Vascular Response of Ruthenium Tetraamines in Aortic Ring from Normotensive Rats

Ana Gabriela Conceição-Vertamatti[1], Luiz Alberto Ferreira Ramos[1], Ivy Calandreli[2], Aline Nunes Chiba[2], Douglas Wagner Franco[3], Elia Tfouni[2], Dora Maria Grassi-Kassisse[1]

Universidade Estadual de Campinas (UNICAMP)[1]; Universidade de São Paulo (USP)[2], Campus Ribeirão Preto; Universidade de São Paulo (USP)[3], Campus São Carlos, São Paulo, SP - Brazil

Abstract

Background: **Ruthenium (Ru) tetraamines are being increasingly used as nitric oxide (NO) carriers. In this context, pharmacological studies have become highly relevant to better understand the mechanism of action involved.**

Objective: **To evaluate the vascular response of the tetraamines trans-[$Ru^{II}(NH_3)_4(Py)(NO)]^{3+}$, trans-[$Ru^{II}(Cl)(NO)(cyclan)](PF_6)_2$, and trans-[$Ru^{II}(NH_3)_4(4\text{-}acPy)(NO)]^{3+}$.**

Methods: **Aortic rings were contracted with noradrenaline (10^{-6} M). After voltage stabilization, a single concentration (10^{-6} M) of the compounds was added to the assay medium. The responses were recorded during 120 min. Vascular integrity was assessed functionally using acetylcholine at 10^{-6} M and sodium nitroprusside at 10^{-6} M as well as by histological examination.**

Results: **Histological analysis confirmed the presence or absence of endothelial cells in those tissues. All tetraamine complexes altered the contractile response induced by norepinephrine, resulting in increased tone followed by relaxation. In rings with endothelium, the inhibition of endothelial NO caused a reduction of the contractile effect caused by pyridine NO. No significant responses were observed in rings with endothelium after treatment with cyclan NO. In contrast, in rings without endothelium, the inhibition of guanylate cyclase significantly reduced the contractile response caused by the pyridine NO and cyclan NO complexes, and both complexes caused a relaxing effect.**

Conclusion: **The results indicate that the vascular effect of the evaluated complexes involved a decrease in the vascular tone induced by norepinephrine (10^{-6} M) at the end of the incubation period in aortic rings with and without endothelium, indicating the slow release of NO from these complexes and suggesting that the ligands promoted chemical stability to the molecule. Moreover, we demonstrated that the association of Ru with NO is more stable when the ligands pyridine and cyclan are used in the formulation of the compound. (Arq Bras Cardiol. 2015; 104(3):185-194)**

Keywords: **Ruthenium; Nitric Oxide; Norepinephrine; Pyridine; Cyclan; Aorta; Rats.**

Introduction

The endothelium plays an important role in the vascular system through the production of vasoactive mediators, such as nitric oxide (NO)[1-3] {Furchgott, 1987 # 14}. The impaired vascular function has been the focus of research on vasoactive compounds, particularly antihypertensive compounds, with the aim to restore the amount of NO necessary to achieve hemodynamic balance[4]. Therefore, complexes capable of delivering NO efficiently and in a controlled manner have been studied not only to understand their chemical nature but also for future medical applications. These studies can significantly contribute to the treatment of vascular diseases.

Mailing Address: Dora Maria Grassi Kassisse •
Rua Monteiro Lobato, 255, Barão Geraldo. Postal Code 13083-862, Campinas, SP – Brazil
E-mail: doramgk@unicamp.br; doramgk@gmail.com
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NO can serve as a ligand for many transition metals, such as iron (Fe), ruthenium (Ru), and chromium (Cr), among others. Metal complexes of Ru (II) have become the focus of research because of its remarkable ability to bind to various compounds. In addition, it is the element that best forms nitrosyl complexes[5,6].

In this respect, the class of tetraamines of Ru (II) *trans*-$Ru^{II}(NO)(NH_3)_4(L)]^{n+}$, reported in the literature as $[Ru^{II}NO^+]$, has significant thermal stability in the Ru–NO bond, ligand L being the guiding element of this stabilization. The disassociation of this bond using a substitution is controlled by the rate constant of NO (K_{NO}) via monoelectronic reduction, wherein the reductive potential of the ligand should lie between 0.320 V and 0.132 V[4-9].

K_{NO} is important because it determines the duration of the vascular effect[4].The kinetic constant of NO (k_{NO}) varies between 0.02 s^{-1} (L = 4-pic) and 4 s^{-1} (L = imC) at 25°C and increases in the order: isn ~ pic ~ nic ~ H_2O ~ py ~ pz < L-His ~ imN < P $(OEt)_3$ < imC[8].

Considering the existing medications and aiming at the improvement of clinical applications for treatment of vascular diseases, this system is advantageous because of

the possibility of the controlled release of NO to specific biological targets[4,8,10-12].Therefore, the present study aimed to investigate the effect of the Ru tetraamines *trans*-[$Ru^{II}(NH_3)_4(Py)(NO)]^{3+}$ (PyNO), *trans*-[$Ru^{II}(Cl)(NO)(Cyclan)](PF_6)_2$ (CyNO), and *trans*-[$Ru^{II}(NH_3)_4(4\text{-acPy})(NO)]^{3+}$ (4-acPyNO) on the vascular response.

Methods

The protocols used were approved by the Ethics Committee on Animal Experimentation of the State University of Campinas (Universidade Estadual de Campinas–UNICAMP) under protocol number 2099-2. The study was conducted in accordance with the standards of the *Guide for the Care and Use of Laboratory Animals* established in 1993[13] and with the Ethical Principles of Animal Experimentation of the Brazilian School of Animal Experimentation (Colégio Brasileiro de Experimentação Animal–COBEA) from 1991 on the use of animals for research and teaching purposes.

Animals

The animals were obtained from the Multidisciplinary Center for Biological Investigation (Centro Multidisciplinar para Investigação Biológica–CEMIB) in the Division for the Study of Laboratory Animals at UNICAMP. The animals were maintained in collective cages (four animals per cage). The room temperature was maintained at 22°C ± 2°C in 12/12 h light–dark cycles with the light cycle starting at 6:30 am. We used 42, 12-week-old, male rats of the Wistar strain (*Rattus norvegicus albinus*, Rodentia, Mammalia), weighing 330 ± 2.45 g.

All normolipidemic animals were fed standard laboratory chow food (Nuvilab CR1; Nuvital Nutrients S.A., Brazil), and food and water were provided daily *ad libitum*.

Histological analysis

After the completion of the experiment, the aortic rings with endothelium (E^+) and without endothelium (E^-) were isolated and placed in formalin solution (200 mL of distilled water, 50 mL of 40% formaldehyde, and 250 mL of 0.2-M phosphate buffer (pH 7.4) for 24 h. Subsequently, the samples were washed with 70% ethanol and stored in formalin solution until paraffin embedding. For inclusion, dehydration was performed using an ascending series of ethyl alcohol solutions until the absolute concentration, clarification was performed in xylol (alcohol-xylol 1:1 and pure xylol), and inclusion was performed in xylol-paraffin (1:1). Inclusion and embedding were performed at 58°C in Paraplast Plus® (a mixture of paraffin, plastic polymers, and dimethylsulfoxide). The embedded aortic rings were glued on wooden blocks and cut into 2-mm-thick sections in a 820 Spencer microtome (American Optical Corporation, USA). Approximately three sections were placed on each slide. After deparaffinization, the sections were stained with hematoxylin and eosin. The images were captured with a Nikon Eclipse 80i optical microscope coupled to a computer and video camera (Nikon Express Series, Shinagawa, Tokyo, Japan) and analyzed using NIS-Elements AR 3.0 software at a magnification of 40× and 100×[14,15].

Measurement of blood pressure

To measure blood pressure, we used 10 rats randomly selected from the experimental groups. The catheterization procedure was performed, in which a cannula (PE 50) was inserted into the right carotid artery and connected to a strain gauge pressure transducer, which in turn was connected to an amplifier (MLS370/7 Blood Pressure Module; ADInstruments, Australia) and to a PowerLab 8/30 data acquisition system (ADInstruments, Australia). For data analysis, the LabChart Pro software (ADInstruments, Australia) was used[14,15].

Analysis of Ru complexes

The complexes were characterized using elemental analysis, electronic spectroscopy in the infrared region, electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR), and electrochemical techniques (PPD, VC) by the research group of Dr. Elia Tfouni from the University of São Paulo (USP) in Ribeirão Preto.

Preparation of isolated aortic rings

The aortic rings were isolated and prepared according to the protocol established by Zanichelli et al[16] The sample size was determined as described by Lenth[17] using the Statistica 7.0 software (StatSoft, Inc., USA), and the following parameters were used: minimum test power of 0.80 and alpha value prefixed at 0.05, and optimal number of experiments per experimental protocol equal to six. Based on these parameters, we used 14 experimental groups divided into aorta samples with endothelium (E^+) and aorta samples without endothelium (E^-), resulting in 28 experimental groups.

The animals were sacrificed under deeper anesthesia. The chest was opened with a midline incision and the thoracic aorta portion was removed and divided into four rings, each with approximately 4 mm.

The endothelium of two rings was removed mechanically from the inner surface of the aorta with the help of a cotton swab whereas the endothelial layer of the other two rings remained intact. Each ring was mounted on two L-shaped stainless steel hooks, and the smaller portion traversed the inside of the ring, after which these hooks were individually placed in a container containing 10 mL of Krebs–Hanseleit physiological solution (115.0 mM of NaCl, 4.6 mM of KCl, 25.0 mM of $NaHCO_3$, 2.5 mM of $MgSO_4.7H_2O$, 2.5 mM of $CaCl_2.2H_2O$, 1.2 mM of KH_2PO_4, 11.0 mM of glucose, and 0.11 mM of ascorbic acid) and coupled to an isometric voltage transducer[16]. The solution was maintained in a water bath at 37°C with the aid of an infusion pump and was constantly bubbled with 95% oxygen and 5% carbon dioxide for maintenance of pH. After placing the samples in the container, a voltage of 1.5 g was induced in the transducer and maintained throughout the experiment for both E^+ and E^- rings. For voltage recording, an isometric voltage transducer (BIOPAC System) containing a four-channel polygraph (MP-100, USA) was used. The rings were stabilized for 50 min and the Krebs–Hanseleit solution was replaced every 20 min.

After the stabilization period, the rings were precontracted with noradrenaline (NA, 10^{-6} M) dissolved in 2% ascorbic acid

and maintained in the bath throughout the assay. After voltage stabilization, a single concentration of the compound to be studied (10^{-6} M) was added to the bath and the recording was made without interruption for 120 min. Immediately after that, a single concentration of acetylcholine (ACh, 10^{-6} M) was added to the assay medium to confirm the presence or absence of endothelial cells and stabilize the response. In addition, sodium nitroprusside (SNP) at 10^{-6} M was added to verify the integrity of the vascular smooth muscle.

To complement the pharmacological investigation of the mechanism of action involved, i.e., after the first analysis of temporal concentration–effect curves, the involvement of the endogenous NO pathways, their mechanism of action via cyclic guanosine monophosphate (cGMP), and possible interference of endogenous eicosanoids were investigated. For this study, assays were conducted using the complexes cyclan NO (CyNO) and pyridine NO (PyNO) at 10^{-6} M using both E^+ and E^- rings, which were previously incubated with the following compounds: 10–30 mM of L-NAME hydrochloride (Enzo Life Sciences International, Inc. Plymouth Meeting, PA, USA)—a NO synthase inhibitor[18,19], 5.6 μM of the cyclooxygenase inhibitor indomethacin (Enzo Life Sciences International, Inc. Plymouth Meeting, PA, USA)[18], 3–10 μM of the soluble guanylate cyclase (GC) inhibitor ODQ (Enzo Life Sciences International, Inc. Plymouth Meeting, PA, USA)[20], 10–300 μM of the NO sequester carboxy-PTIO (Enzo Life Sciences International (Plymouth Meeting, PA, USA).

To further evaluate the effect caused by these complexes, assays were performed using 10^{-6} M PyNO interacting with more than one enzyme inhibitor, e.g., by the preincubation with L-NAME and indomethacin or with L-NAME, indomethacin, and ODQ, maintaining the specific concentration for each inhibitor.

All salts used for the preparation of the Krebs–Hanseleit solution were of American Chemical Society (ACS) standard. The NA stock solutions were prepared in 2% ascorbic acid and stored at −20°C for a maximum of 7 days. For the preparation of the indomethacin solution, a 5% sodium bicarbonate buffer was used. The dilutions were made in Krebs–Hanseleit buffer immediately before use and then discarded.

Statistical analysis

The results are presented as mean ± standard error of mean (SEM) of the percentage of response. Normality was confirmed using the Kolmogorov–Smirnov test. Student's t test was used to compare the different experimental protocols for the following variables: response in the presence of vascular tone induced by NA, response before the addition of the compound, and response after different assay periods in the presence and absence of antagonists and enzyme inhibitors. Analysis of variance (ANOVA) followed by Dunnet test was performed to compare the areas under the curve. In all cases, p values of $< 5\%$ were accepted as indicating statistically significant differences. The curves were performed using GraphPad Prism software (GraphPad Software, San Diego, California, USA).

Results

Blood pressure

The values of blood pressure of the study animals were similar to those previously reported for young adult rats with average weight and following anesthesia: systolic pressure of 119.4 ± 3.862 mmHg, diastolic pressure of 92.75 ± 6.125 mmHg, and mean arterial pressure of 104.5 ± 4.29 mmHg, indicating that these animals were normotensive[21-24].

Histological analysis

Histological analysis confirmed the experimental data, which indicated the presence or absence of endothelial cells (Figure 1).

Vascular reactivity

Corroborating histological data, the presence of endothelial cells was confirmed by the significant relaxation effect of ACh

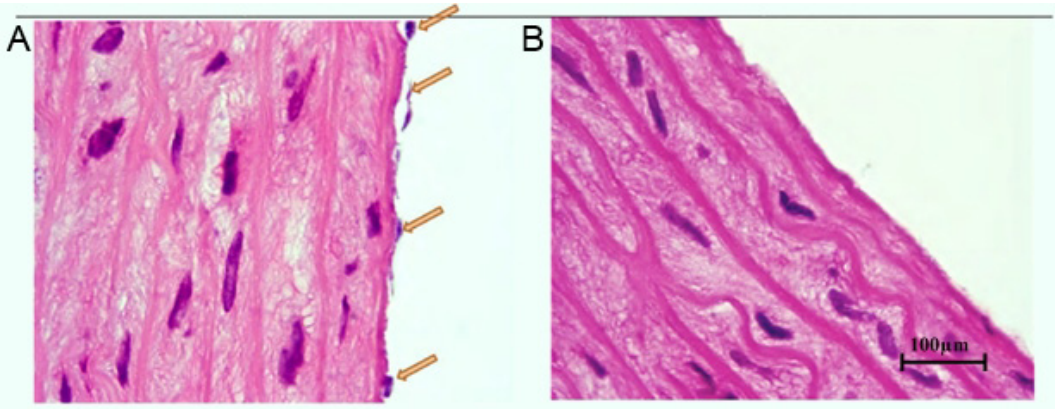

Figure 1 – *Photomicrographs of (A) aortic rings with endothelium (E^+) and (B) without endothelium (E^-) isolated from normotensive rats (100×). The arrows indicate the presence of endothelial cells.*

on E^+ aortic rings, and the integrity of the smooth muscle was confirmed by observing the relaxation in both E^+ and E^- aortic rings caused by sodium nitroprusside (SNP)[18].

In contrast to the results of previous experiments demonstrating the induction of vascular tone only by NA, all tetraamines analyzed caused a significant decrease in the vascular tone (Figure 2).

After precontraction was performed with NA, the analyzed complexes caused increased vascular tone within 1 h after treatment, in both E^+ and E^- aortic rings, and decreased vascular tone 90 min after treatment (Figure 3).

In E^- aortic rings, inhibition of GC significantly altered the contractile response induced by CyNO, causing vascular relaxation within 30 min. No significant responses were observed in E^+ aortic rings (Figure 4).

The contractile response induced by PyNO in E^+ aortic rings significantly decreased after the inhibition of endothelial NO synthase (eNOS), cyclooxygenase, and GC. The inhibition of GC exerted a reducing effect within the first 60 min whereas the inhibition of eNOS and cyclooxygenase induced a contractile response after 120 min of incubation. In the absence of endothelial cells, only the inhibition of GC exerted a reducing effect (Figure 5).

With the aim to better understand the effect of such complexes, assays with the PyNO complex (10^{-6} M) were performed in the presence of more than one enzyme inhibitor, e.g., preincubation of aorta samples with L-NAME and indomethacin, with the aim to block the activity of endogenous NO and eicosanoids, such as prostacyclin (PGI_2 and TXA_2). The simultaneous incubation of samples with L-NAME, indomethacin, and ODQ was also performed to eliminate the presence of other compounds in the same assay, including endogenous NO (synthesis and action) and endogenous eicosanoids (PGI_2 and TXA_2).

Therefore, when we blocked the endothelial function by inhibiting eNOS and cyclooxygenase, we observed a significant decrease in vascular tone in E^+ aortic rings, confirming the direct action of the PyNO complex in smooth muscle. In contrast, no changes were observed in the vascular response using E^- aortic rings.

When we blocked potential interferences in the activity of these complexes, i.e., preventing the synthesis of NO by blocking eNOS and preventing the action of eNOS by blocking GC as well as blocking potential interferences in the activity of PGI_2 and TXA_2 by blocking cyclooxygenases, we observed a decreased contractile response in both E^+ and E^- rings, corroborating the effect of PyNO directly on the smooth muscle (Figure 6).

The inhibiting effect of PyNO on the contractile function was not significantly different from that caused by the acetylation of 4-acPyNO in both E^+ and E^- aortic rings.

The results were also analyzed by calculating the areas under the curve (AUC). The response induced by PyNO, CyNO, and 4-acPyNO did not significantly differ for E^+ and E^- aortic rings (Table 1). On the other hand, the inhibition of eNOS by L-NAME caused a significant decrease in the response induced by PyNO and CyNO in E^+ rings but not in E^- rings. A similar effect was observed when we inhibited the cyclooxygenase pathway, resulting in a significant decrease in the response in E^+ rings but not in E^- rings. The GC inhibition by ODQ caused a significant decrease in the response induced by PyNO and CyNO in E^- rings. Moreover, the sequestration of NO by C-PTIO did not affect the effect of the complexes evaluated.

Discussion

The nitrosyl complexes of the class *trans*-$[Ru^{II}(NH_3)_4(L)(NO)]^{3+}$ are considered important molecules because of their low toxicity, good solubility in water, and ability to modulate the release of NO as a function of the *trans* effect played by the choice of ligand L, along with the fact that the reductive potential of NO^+ is accessible to many reducing agents found in biological processes[5,6]. The nature of the ligand L is exactly what controls the strength of the Ru–NO bond so that the higher the binding property of the receptor, the weaker is the strength of the NO bond[9]. According to Tfouni et al[4], the release or retention of NO is selective to the biological target, and a possible alternative would be the immobilization of complexes to silica, which could facilitate the action of reducing agents, possibly forming more stable compounds.

However, it was shown that the immobilization to silica does not modify compound reactivity, indicating that the properties of the Ru–NO bond may change depending on the nature of the ligand[4].

A study conducted by Caramoni and Frenking[26] indicated that the use of tretraaza macrocyclic ligands, such as *trans*-[RuCl(NO)(Cyclan), as equatorial ligands promoted greater stability of the Ru–NO bond and thereby could be used as vasodilating agents[9]. However, studies in hypertensive rats using the complex *trans*-$[Ru^{II}(NO^+)(Cyclan)Cl(PF_6)_2$ indicated differences in the relaxation time when activated thermally (595 s) or by light irradiation (50 s)[4].

Studies conducted in rat aortas demonstrated that the relaxation induced by the compound *trans*-$[Ru^{II}(NO^+)(Cyclan)Cl(PF_6)_2$ was inhibited under light irradiation and the amount of NO released was insufficient to affect the biological pathways[4,27]. Therefore, it is essential to evaluate the intensity and duration of relaxation, and for this reason, the measurement of K_{NO} is important when assessing the duration of the vasorelaxant effect[4].

Our results indicate that the vascular effect of the complexes tested involved decreased contractile tone followed by a relaxation effect after 90–120 min of incubation, suggesting that the assay time was not sufficient to effectively release NO. In addition, we can consider that the influence of the ligands pyridine and cyclan on the compounds helped to measure K_{NO}, and consequently, the stabilization of the Ru–NO bond in order to release NO from the metallic complex more rapidly or more slowly.

The relaxation promoted by Ru II appears to be mediated by GC stimulation but has also been associated with the direct activation of K^+ channels independently of cGMP, which indicates that Ru II is directly involved in the vascular relaxation promoted by NO. NO has a cGMP-dependent and

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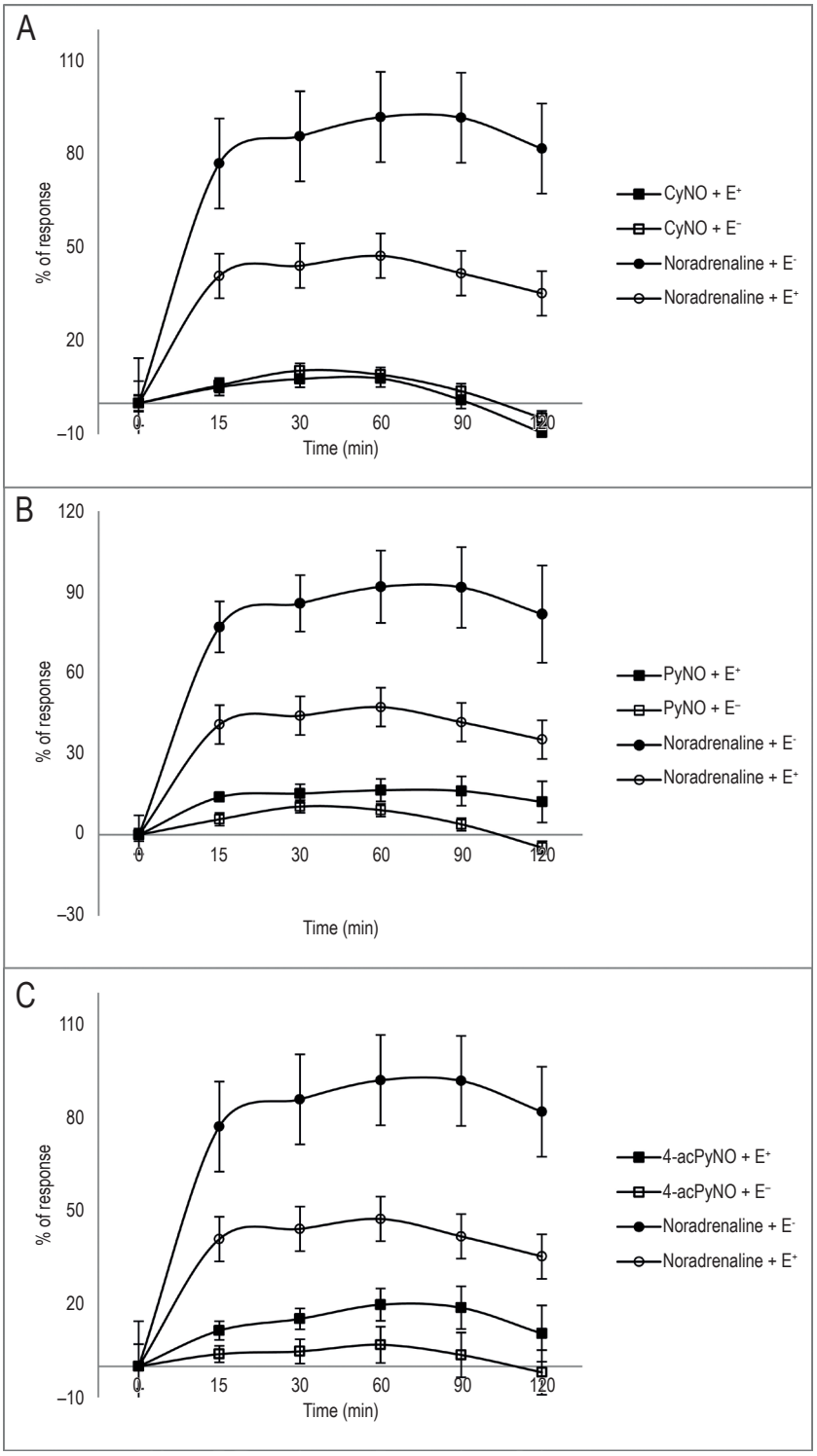

Figure 2 – *Effect of tetraamines (A) CyNO–trans-[Ru^{II}(Cl)(NO)(Cyclan)](PF$_6$)$_2$, (B) PyNO–trans-[Ru^{II}(NH$_3$)$_4$(Py)(NO)]$^{3+}$, and (C) 4-acPyNO–trans-[Ru^{II}(NH$_3$)$_4$(4-acPy)(NO)]$^{3+}$ 10^{-6} M in E$^+$ aortic rings (■) and E$^-$ aortic rings (□) compared with control assays. *p value of < 0.05 using unpaired Student's test for the values of vascular tone induced by NA (10^{-6} M). E$^+$: PyNO, p = 0.0036; CyNO, p = 0.0008; 4-acPyNO, p = 0.0026; E$^-$: PyNO, p = 0.0022; CyNO, p = 0.0022; 4-acPyNO, p = 0.0014.*

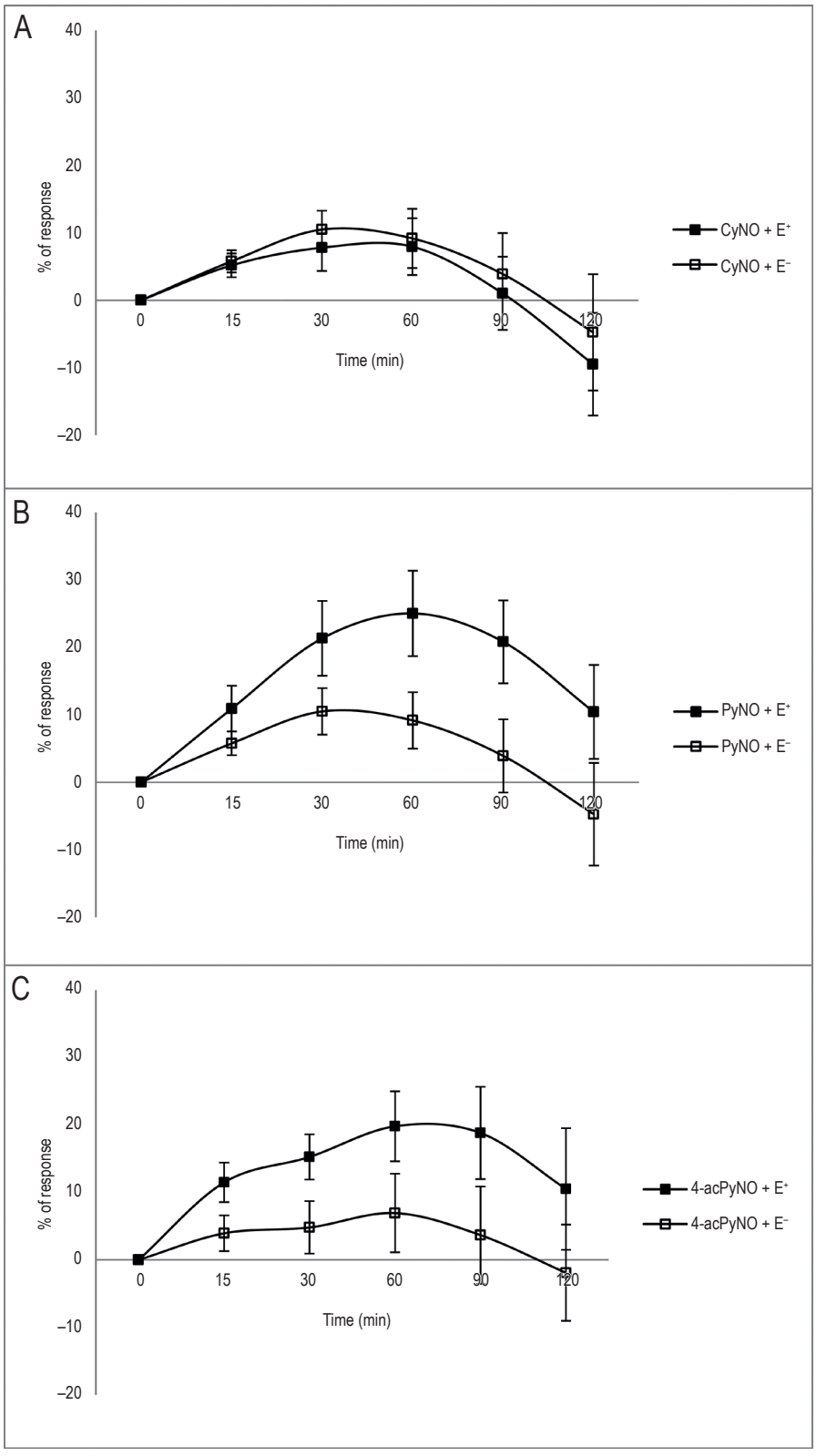

Figure 3 – *Effect of tetraamines (A) CyNO–trans-[Ru^{II}(Cl)(NO)(Cyclan)](PF$_6$)$_2$, (B) PyNO–trans-[Ru^{II}(NH$_3$)$_4$(Py)(NO)]$^{3+}$, and (C) 4-acPyNO–trans-[Ru^{II}(NH$_3$)$_4$(4-acPy)(NO)]$^{3+}$ 10^{-6} M after a single concentration of NA (10^{-6} M) in E$^+$ aortic rings (■) and E$^-$ aortic rings (□). *p value of < 0.05 using unpaired Student's t test for the values obtained immediately after administration of the compounds. E$^+$: PyNO, p = 0.0195; CyNO, p = 00241; 4-acPyNO, p = 0.0116; E$^-$: PyNO, p = 0.0216; CyNO, p = 0.0377; 4-acPyNO, p = 0.0179.*

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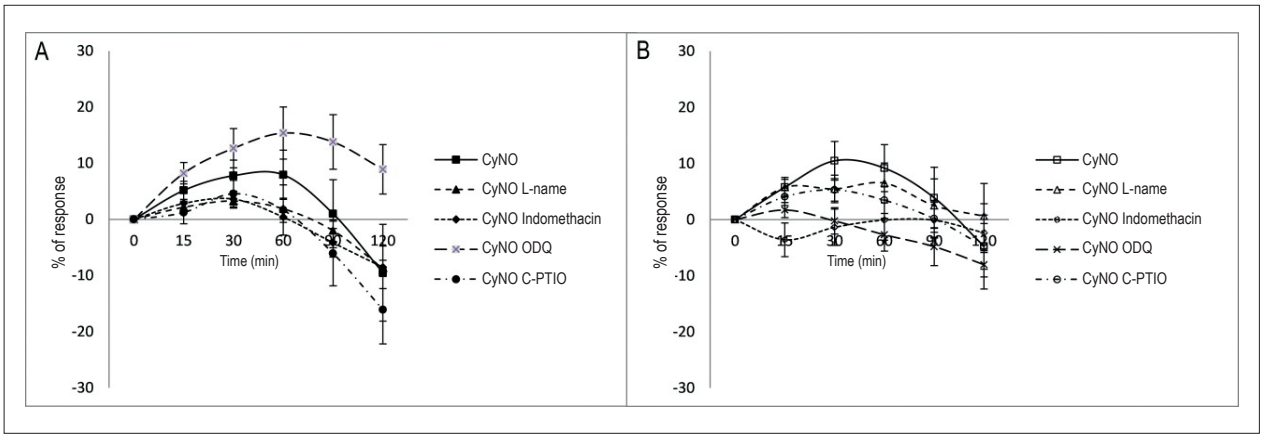

Figure 4 – *Effect of trans-[Ru^{II}(Cl)(NO)(Cyclan)](PF$_6$)$_2$−CyNO in (A) E$^+$ aortic rings and (B) E$^-$ aortic rings after treatment with enzyme inhibitors L-NAME with E$^+$ (▲) and with E$^-$ (△), indomethacin with E$^+$ (♦) and with E$^-$ (◇), ODQ with E$^+$ (⊠) and with E$^-$(×), C-PTIO with E$^+$ (●) and with E$^-$ (○). *p value of < 0.05 using unpaired Student's t test compared with the values obtained in the absence of antagonists or enzyme inhibitors CyNO vs. ODQ with E$^-$, p = 0.0152.*

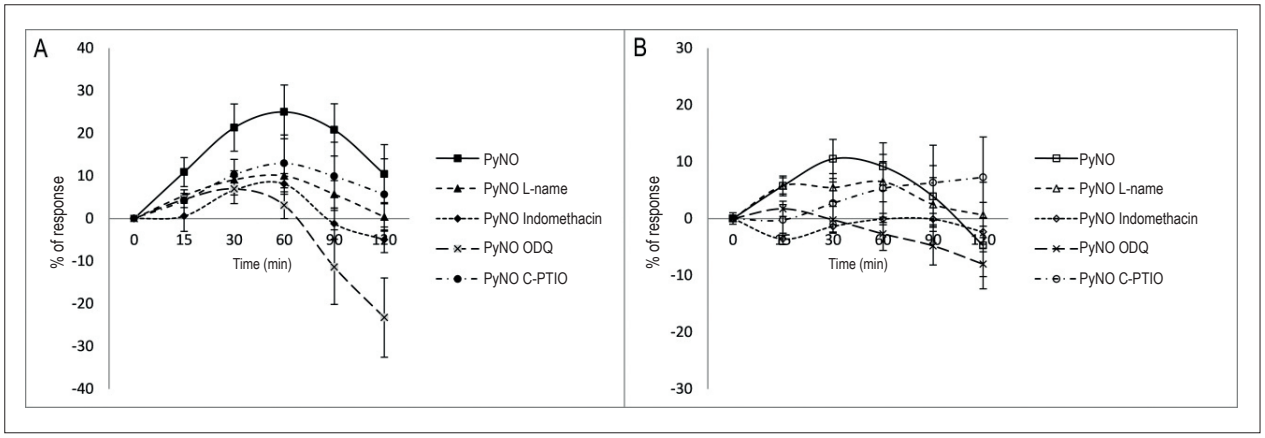

Figure 5 – *Effect of PyNO−trans-[Ru^{II}(NH$_3$)$_4$(Py)(NO)]$^{3+}$ on (A) E$^+$ aortic rings and (B) E$^-$ aortic rings after treatment with enzyme inhibitors: L-NAME with E$^+$ (▲) and with E$^-$ (△), indomethacin with E$^+$ aorta (♦) and with E$^-$ (◇), ODQ with E$^+$ (⊠) and with E$^-$(×), C-PTIO with E$^+$ (●) and with E$^-$ (○). *p value of < 0.05 using unpaired Student's t test compared with the values obtained in the absence of antagonists or enzyme inhibitors E$^+$: PyNO vs. L-NAME, p = 0.0056; PyNO vs. indomethacin, p = 0.0459; PyNO vs. ODQ, p = 0.0043; E$^-$: PyNO vs. ODQ, p = 0.0140.*

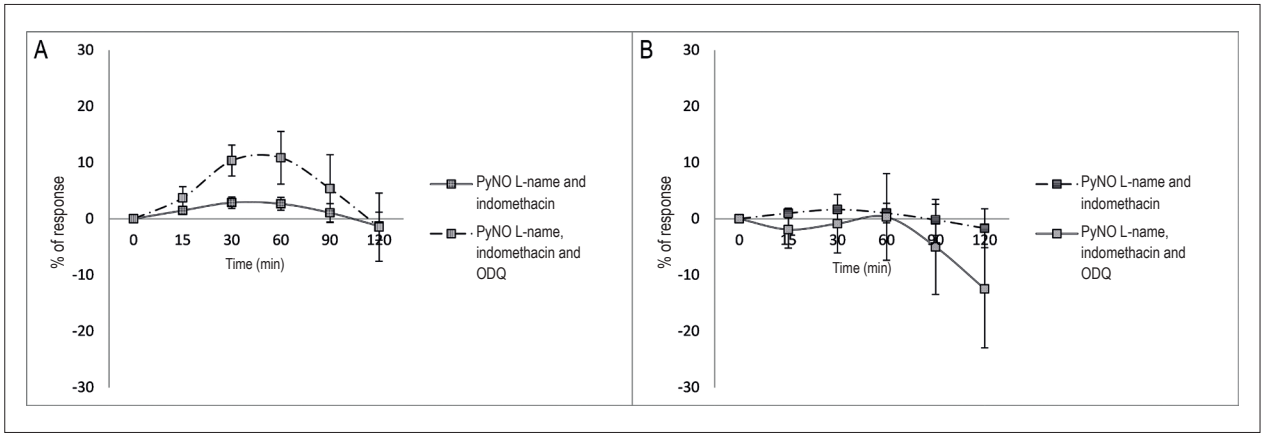

Figure 6 – *Effect of PyNO–trans-[Ru^{II}(NH$_3$)$_4$(Py)(NO)]$^{3+}$ 10^{-6} M on (A) E$^+$ aortic rings and (B) E$^-$ aortic rings after incubating with the enzyme inhibitors L-NAME, indomethacin, and ODQ. *p value of < 0.05 using unpaired Student's t test compared with the values obtained in the absence of antagonists or enzyme inhibitors. E$^+$: PyNO vs. PyNO + L-NAME + indomethacin, p = 0.0056, and PyNO vs. PyNO + L-name + indomethacin + and ODQ, p = 0.0454.*

Table 1 – Area under the curve of the Ru tetraamines *trans*-[Ru^{II}(Cl)(NO)(Cyclan)]$(PF_6)_2$, *trans*-[$Ru^{II}(NH_3)_4$(Py)(NO)]$^{3+}$, and *trans*-[$Ru^{II}(NH_3)_4$(4-acPy)(NO)]$^{3+}$ in E^+ aorta rings and E^- aorta rings isolated from normotensive rats

	trans- [Ru^{II}(Cl)(NO)(Cyclan)]$(PF_6)_2$		*trans*-[$Ru(NH_3)_4$(Py)(NO)]$^{3+}$		*trans*-[$Ru(NH_3)_4$(4-acPy)(NO)]$^{3+}$	
	E^+	E^-	E^+	E^-	E^+	E^-
-	1.110,6 ± 225,6	1.068,7 ± 317,5	2.292,2 ± 520,6	1.069,4 ± 317,2	1.949,0 ± 542,4	1.292,9 ± 312,8
L-NAME (10-30 µM)	439,9 ± 75,0*	898,7 ± 250,6	852,4 ± 245,4*	898,6 ± 250,3	-	-
Indomethacin (5,6 µM)	496,3 ± 92,4*	741,1 ± 161,9	796,07 ± 119,1*	740,7 ± 161,9	-	-
ODQ (3-10 µM)	1.424,5 ± 453,1	646,3 ± 149,8	1.109,4 ± 388,4	646,7 ± 149,9	-	-
C-PTIO (10-300 µM)	1.062,7 ± 159,9	646,3 ± 109,6	1.718,9 ± 276,8	1.223,7 ± 327,6	-	-
L-NAME (10-30 µM), indomethacin (5,6µM) and ODQ (3-10µM)	-	-	1.111,850 ± 350,891	1.329,567 ± 510,506	-	-

The area under the curve of Ru tetraamines (AUC) trans-[Ru^{II}(Cl)(NO)(Cyclan)]$(PF_6)_2$, trans-[$Ru^{II}(NH_3)_4$(Py)(NO)]$^{3+}$, trans-[$Ru^{II}(NH_3)_4$(4-acPy)(NO)]$^{3+}$, trans-[Ru^{II}(Cl)(NO)(Cyclan)]$(PF_6)_2$, and trans-[$Ru^{II}(NH_3)_4$(Py)(NO)]$^{3+}$ are shown in the absence and presence of L-NAME, indomethacin, ODQ, and carboxy-PTIO in E^+ aortic rings and E^- aortic rings.
**p value of < 0.05; Analysis of variance (ANOVA) followed by Dunnet and Student's t test where appropriate. (−) Indicates the absence of assays with the enzyme inhibitors evaluated; AUC is shown as mean ± SEM as gF/min.*

a cGMP-independent signaling pathway, which could directly activate the K^+ channels[28].

Another important factor involved in the onset of vasodilation in smooth muscle is the decreased concentration of cytosolic calcium through inhibition of calcium entry[29]. Previous studies have indicated that the NO/cGMP pathway can decrease the intracellular calcium concentration and thereby decrease the contractile sensitivity, resulting in smooth muscle relaxation[4].

According to a study conducted by Lunardi et al[30], confocal microscopy experiments indicated that *trans*-[$Ru^{II}(NO^+)$([15]aneN4)Cl]$^+$, [$Ru^{II}(NO^+)(NH_3NHQ)$(terpy)]$^{3+}$ and *cis*-[$Ru^{II}(NO^+)(bpy)_2$Cl](PF6)$_2$ decreased calcium concentrations in the vasculature[4].

Another study demonstrated that the relaxation induced by the complexes *trans*-[$Ru^{II}(NO^+)$(cyclan)Cl]$^{3+}$ and *trans*-[$Ru^{II}(NO^+)(NH_3)_4P(OEt)_3$]$^{3+}$ was completely blocked by the use of a NO sequester and GC inhibitors, suggesting that the mechanism of action is related to the NO/cGMP pathway[4].

The present study corroborates the occurrence of changes associated with the NO/cGMP pathway, considering that GC inhibition promoted faster vascular relaxation in E^- aortic rings by the CyNO complex, and this inhibition was also observed using the PyNO complex, suggesting a strong influence of the NO/cGMP pathway in the vascular effect induced by the compounds analyzed.

NO is the common mediator released from all vasodilator complexes, but its mechanism of action is distinguished by the specificity of activation of GC, which is different for each donor compound[30]. Considering that NO may also exist in a variety of forms, such as ion, and nitrosyl, and nitronium free radicals, NO released from Ru complexes may differ from NO produced by endothelial cells. This would explain the difference in potency and efficacy of NO donors in the induction of vascular relaxation[30].

Conclusion

The results presented herein indicate that the vascular effect of the complexes evaluated involved decreased vascular tone induced by norepinephrine (10^{-6} M) at the end of the incubation period in rings with and without endothelium, indicating the slow release of NO from these complexes and suggesting that the ligands promoted chemical stability in the molecule. In addition, we demonstrated that the Ru–NO bond was more stable when pyridine and cyclan ligands were used in the formulation of the compound.

Considering the protocol used, the effect induced by the compounds investigated on the vascular function of aortic rings with endothelium is partially dependent on the cyclooxygenase, guanylate cyclase, and eNOS pathways. On the other hand, only the guanylate cyclase pathway modulated the activity of these compounds on the aortic rings without endothelium.

To date, several Ru complexes have been synthesized and tested for their potential therapeutic use and their effects and mechanisms of action are being intensely studied by different research groups. However, many details remain unknown and will be elucidated using multidisciplinary studies.

Author contributions

Conception and design of the research: Franco DW, Grassi-Kassisse DM; Acquisition of data and Critical revision of the manuscript for intellectual content: Conceição-Vertamatti AG, Ramos LAF, Calandreli I, Chiba AN, Franco DW, Tfouni E, Grassi-Kassisse DM; Analysis and interpretation of the

data: Conceição-Vertamatti AG, Ramos LAF, Grassi-Kassisse DM; Statistical analysis and Writing of the manuscript: Conceição-Vertamatti AG, Grassi-Kassisse DM; Obtaining financing: Franco DW, Tfouni E, Grassi-Kassisse DM.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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