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New nitric oxide donors based on ruthenium complexes

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Nitric oxide (NO) donors produce NO-related activity when applied to biological systems. Among its diverse functions, NO has been implicated in vascular smooth muscle relaxation. Despite the great importance of NO in biological systems, its pharmacological and physiological studies have been limited due to its high reactivity and short half-life. In this review we will focus on our recent investigations of nitrosyl ruthenium complexes as NO-delivery agents and their effects on vascular smooth muscle cell relaxation. The high affinity of ruthenium for NO is a marked feature of its chemistry. The main signaling pathway responsible for the vascular relaxation induced by NO involves the activation of soluble guanylyl-cyclase, with subsequent accumulation of cGMP and activation of cGMP-dependent protein kinase. This in turn can activate several proteins such as K^+ channels as well as induce vasodilatation by a decrease in cytosolic Ca^{2+}. Oxidative stress and associated oxidative damage are mediators of vascular damage in several cardiovascular diseases, including hypertension. The increased production of the superoxide anion (O_2^-) by the vascular wall has been observed in different animal models of hypertension. Vascular relaxation to the endogenous NO-related response or to NO released from NO deliverers is impaired in vessels from renal hypertensive (2K-1C) rats. A growing amount of evidence supports the possibility that increased NO inactivation by excess O_2^- may account for the decreased NO bioavailability and vascular dysfunction in hypertension.

Key words: Nitric oxide donor; Ruthenium complex; Nitrovasodilator; Vasodilatation

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Introduction

Endothelium dysfunction that leads to nitric oxide (NO) deficiency is present in several cardiovascular diseases (1). The discovery that NO is involved in multiple physiological and pathophysiological processes has promoted a large amount of pharmacological research into the design of new drugs that are capable of influencing NO production, directly and/or indirectly, for therapeutic purposes. In particular, the so-called NO donor drugs could have an important therapeutic effect on the treatment of many cardiovascular diseases such as angina and hypertension. The classic nitrovasodilators organic nitrate and nitrite esters, including nitroglycerin, amyl nitrite, isosorbide dinitrate, isosorbide 5-mononitrate and nicorandil, have been used in the treatment of cardiovascular diseases for many years (2).

NO donors produce NO-related activity when applied to biological systems, so they are mainly suited to either mimic an endogenous NO-related response or substitute an endogenous NO deficiency (1). NO is also the pharmacological principle of a number of drugs collectively termed

nitrovasodilators, which are clinically used to control hypertensive crisis, protect patients from attacks of angina pectoris, and unload the heart during acute heart failure. Numerous other compounds, including NONOates, are available for the experimental generation of NO (3,4). Several NO donors have been used in clinical settings for decades (e.g., nitroglycerin and sodium nitroprusside). However, the growing interest in NO physiology since the mid 1980's has led to the development of a variety of new NO donors that offer several advantages over conventional NO donors (1,5). Recently, several new metal complexes have been studied as NO donors or scavengers, including nitrosyl ruthenium complexes, nitrite complexes and related species (6-17).

The chemistry of metal nitrosyl complexes has taken on added significance in recent years because of the important role involving transition metal in the biological process of NO, as well as the possibility of producing thermodynamically stable and kinetically labile species (18-20). Such strategies have focused on the development of pharmacological substances capable of releasing NO at specific rates in tissues, in order to overcome NO deficiency. NO scavengers that reduce NO levels where excessive production is observed have also been designed. In recent years, there has been increasing interest in the chemistry of ruthenium complexes and their suitability as potential pharmaceutical agents because of their usually low cytotoxic characteristics (10,21). Ruthenium (II) and ruthenium (III) are the most commonly employed ions and they are generally surrounded by six coordination spheres.

The high affinity of ruthenium for NO is a marked feature of its chemistry. Because this system has proven to be an NO donor agent under external stimulation, a large number of ruthenium complexes are currently being investigated. Compared with the existing NO-donor drugs, this system has the advantage that NO could be released at a specific biological target. Although several nitrosyl ruthenium complexes have been synthesized and their chemical and physicochemical properties have been described, only few examples involve the above-discussed pharmacological aspects. In the present report, we will focus our attention on the compounds shown in Figure 1. A strategy that we have adopted in the design of nitrosyl ruthenium complexes is to bind ruthenium (II) with ligands that confer high thermodynamic stability and also water solubility to the resulting complex.

The co-ligands (L) in Figure 1 are named 1,4,8,12-tetraazacyclopentadecane ([15]aneN$_4$) (I); 2,2'-bipyridine (bpy) (II) and 1,2-benzoquinonediimine-5-carboxylate (bdqi-COO) (III). Species (I) and (III) are characterized by a $\{Ru^{II}\text{-}NO^{+}\}^{3+}$ bond, while compound (II) consists of $\{Ru^{II}\text{-}NO^{0}\}^{2+}$. All the species are stable at physiological pH, although at pH ≥9.0 equilibrium with an appreciable amount of nitrite bound to ruthenium (II) takes place, as depicted in Equation 1.

$$[RuL_5NO]^{3+} + 2OH^- \leftrightarrow [RuL_5(NO)_2]^{+} + H_2O \qquad \text{(Equation 1)}$$

Compounds (I) and (III) produce NO under light irradiation (Equation 2) or reducing medium (Equation 3) while compound (II) is an NO donor only under light stimulation.

$$[RuL_5NO]^{3+}\ h\nu \rightarrow [RuL_5(H_2O)]^{3+} + NO \qquad \text{(Equation 2)}$$

$$[RuL_5NO]^{3+} + e^- \rightarrow [RuL_5(H_2O)]^{2+} + NO \qquad \text{(Equation 3)}$$

On the basis of these properties, we have performed a series of pharmacological assays with these nitrosyl ruthe-

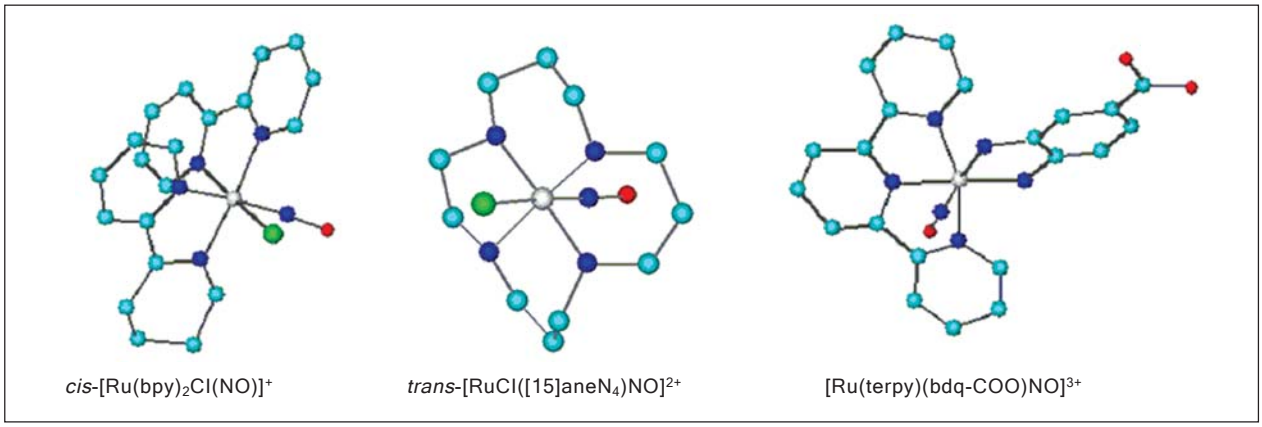

Figure 1. Molecular structure of *cis*-[Ru(bpy)$_2$Cl(NO)]$^+$, *trans*-[RuCl([15]aneN$_4$)NO]$^{2+}$ and [Ru(terpy)(bdq-COO)NO]$^{3+}$ (silver = ruthenium, dark blue = nitrogen, light blue = carbon, and red = oxygen; hydrogen atoms have been omitted in the structure).

nium complexes as new NO donor species. Our results suggest a new class of metal-based drugs.

Nitrosyl ruthenium complex is an NO donor that induces rat aorta relaxation

The mechanism by which NO induces relaxation is the subject of much investigation. When the signaling molecule NO enters a vascular smooth muscle cell, it activates an enzyme called guanylyl cyclase, which produces the second messenger 3'5'guanosine-monophosphate (cGMP) (22,23). Guanylyl cyclase is a heterodimer that consists of two subunits, termed α and β, which are linked by disulfide bonds, and it has a prosthetic heme group covalently bound to the heterodimer (23,24). NO activates cGMP synthesis by binding to the heme group, leading to the conversion of guanosine triphosphate to cGMP. cGMP activates a serine/threonine kinase, the cGMP-dependent protein kinase G (PKG), leading to the phosphorylation of key regulatory proteins (25). PKG activation has been associated with isoform-specific autophosphorylation, which results in changes in the properties of the enzyme, including increased sensitivity to cyclic nucleotides and increased constitutive activity (26,27). An increase in cellular cGMP levels occurs in response to the activation of the membrane guanylyl-cyclase or more commonly by activation of soluble guanylyl-cyclase by NO (28). Numerous studies have inferred activation of PKG in response to diverse ligands, presumably downstream of NO production (29). NO has two signaling pathways, a cGMP-dependent one and a cGMP independent one, which could directly activate K^+ channels (30).

Potassium channel

Potassium channels are a group of transmembrane proteins that have several functions (31,32). The activation of K^+ channels in the vascular smooth muscle cell membrane stimulates K^+ efflux, membrane hyperpolarization, decreased Ca^{2+} entry through voltage-operated channels, and vasodilatation. Changes in the activity of the K^+ channels regulate the vascular tone.

The main K^+ channel subtypes consist of large and small conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), which are selectively blocked by iberiotoxin and apamin, respectively. The K^+ channel group that appears to be involved in vascular tone regulation also includes ATP-sensitive potassium channels, which are blocked by glibenclamide voltage-dependent channels (K_V), which in turn are blocked by 4-aminopyridine and inward rectifier channels. The expression of these channels has been reported to vary among vessels of different sizes and vascular beds (33). However, the presence of K_V and BK_{Ca} channels has been demonstrated in all the vascular myocytes tested (34).

Altered function of K^+ channels is associated with hypertension, and K_V and BK_{Ca} are the most often studied channels involved in the vascular tone regulation. Increased vascular tone, a typical feature of most forms of hypertension, has been associated with impaired NO signaling. Although the main mechanism of vasodilatation induced by the ruthenium-derived NO donors is due to K^+ channel activation and membrane hyperpolarization in normotensive rat aorta, we have reported that none of the K^+ channel subtypes are activated by the NO donor *trans*-[RuCl([15]aneN$_4$)NO]$^{2+}$ in the aorta from renal hypertensive rats (35). These results demonstrate that in this model of hypertension, impaired K^+ channels can contribute to decreased vasodilatation in response to NO.

Vascular relaxation induced by the NO donors

The new NO donors like *trans*-[RuCl([15]aneN$_4$)NO]$^{2+}$ (15-ane), [Ru(NH.NHq) (terpy)NO$^+$]$^{3+}$ (TERPY), *cis*-[RuCl (bpy)(2)(NO)](PF(6)) (RUNOCL), and sodium nitroprusside (SNP) induce relaxation in a concentration-dependent manner in denuded aortic rings pre-contracted with norepinephrine (6,7). An illustration of this effect can be seen in Figure 2, which demonstrates the similar concentration dependent for all the new NO donors.

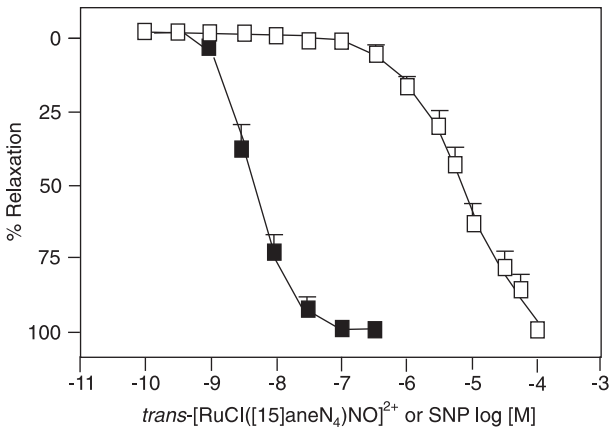

Figure 2. Effects of *trans*-[RuCl([15]aneN$_4$)NO]$^{2+}$ and sodium nitroprusside (SNP) on rat thoracic aorta pre-contracted with norepinephrine. The arteries were pre-contracted with 0.1 µM norepinephrine and *trans*-[RuCl([15]aneN$_4$)NO]$^{2+}$ (open squares, N = 6) or SNP (filled squares, N = 6) was cumulatively added (0.1 nM to 100 µM and 0.1 nM to 0.3 µM, respectively). Data are reported as means ± SEM of N experiments performed on preparations obtained from different animals. From Ref. 36 (with permission).

NO released from all the NO donors is the common mediator that elicits their vasodilatory effects. The activation of guanylyl cyclase and accumulated cGMP lowers cytosolic Ca^{2+}, leading to vasodilatation. Regarding the vascular relaxation induced by these new NO donors, all the new NO compounds induce vascular relaxation in a concentration-dependent way in denuded rat aortas precontracted with 0.1 µmol/L phenylephrine. The specificity of soluble guanyl cyclase activation should be different for the different NO donors. NO can exist in a variety of forms such as free radical and nitrosyl or nitronium ions, and its form depends on the NO source. Therefore, the NO released from NO donors can differ from the NO produced in endothelial cells. This could explain the differences in potency and efficacy of the known NO donors in inducing vasodilatation. The NO donor SNP also induces concentration-dependent relaxation, but it is more potent (pD_2: 7.97 ± 0.07, N = 6) than 15-ane (pD_2: 5.03 ± 0.2, N = 6), TERPY (pD_2: 6.47 ± 0.13, N = 6) and RUNOCL (pD_2: 6.62 ± 0.16, N = 7). In relation to the maximum relaxant effect, however, all the compounds induce a similar effect, as shown by E_{max} (105.86 ± 3.34%, N = 6 for SNP; 98.35 ± 1.22%, N = 6 for 15-ane; 102.38 ± 0.38%, N = 6 for TERPY, and 101.2 ± 3.7%, N = 7 for RUNOCL). When mouse aorta relaxation is concerned, NO gas, spermine NONOate and Angeli's Salt are equipotent, with pD_2 in the range of 6.0. All these new NO donors display an important ability to induce vascular relaxation (36-40). The potency and efficacy of these NO donors and those of the authentic NO gas are comparable.

Time-course of the relaxation induced by 15-ane, TERPY, RUNOCL, and SNP

The time to reach the maximum relaxation has been evaluated for the new NO donors. Table 1 shows the values obtained in denuded aortic rings from normotensive rats. According to the structure and the particular characteristics of each new NO donor such as extra/intracellular release (6,35-40), reduction potential (32), and light activation (6), the time course of the maximum relaxation can be modulated to a fast/slow event.

Table 1. Time to reach maximum relaxation of aorta ring induced by nitric oxide (NO) donors (modified Krebs solution, 37°C, [NO donors] = 0.1 mmol/L).

NO donor	Time (s)
Sodium nitroprusside	195
TERPY	255
15-ane	595
RUNOCL	1630

TERPY = $[Ru(NH.NHq)\ (terpy)NO^+]^{3+}$; 15-ane = *trans*-$[RuCl([15]aneN_4)NO]^{2+}$; RUNOCL = *cis*-[RuCl(bpy)(2)(NO)] (PF(6)).

Calcium measurements

NO is an important regulator of vascular smooth muscle relaxation. It is well known that changes in the cytosolic calcium concentration ($[Ca^{2+}]_c$) play a critical role in the relaxation of vascular smooth muscle cells (41). When activated by its second messenger cGMP, PKG modulates cellular function via phosphorylation of substrate proteins (42).

It has been shown that the mechanism of NO-dependent relaxation of the vascular smooth muscle cells involves a decrease in $[Ca^{2+}]_c$ by inhibition of Ca^{2+} entry (43). It has also been postulated that NO and cGMP may relax vascular smooth muscle cells by a cGMP-dependent K^+ channel activation (44). In the same way, a novel mechanism that operates by a direct effect of NO on Ca^{2+}-dependent K^+ channels (45) and L-type calcium current (44) and does not require cGMP has been demonstrated.

Studies on other types of smooth muscle preparations indicate that NO/cGMP may decrease $[Ca^{2+}]_c$ and reduce the sensitivity of contractile proteins to Ca^{2+}, thereby resulting in the relaxation of smooth muscle.

Our confocal microscopy studies have shown that NO donors like 15-ane, TERPY and RUNOCL decrease $[Ca^{2+}]_c$ in the vascular smooth muscle cells. We have observed a decrease in $[Ca^{2+}]_c$ measured as fluorescence intensity of the Ca^{2+} sensitive dye Fluo-3AM after addition of the NO donors 15-ane (%ΔFI: 14.1 ± 3.5%, N = 4), TERPY (%ΔFI: 55.7 ± 4.8%, N = 5), RUNOCL (%ΔFI: 40.0 ± 10.0%), and SNP (%ΔFI: 18.6 ± 4.7%, N = 4) (6,7,35). Among these new NO donors, TERPY leads to a more effective $[Ca^{2+}]_c$ reduction (Figure 3) (6,40,46-49).

Superoxide can contribute to reduced NO bioavailability and vascular relaxation in renal hypertensive rats

It is well recognized that reactive oxygen species (ROS) play an important role in hypertension and in other cardiovascular diseases. Vascular cell types such as endothelial and vascular smooth muscle cells can produce ROS via cell membrane-associated NAD(P)H oxidase. ROS include superoxide (O_2^-), hydrogen peroxide and hydroxyl anion. Reactive nitrogen species such as NO and peroxynitrite are also biologically important O_2 derivatives.

We have reported that relaxation induced by NO re-

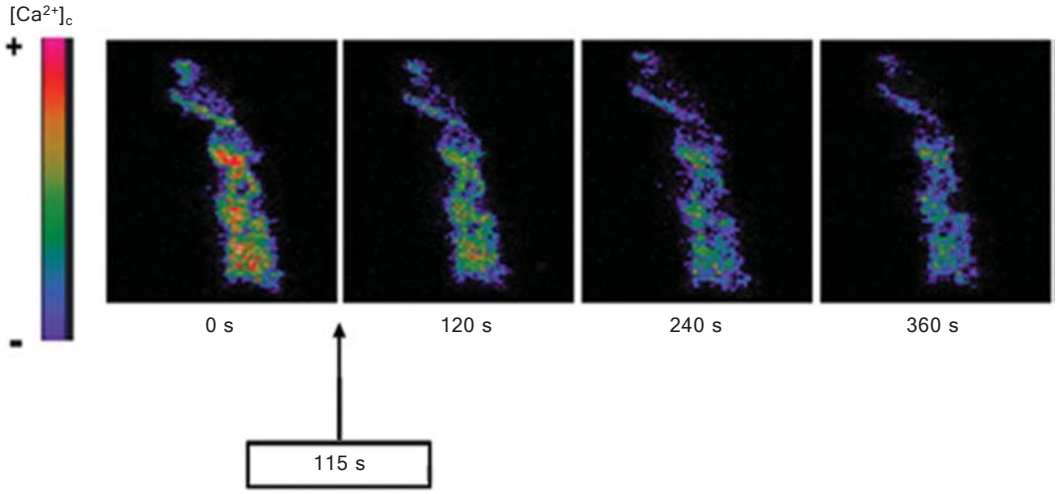

Figure 3. Decrease in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) induced by $[Ru(NH.NHq)(terpy)NO^+]^{3+}$ (TERPY) in vascular smooth muscle cells from rat aortas. Cells were preloaded with Fluo-3AM (10 µM) and stimulated with TERPY (10 µM). Temporal effect of TERPY in a single vascular smooth muscle cell was recorded with a confocal microscope. TERPY was added after 115 s. The intensity of the colors indicate higher $[Ca^{2+}]_c$ (as shown in the left bar). From Ref. 49 (with permission).

leased from the complexes TERPY and 15-ane is impaired in the aortic rings from 2K-1C rats. This impairment has been attributed to increased ROS production. The impaired relaxation to TERPY in 2K-1C aortic rings is normalized in the presence of the antioxidant agent vitamin C. According to Oliveira-Sales et al. (50) the overactivity of NAD(P)H oxidase-derived ROS and lowered activity of CuZnSOD, an endogenous antioxidant, could contribute to 2K-1C hypertension. These investigators have hypothesized that the increased angiotensin II characteristic of this hypertension model would activate NAD(P)H oxidase. An imbalance between ROS production and the antioxidant levels could lead to a significant increase in oxidative stress and hypertension.

We have shown that the basal cytosolic NO concentration ($[NO]_c$) is lower in 2K-1C than in normotensive (2K) aortic rings (49). After stimulation with the NO donor TERPY, $[NO]_c$ is also lower in 2K compared with 2K-1C aortic rings. These results indicate that NO bioavailability under basal conditions or bioavailability of NO released from TERPY is larger in the normotensive than in the renal hypertensive rat aorta.

Concluding remarks

NO released from the ruthenium complexes induces vascular smooth muscle relaxation via activation of cGMP production and K^+ channel activation in normotensive rat aorta, as well as decreased cytosolic Ca^{2+} concentrations. Vascular smooth muscle relaxation induced by the new NO donors is impaired in renal hypertensive rat aortas due to vascular dysfunction by decreased NO bioavailability and impaired K^+ channel activation.

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