showed that soluble smoke-derived products, most likely quinones, can be absorbed at the lung towards the blood and facilitate vascular $O_2^{\bullet-}$ formation by redox cycling, among other potential mechanisms. Oral administration of ascorbic acid and α-tocopherol (for 165 days) led to an increase of their plasma levels and, in parallel, reversed the endothelial dysfunction in this population of smokers. This translational study provided a "proof-of-concept" on the role of smoke-related vascular oxidative stress and deficits in endothelial-derived $^{\bullet}NO$ bioactivity *via* its oxidative "inactivation" as a central cause of endothelial dysfunction in very early stages of vascular degeneration.

10. Redox-based therapeutics to neutralize peroxynitrite

In the mid to late 90's we initiated work in collaboration with Ines Batinic-Haberle, Irwin Fridovich and collaborators at Duke University to assess the reactions of Mn-porphyrins (MnP) with peroxynitrite. At the time MnP were seen as "SOD mimics" [96] but it readily became apparent that the redox and cytoprotective actions that MnP exerted in cellular systems and even *in vivo*, were likely due to redox mechanisms

[1] Gonzalo Peluffo, MD, PhD (1970–2015), was an Associate Professor of Biochemistry, a brilliant scientist and wonderful human being. He played a central role in building up the Montevideo nitric oxide/free radical research group for two decades and was one of its top co-investigators. He passed away at the peak of his career due to a debilitating disease, leaving the group, and the many scientists that knew him nationally and internationally, with an exemplary legacy of courage and commitment to science.

other than simply $O_2^{\bullet-}$ dismutation, due to kinetic considerations. In this context, we initiated studies assessing the role of MnP in the catalytic decomposition of peroxynitrite at the expense of reducing equivalents provided by low molecular weight reductants such as ascorbate and glutathione and even by electrons coming from the mitochondrial electron transport chain [97–99]. Indeed, in different cell and disease models, MnP were shown to more readily act as a peroxynitrite decomposition catalysts than as "SOD mimics" [100]. These compounds have been extensively used to pharmacologically modulate oxidative stress conditions, and peroxynitrite reduction seems to be one of the various potential redox-dependent processes that MnP can support. In recent work, and with the use of Raman Resonance based methods (again in collaboration with Daniel Murgida and associates at Universidad de Buenos Aires, Argentina), the intracellular reduction of MnP from the Mn^{3+} to the Mn^{2+} state and how the intracellular formation or extracellular challenge with peroxynitrite could affect the redox states of the MnP were shown [101]. MnP are capable of interacting with mitochondria and protect them from the disruptive actions of peroxynitrite [101]. Interestingly, we previously reported the protective role of MnP on mitochondria in the animal model of ALS, where mitochondrial oxidative damage and dysfunction was attenuated *in vivo* [48].

Recent studies in collaboration with Andreza Fabro de Bem[2] and associates found that organo-selenium compounds could also exert potent cytoprotective actions against peroxynitrite. However, in spite of an existing and relatively fast direct reaction of peroxynitrite with Se-containing compounds, the pharmacological mechanism of action at the non-toxic compound concentration reaching cells (*i.e.* μM levels) are likely related to redox signaling with the activation of the Nfr-2 and Foxo pathways [102,103]; these signaling pathways increase the expression levels of cytosolic peroxiredoxins and glutathione peroxidase and mitochondrial peroxiredoxin and MnSOD. The *in-tandem* increase of these antioxidant enzymes results in a decrease of the cellular steady-state levels of peroxynitrite and cellular protection. Further work in this area is currently underway.

Through over two decades of research we were able to characterize reaction mechanisms and kinetics of a large number of biomolecules and compounds with peroxynitrite and this information turned to be useful to rationalize peroxynitrite therapeutics [14,104] and detection [105–108]. It is important to recognize that while some molecules (*e.g.* peroxiredoxins) exert protective effects *via* direct reactions with peroxynitrite, others (*e.g.* uric acid, glutathione, ubiquinone) protect against peroxynitrite-derived radicals [14,109].

11. Concluding remarks

A research journey on $^{\bullet}NO$ and peroxynitrite research started in Uruguay over 25 years ago. While of strong biochemical basis, the studies evolved through more than two decades encompassing several disciplines ranging from physical-chemistry to translational medicine. The body of accumulated research allowed to create and solidify a hypothesis that links $^{\bullet}NO$ with redox metabolism in relation to human disease conditions and the process of aging. This research flourished in Uruguay with now several independent investigators[3] and groups successfully working at various local institutions,[4] all of which still keep tight connections, interactions and collaborations through our Center for Free Radical and Biomedical Research (CEINBIO) at Universidad de la República. It is also important to recognize the many visiting scientists and scholars that came to our labs to participate in a wide variety of academic activities throughout the period and that have helped to establish an active and visible international collaborative research network; last but not least, the support of several national and international funding agencies was essential to make our work possible and keep our labs up and running (see under Acknowledgements), notably during the quite difficult economic times of Uruguay in the early 2000's. The influence that the Uruguayan discoveries on nitric oxide and peroxynitrite research have had in the scientific community is exemplified in the bibliometric analysis shown in Fig. 1.

With a more general perspective, free radical and redox research in Uruguay bridges 60 years of Uruguayan biochemical and biomedical investigations. We are fortunate to say that this research program stays vibrant and dynamic with the participation of a new generation of Uruguayan and regional scientists eager to understand how free radicals, $^{\bullet}NO$ and peroxynitrite participate in human biology and medicine [17,110].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.03.003.

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