Gold nanoparticle modifies nitric oxide release and vasodilation in rat aorta

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Abstract Nitric oxide (NO) plays an important role on several biological functions. Recently, it has been reported the possibility of modifying the NO release profile from the NO donors through its coupling to gold nanoparticles (AuNPs). Thus, AuNPs were synthesized and they were exposed to the NO donor ruthenium complex $\text{Cis-}[\text{Ru}(\text{bpy})_2(\text{NO})(4\text{PySH})](\text{PF}_6)_3$ termed (Ru-4PySH)—forming $\text{AuNPs-}\{\text{Ru-4PySH}\}_n$ cluster. Our results indicate that AuNPs do not modify the maximum effect (ME) and potency (pD_2) in the vasodilation induced by Ru-4PySH. Both complexes induce similar vascular relaxation in concentration-dependent way. However, the NO released from the complex

$\text{AuNPs-}\{\text{Ru-4PySH}\}_n$ is lower than Ru-4PySH. Both complexes release only NO^0 specie, but $\text{AuNPs-}\{\text{Ru-4PySH}\}_n$ releases NO in constant way and exclusively in the extracellular medium. In time-course, Ru-4PySH was faster than $\text{AuNPs-}\{\text{Ru-4PySH}\}_n$ in inducing the maximum vasodilation. Inhibition of soluble guanylyl cyclase (sGC) abolished the vasodilation induced by Ru-4PySH, but not by $\text{AuNPs-}\{\text{Ru-4PySH}\}_n$. Non-selective potassium (K^+) channel blocker TEA had no effect on the vasodilation induced by $\text{AuNPs-}\{\text{Ru-4PySH}\}_n$, but it reduced the potency to Ru-4PySH. In conclusion, our results suggest that AuNPs can reduce the permeability of NO donor Ru-4PySH due to $\text{AuNPs-}\{\text{Ru-4PySH}\}_n$ cluster formation. AuNPs reduce NO release, but they do not impair the vasodilator effect induced by the NO donor. Ru-4PySH induces vasodilation by sGC and K^+ channels activation, while $\text{AuNPs-}\{\text{Ru-4PySH}\}_n$ activates mainly sGC. Taken together, these findings represent a new pharmacological strategy to control the NO release which could activate selective biological targets.

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Introduction

In the recent years, the diatomic free radical nitric oxide (NO) has been shown to be involved in many important biological events, such as platelet adhesion and aggregation, metabolism, neuronal activity, immune response, and vasodilation [1]. Among its diverse functions, NO has been widely implicated in the vascular smooth muscle relaxation [2–5]. The predominant signaling pathway responsible for the vascular

relaxation induced by NO appears to involve the activation of soluble guanylyl cyclase (sGC) with the production of cyclic guanosine monophosphate (cGMP) and activation of protein kinase G [6]. Nevertheless, some authors have reported that vascular relaxation to NO may occur through a cGMP independent pathway in the smooth muscle by the direct activation of K^+ channels [7, 8].

Recently, it is increasing the number of disease states involving NO imbalance [9, 10] and this has stimulated extensive research activity into the chemistry, biology, and pharmacology of NO. The proposal and achievement of new drugs can represent an advance in this field. In this way, the discovery of the several bioregulatory roles of NO demands new methods for generating NO in a controlled way to facilitate an improved understanding of the physiological and physiopathological function of NO. Bonaventura et al. [11] by comparing the mechanism underlying the relaxation induced by two NO donors sodium nitroprusside (SNP) and a ruthenium complex verified that the ruthenium complex can be used as pharmacological tool without toxicity, in contrast to SNP that is toxic since it releases cyanide.

Beyond the NO donor ruthenium complex represents an exogenous source of NO apparently compatible with biological system, another new strategy is try to modify the NO release from this synthetic drug. Gold nanoparticles (AuNPs) have recently emerged as attractive novel platform for target-specific delivery of therapeutic agents [12]. Gold nanoparticles are essentially inert, non-toxic, and easy synthesis [13]. Rothrock et al. [14] reported the synthesis of gold nanoparticles that was designed to control the NO release. A few years later, Polizzi et al. [15] reported the synthesis of water-soluble NO-releasing gold nanoparticles with significantly enhanced NO payloads.

In this way, we synthesized a NO donor complex, $\text{cis}[\text{Ru}(\text{bpy})_2(\text{NO})(4\text{PySH})](\text{PF}_6)_3$, also called Ru-4PySH, that it was adsorbed to gold nanoparticles (AuNPs) forming $\text{AuNPs}\{-\text{Ru-4PySH}\}_n$ cluster. In contrast to the NO donor coupled to AuNPs in DMSO obtained by Diaz-Garcia et al. [16], we have proposed its synthesis and studies in aqueous solution. The potential of this nitro ruthenium complex has been explored as NO deliver agent in physiological conditions [17–21]. Thus, the current study aimed to characterize the profile of NO release by ruthenium complexes without (Ru-4PySH) or with gold nanoparticles ($\text{AuNPs}\{-\text{Ru-4PySH}\}_n$), and the cellular mechanisms that are activated by these complexes to induce vascular relaxation in rat aorta.

Materials and methods

The UV–visible spectra were recorded in the Hitachi U-3501. Infrared (IR) spectra were recorded on a Protegé 460 series FT-IR spectrometer, by using solid samples

pressed in KBr pellets. The NO measurements were performed by using the ISO-NO NO-meter and the DUO-18 acquisition board. The sensitivity of this apparatus ranges between 1 nmol/L and 20 $\mu\text{mol/L}$, with a 2-mm sensor, which directly detects NO concentration by amperometric technique. The setup was calibrated following the procedure previously reported [22–24]. The recordings were performed in organ chamber with aortic rings in Krebs solution maintained at pH 7.4 gassed with 95 % O_2 and 5 % CO_2 at 37 °C under ambient light to guarantee similar conditions of the functional studies.

Synthesis and characterization of the complexes

The recrystallized complex salt $\text{Cis}[\text{Ru}(\text{bpy})_2(\text{NO})(\text{L})](\text{PF}_6)_3$ was prepared as previously described by Sauer et al. [25]. The ligand “L” was replaced by thiol 4-aminopyridine (4PySH) forming $\text{Cis}[\text{Ru}(\text{bpy})_2(\text{NO})(4\text{PySH})](\text{PF}_6)_3$ also called Ru-4PySH. The Ru-4PySH complex was characterized by UV–Vis and infrared spectroscopy, and then compared to the published results [25].

Gold nanoparticles (AuNPs) were synthesized by the technique described by Turkevitch and adapted by Frens [26]. This method consists in the reduction of HAuCl_4 by citrate anion in aqueous solution and the obtainment of AuNPs was characterized by plasmon band formation in 520 nm wavelength and ruby red color. We obtained AuNPs around 10 nm measured by intensity of size distribution using Zeta Sizer (Malvern Instruments). AuNPs and thiol 4-aminopyridine (4PySH) exhibit high chemical affinity [27]. Therefore, the $\text{AuNPs}\{-\text{Ru-4PySH}\}_n$ cluster formation was monitored by UV–Vis spectroscopy of 3.32 mL AuNPs before (plasmon band in 520 nm at time zero) and after the addition of 10 μL Ru-4PySH 1.38 mmol/L.

After 60 min of interaction and stabilization, the sample (AuNPs plus Ru-4PySH) assumed violet staining and a new size measurement was performed. The size of AuNPs along Ru-4PySH was in the range from 10 nm to around 1,000 nm. The $\text{AuNPs}\{-\text{Ru-4PySH}\}_n$ complex was always prepared 60 min before the use. The vasodilator effect of both complexes was evaluated in ambient light conditions.

Vessel preparation

Male Wistar rats (180–200 g) were killed by decapitation, and all the procedures were performed in accordance with the Ethical Animal Committee of the University of São Paulo, Brazil. The thoracic aorta was quickly removed, dissected free, and cut into 4-mm long. In order to avoid the interference of the endothelium factors, in the present study, we investigated the vascular relaxation induced by Ru-4PySH, simple AuNPs, and $\text{AuNPs}\{-\text{Ru-4PySH}\}_n$ in denuded aortic rings which were placed between two stainless-steel stirrups and

connected to an isometric force transducer (Letica Scientific Instruments; Barcelona, Spain) to measure tension. The aortic rings were placed in a 10 mL organ chamber containing Krebs solution with the following composition (mmol/L): NaCl 130, KCl 4.7, KH_2PO_4 1.2, MgSO_4 1.2, NaHCO_3 14.9, glucose 5.5, CaCl_2 1.6. The solution was maintained at pH 7.4 gassed with 95 % O_2 and 5 % CO_2 at 37 °C at ambient light. The aortic rings were initially stretched to a basal tension of 1.5 g before allowing them to equilibrate for 60 min in the bath fluid. Endothelial integrity was qualitatively assessed by the degree of relaxation caused by acetylcholine (1 $\mu\text{mol/L}$) in the presence of contractile tone induced by 0.1 $\mu\text{mol/L}$ phenylephrine. Because our studies required endothelium-denuded aortas, the rings were discarded if there was any degree of relaxation.

Experimental protocols

Vasodilator effect induced by Ru-4PySH, AuNPs-{Ru-4PySH}_n, and simple AuNPs

To examine the vasorelaxation induced by the NO donors, the aortic rings were contracted with 0.1 $\mu\text{mol/L}$ phenylephrine (EC_{50}). When the contraction had reached the plateau, Ru-4PySH (0.3 nmol/L to 10 $\mu\text{mol/L}$), AuNPs-{Ru-4PySH}_n (0.3 nmol/L to 10 $\mu\text{mol/L}$), or AuNPs control (in similar concentrations of AuNPs-{Ru-4PySH}_n) were cumulatively added.

Time-course for vascular relaxation induced by Ru-4PySH and AuNPs-{Ru-4PySH}_n

Ru-4PySH and AuNPs-{Ru-4PySH}_n (5 $\mu\text{mol/L}$) were added to the organ chamber to induce the maximum vasodilator effect when a stable contraction in response to 0.1 $\mu\text{mol/L}$ phenylephrine was achieved.

Effect of the extracellular NO scavenger oxyhemoglobin (HbO_2), NO^0 scavenger hydroxocobalamin, or NO^- scavenger L-cysteine on the vascular relaxation induced by Ru-4PySH and AuNPs-{Ru-4PySH}_n

The oxidation of hemoglobin was performed as previously described [28]. Oxyhemoglobin (HbO_2 10 $\mu\text{mol/L}$), hydroxocobalamin (0.1 mmol/L), or the NO^- scavenger L-cysteine (1 mmol/L) was added 30 min before the addition of the contractile agonist 0.1 $\mu\text{mol/L}$ phenylephrine. Then, cumulative concentration–response curves to Ru-4PySH or AuNPs-{Ru-4PySH}_n (0.3 nmol/L to 10 $\mu\text{mol/L}$) were obtained.

Effect of the soluble guanylyl cyclase inhibitor (ODQ) and potassium (K^+) channels blocker (TEA) on the relaxation induced by Ru-4PySH and AuNPs-{Ru-4PySH}_n

To examine the contribution of soluble guanylyl cyclase or K^+ channels to the relaxation induced by Ru-4PySH and AuNPs-{Ru-4PySH}_n, ODQ (1 $\mu\text{mol/L}$), or tetraethylammonium (TEA 1 mmol/L), non-selective K^+ channel blocker was incubated for 30 min before the addition of phenylephrine (0.1 $\mu\text{mol/L}$). Then, cumulative concentration–response curves for Ru-4PySH or AuNPs-{Ru-4PySH}_n (0.3 nmol/L to 10 $\mu\text{mol/L}$) were obtained.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Each set of experiments were studied at least five aortic rings isolated from different rats. The pharmacological parameters, maximum effect (ME) and potency (pD_2), obtained from concentration–response curves for Ru-4PySH and AuNPs-{Ru-4PySH}_n were used to express the data. ME was considered as the maximal amplitude response reached in the concentration–effect curves for relaxant agents. The concentrations of the agents producing a half-maximal relaxation amplitude (EC_{50}) were determined after logit transformation of the normalized concentration–response curves, and they were reported as the negative logarithm (pD_2) of the mean of individual values for each tissue using the GraphPad Prism version 3.0 (GraphPad Software Corporation, San Diego, CA). Statistical significance was tested by one way ANOVA (post-test: Newman–Keuls) and Student's *t* test, and values of $P < 0.05$ were considered to be significant.

Drugs

Phenylephrine, acetylcholine, hydroxocobalamin, L-Cysteine, tetraethylammonium (TEA), ODQ, and MTT were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Hemoglobin was purchased from Calbiochem–Novabiochem (La Jolla, CA, USA).

Reagents

$\text{RuCl}_3 \cdot 3\text{H}_2\text{O}$ and thiol 4-aminopyridine were purchased as a highly pure reagent from Sigma-Aldrich Chemical and used as supplied; sodium nitrite purchased from Mallinckrodt. Double distilled H_2O was used for all experiments. All chemical preparations and measurements were carried out under an argon atmosphere and protected from light.

Fig. 1 UV–Vis spectrum of $\text{Cis-}[\text{Ru}(\text{bpy})_2(\text{NO})(4\text{PySH})].(\text{PF}_6)_3$ (Ru-4PySH) in phosphate buffer pH 7.4. *Inset*: Infrared spectrum. NO frequency of $\text{Cis-}[\text{Ru}(\text{bpy})_2(\text{NO})(4\text{PySH})].(\text{PF}_6)_3$ (Ru-4PySH) in $1,942\text{ cm}^{-1}$ indicates that formally a linear NO^+ is coordinated to the ruthenium(II) center

Results and discussion

In the last years, it has been demonstrated that several ruthenium-derived NO donors induce vascular relaxation,

Fig. 2 UV–Vis spectrum of 3.32 mL AuNPs before (plasmon band in 520 nm at time zero) and after the addition of 10 μL $\text{Cis-}[\text{Ru}(\text{bpy})_2(\text{NO})(4\text{PySH})].(\text{PF}_6)_3$ (Ru-4PySH) 1.38 mmol/L. Time-dependent spontaneous interaction between AuNPs and Ru-4PySH shown by decrease plasmon band intensity in 520 nm and increase absorbance intensity in wavelengths higher than 650 nm with stabilization between 30 and 60 min

without vascular cytotoxicity on different conditions and by different mechanisms [11, 17, 18, 20, 21, 29]. In the present study, we have verified that AuNPs, Ru-4PySH, and $\text{AuNPs-}\{\text{Ru-4PySH}\}_n$ in the concentrations that induce the maximum effect did not present cytotoxicity to the smooth muscle cells (data not shown). These results are according to data previously reported by Bonaventura and colleagues for other NO donor complex [29].

The ruthenium complex Ru-4PySH was characterized by elemental analysis, UV–Visible spectrum (UV–Vis) and infrared spectrum (FT–IR). FT–IR is frequently used to characterize the oxidation state of the metal and NO ligands in ruthenium complexes [30]. The NO frequency of $\text{Cis-}[\text{Ru}(\text{bpy})_2(\text{NO})(4\text{PySH})].(\text{PF}_6)_3$ (Ru-4PySH) reported in this study is $1,942\text{ cm}^{-1}$ which indicates that formally a linear NO^+ is coordinated to the ruthenium(II) center. Thus, the ruthenium complex was characterized in situ by UV–Vis spectrum and it was attributed to the formation of $\text{Cis-}[\text{Ru}(\text{bpy})_2(\text{NO})(4\text{PySH})].(\text{PF}_6)_3$ simplified termed (Ru-4PySH) (Fig. 1).

Gold nanoparticles were characterized in situ by UV–Vis spectrum by plasmon band formation in 520-nm wavelength with maximum absorbance intensity around 1 at time zero (Fig. 2). The complex $\text{AuNPs-}\{\text{Ru-4PySH}\}_n$ was obtained by spontaneous interaction between AuNPs and Ru-4PySH. It was monitored at 1, 2, 5, 10, 20, 30, and 60 min by UV–Vis spectroscopy (Fig. 2). It shows a decrease in the absorbance in

Fig. 3 Representative scheme of the steps to obtainment of the complexes Ru-4PySH and AuNPs- $\{Ru-4PySH\}_n$. 4PySH thiol 4-aminepyridine, *L* ligand, *Ru* *Cis*- $[Ru(bpy)_2(NO)(L)](PF_6)_3$; *Ru-4PySH* $[Ru(bpy)_2(NO)(4PySH)](PF_6)_3$, *AuNPs* gold nanoparticles, *AuNPs*- $\{Ru-4PySH\}_n$, AuNPs+ $[Ru(bpy)_2(NO)(4PySH)](PF_6)_3$, *n* undetermined number of Ru-4PySH that interact with AuNPs

520 nm followed by an increase in the intensity absorbance in wavelength higher than 650 nm. After 30 min, the interaction was stabilized and it was not different from 60 min. The decreased peak in 520 nm (UV-Vis) indicates consumption of single AuNPs due its possible interaction with thiol group from Ru-4PySH.

The representative scheme of the steps to obtain the complexes Ru-4PySH and AuNPs- $\{Ru-4PySH\}_n$ is shown in the Fig. 3.

The NO released from the ruthenium complexes Ru-4PySH and AuNPs- $\{Ru-4PySH\}_n$ was analyzed by a selective NO-sensor, which directly detects NO concentrations by amperometric technique. As shown in the Fig. 4, the NO released from the AuNPs- $\{Ru-4PySH\}_n$ complex was lower than NO released from Ru-4PySH, but its NO release was constant up to 500 s.

Vascular relaxation induced by Ru-4PySH, AuNPs- $\{Ru-4PySH\}_n$, and simple AuNPs

As shown in the Fig. 5, the relaxation induced by the complexes Ru-4PySH and AuNPs- $\{Ru-4PySH\}_n$ was

concentration-dependent in denuded aortic rings contracted with 0.1 $\mu\text{mol/L}$ phenylephrine. Ru-4PySH and AuNPs- $\{Ru-4PySH\}_n$ demonstrated similar maximum effect and potency as shown by the values of ME and pD_2 , respectively (Table 1). On the other hand, AuNPs did not induce vascular relaxation. The absence of vasodilator effect for single AuNPs is also an indicative of non-cytotoxicity. Gold particles have been also used in medicine to improve the therapeutic effects of drugs designed to treat arthritis. Currently, due to its inert chemical and physical properties, gold cores are considered to be biocompatible and non-toxic which has enlarged its use for disease diagnosis and therapy [31, 32].

Time-course for vascular relaxation induced by Ru-4PySH and AuNPs- $\{Ru-4PySH\}_n$

The time to reach the maximum relaxation was longer for AuNPs- $\{Ru-4PySH\}_n$ (750 s, $n=5$) than for Ru-4PySH (450 s, $n=5$) as shown in the Fig. 6.

Fig. 4 Time-dependent NO release from Ru-4PySH and AuNPs- $\{Ru-4PySH\}_n$ (5 $\mu\text{mol/L}$) in Krebs solution at pH 7.4 in the presence of the aortic rings

Fig. 5 Effect of AuNPs, Ru-4PySH, and AuNPs-{Ru-4PySH}_n on denuded aortic rings contracted with phenylephrine (PE 0.1 μ mol/L). In the maintained contractions, Ru-4PySH (0.3 nmol/L to 10 μ mol/L, $n=10$), AuNPs-{Ru-4PySH}_n (0.3 nmol/L to 10 μ mol/L, $n=10$), or AuNPs (in proportional concentration to AuNPs-{Ru-4PySH}_n, $n=5$) was cumulatively added. Data are mean $\pm$ SEM of n experiments performed on preparations obtained from different animals. Asterisk denotes difference from Ru-4PySH and AuNPs-{Ru-4PySH}_n ($P<0.001$)

Our work indicates that the coupling of AuNPs with Ru-4PySH contributes to reduce the termed “burst release” [33], characterized by an initial large *bolus* of NO released before its rate reaches a stable profile. Consequently, time-course for vascular relaxation induced by AuNPs-{Ru-4PySH}_n also was increased as compared to Ru-4PySH.

Effect of oxyhemoglobin, hydroxocobalamin, and L-cysteine on vascular relaxation induced by Ru-4PySH and AuNPs-{Ru-4PySH}_n

One of the purposes of this work was to evaluate whether the resizing of the AuNPs along with Ru-4PySH, from 10 nm to around 1,000 nm, forming AuNPs-{Ru-4PySH}_n would modify the localization where the NO is released. The incubation with the extracellular NO scavenger oxyhemoglobin (HbO₂ 10 μ mol/L) shifted to the right the concentration-effect curve for Ru-4PySH complex reducing the potency (Fig. 7b), but it

did not reduce its ME (Fig. 7a and Table 1). On the other hand, HbO₂ abolished the relaxation induced by AuNPs-{Ru-4PySH}_n (Fig. 7a, b and Table 1).

Our results show that the relaxation induced by Ru-4PySH was less potent in presence of HbO₂ as compared to the control in absence of the extracellular NO scavenger. It indicates that Ru-4PySH induces vascular relaxation due to intracellular and extracellular NO release similarly to other NO donors [11, 34]. However, HbO₂ abolished the vascular relaxation induced by AuNPs-{Ru-4PySH}_n. It suggests that due to its large size, AuNPs-{Ru-4PySH}_n probably does not permeate the plasma membrane. Thus, it releases NO only in the extracellular medium.

Another important point for the vasodilator effect is the NO specie released by the NO donors as free radical (NO⁰) or nitroxyl ions (NO⁻). Incubation with the NO⁰ scavenger hydroxocobalamin shifted to the right the concentration-effect curve for Ru-4PySH complex reducing the potency (Fig. 7b), but it did not reduce its ME (Fig. 7a and Table 1). Nevertheless, hydroxocobalamin abolished the relaxation induced by AuNPs-{Ru-4PySH}_n (Fig. 7a, b and Table 1). On the other hand, the NO⁻ scavenger L-cysteine (1 mmol/L) had no effect on vasodilator effect induced by both NO donors (Fig. 7a, b and Table 1). These data indicate that NO⁰ plays an important role in vasodilator effect induced by both NO donors, but its role is more important for AuNPs-{Ru-4PySH}_n than Ru-4PySH.

Effect of ODC and TEA on vascular relaxation induced by Ru-4PySH and AuNPs-{Ru-4PySH}_n

In relation to the cellular mechanism induced by both complexes Ru-4PySH and AuNPs-{Ru-4PySH}_n, some pharmacological tools were used. ODC was used as a selective sGC inhibitor, the main target to the vasodilator effect of NO responsible by cGMP production [35].

Table 1 Effect of the extracellular NO scavenger oxyhemoglobin (HbO₂, 10 μ mol/L), NO⁰ scavenger hydroxocobalamin (0.1 mmol/L), NO⁻ scavenger L-cysteine (1 mmol/L), non-selective potassium channel

blocker tetraethylammonium (TEA, 1 μ mol/L), and soluble guanylyl cyclase inhibitor (ODQ 1 μ mol/L) on vascular relaxation induced by NO donors Ru-4PySH and AuNPs-{Ru-4PySH}_n in denuded aortic rings

	Ru-4PySH			AuNPs-{Ru-4PySH} _n		
	ME (%)	pD ₂	<i>n</i>	ME (%)	pD ₂	<i>n</i>
Control	104.6 $\pm$ 0.9	6.22 $\pm$ 0.13	10	102.2 $\pm$ 2.2	6.34 $\pm$ 0.07	10
HbO ₂	91.4 $\pm$ 4.2	4.70 $\pm$ 0.15*	5	—	—	5
Hydroxocobalamin	94.4 $\pm$ 7.2	5.00 $\pm$ 0.26*	5	62.7 $\pm$ 9.8*	4.62 $\pm$ 0.19*	6
L-cysteine	101.7 $\pm$ 1.9	6.14 $\pm$ 0.04	5	103.9 $\pm$ 6.0	6.23 $\pm$ 0.05	5
TEA	108.6 $\pm$ 3.9	5.51 $\pm$ 0.12*	7	99.9 $\pm$ 3.9	6.22 $\pm$ 0.05	7
ODQ	—	—	7	25.4 $\pm$ 8.0*	—	7

* $P<0.001$, denotes difference from control

Fig. 6 Time-course for Ru-4PySH and AuNPs-{Ru-4PySH}_n-induced relaxation. Denuded thoracic aortic rings were pre-contracted with 0.1 mmol/L phenylephrine and 0.1 and 5 μ mol/L Ru-4PySH or AuNPs-{Ru-4PySH}_n were added. Data are mean $\pm$ SEM of five experiments performed on preparations obtained from different animals

As shown in Fig. 8a and Table 1, the sGC-inhibitor ODQ abolished the vascular relaxation induced by Ru-4PySH. However, ODQ only partially reduced the vascular relaxation induced by AuNPs-{Ru-4PySH}_n. On the other hand, the K⁺ channel blocker TEA reduced the potency to Ru-4PySH, but it did not modify the ME (Fig. 8a and Table 1). In contrast, TEA did not alter the ME neither pD₂ for AuNPs-{Ru-4PySH}_n (Fig. 8b and Table 1).

Our results show that the sGC inhibition abolished the vascular relaxation induced by Ru-4PySH, but it did not abolish the vasodilation induced by AuNPs-{Ru-4PySH}_n. These data suggest that NO released from both complexes Ru-4PySH and AuNPs-{Ru-4PySH}_n distinctly activate sGC. On the other hand, the use of non-selective K⁺ channel blocker TEA inhibited the vascular relaxation induced by Ru-4PySH as observed for the other NO donors Terpy and SNP [11]. However, TEA and the selective K⁺ channel blockers (data not shown) had no effect on vascular relaxation induced by AuNPs-{Ru-4PySH}_n.

The main finding of this work was that the association of the NO donor Ru-4PySH to AuNPs forming AuNPs-{Ru-4PySH}_n cluster did not modify the efficacy and potency of these compounds in inducing vascular relaxation, although it modified the NO release and the cellular mechanism. For our surprise, this procedure reduced the NO release. These results are different of the results obtained by Polizzi et al. [15]. In accordance to our finding, Rothrock et al. [14] reported that at 37 °C the NO donor coupled to AuNPs released low levels of NO. These results suggest the possible chemical and thermal mechanisms involved in the NO release.

As reported by Thomas et al. [36], the steady-state NO concentrations seem to be a key determinant of its biological function. Precise cellular responses are differentially regulated by specific NO concentration. The direct effects of NO are those chemical reactions that occur in low concentrations enough to allow NO to react directly with a biological target molecule.

The NO⁰ species has been described to activate mainly sGC, while NO⁻ species could activate directly K⁺ channels [7, 37]. In addition, the present work shows that the amount and duration of NO⁰ released by NO donor also determine its pharmacological properties. The degree of activation of K⁺ channels and sGC may depend on the amount of NO⁰ released

Fig. 7 Effect of extracellular NO scavenger oxyhemoglobin (HbO₂, 10 μ mol/L), NO⁰ scavenger hydroxocobalamin (0.1 mmol/L) or NO⁻ scavenger L-cysteine (1 mmol/L) on the relaxation induced by the NO donors Ru-4PySH (a) or AuNPs-{Ru-4PySH}_n on denuded aortic rings pre-contracted with phenylephrine (PE 0.1 μ mol/L) after incubation or in the absence of scavenger (control). Data are mean $\pm$ SEM of $n=5-10$ experiments performed on preparations obtained from different animals. Asterisk denotes difference of maximum effect (ME) (a) and potency (pD₂) (b) from control ($P<0.001$)

Fig. 8 Effect of non-selective potassium (K^+) channel blocker tetraethylammonium (TEA 1 $\mu\text{mol/L}$) or soluble guanylyl cyclase inhibitor (ODQ 1 $\mu\text{mol/L}$) on the relaxation of denuded aortic rings induced by Ru-4PySH (**a**) or AuNPs- $\{\text{Ru-4PySH}\}_n$ (**b**) in the absence (control) or after incubation. Data are mean $\pm$ SEM of $n=7$ –10 experiments performed on preparations obtained from different animals. Asterisk denotes difference from control ($P<0.001$)

from NO donor. Although we did not quantify the NO molar concentration released by both NO donors, in accordance our findings, Hall and Garthwaite [38] reported that low NO concentration, a range of 100 pmol/L (or below), seems contribute to selective *in vivo* sGC activation.

Conclusion

Taken together, our results suggest that Ru-4PySH induces vasodilator effect by activation of sGC and K^+ channels while AuNPs- $\{\text{Ru-4PySH}\}_n$ activates mainly sGC, possibly because the AuNPs- $\{\text{Ru-4PySH}\}_n$ releases low NO levels, but only NO^0 specie in constant way. It would activate sGC–cGMP-mediated processes and possibly independent of K^+ channel activation. In conclusion, this study provides evidence that NO donor coupled to AuNPs represents a new pharmacological strategy to control the NO release and, consequently, modify its cellular mechanisms without impairing its effectiveness.

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Conflict to interest None declared.

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