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Nitric Oxide Releasing Metal–Diazeniumdiolate Complexes Strongly Induce Vasorelaxation and Endothelial Cell Proliferation

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The preparation, characterization, and NO-releasing properties of metal complexes derived from N-*aminoethylpiperazine*-N-*diazeniumdiolate (HPipNONO), [Cu(PipNONO)Cl] and [Ni(PipNONO)Cl], and the Ni^{II} complex derived from the Schiff base between HPipNONO and salicylaldehyde, [Ni(SalPipNONO)], are described. Compounds [Cu(PipNONO)Cl] and [Ni(SalPipNONO)] release NO at a much slower rate than HPipNONO in aqueous buffer in the pH range between 6.8 and 8.0. The kinetics of NO release by [Ni(SalPipNONO)] is complex, with an apparent stepwise release of NO molecules. Both [Cu(PipNONO)Cl] and [Ni(SalPipNONO)] are effective vasorelaxant agents of precontracted rabbit aorta rings, and are active in the nM concentration range. In addition, these complexes promote the proliferation of endothelial cells. Both vascular activities were inhibited by a specific inhibitor of guanylate cyclase, suggesting the involvement of the cGMP pathway. In all biological assays, the reference agent sodium nitroprusside was shown to be 10–1000-fold less potent than the two metal–NONOates.*

Introduction

Research in the last two decades has identified nitric oxide as an important signaling molecule in the cardiovascular, nervous, and immune systems.[1–3] Endogenous NO is synthesized from L-arginine by three isoforms of the enzyme NO synthase (NOS),[4] which are either constitutively expressed, and therefore require activation by Ca^{2+}-calmodulin for transcellular signaling,[5] or are induced in response to pathogenic challenge,[6] or in cardiovascular pathologies such as atherosclerosis and heart failure.[7] Dysfunction in NO synthesis or the decreased availability of NO through its inactivation by reactive oxygen species have been implicated as major factors in the development of a variety of diseases.[8–10] Therefore, the development of molecules that release NO to areas where the delivery of endogenous NO is decreased has invoked great interest as a potential therapeutic option. Several families of NO donor molecules are known and many of them have been tested in pharmacological and clinical studies.[11,12] Among these, organic nitrates such as glyceryl trinitrate,[11] metal nitrosyls such as sodium nitroprusside ($Na_2[Fe(CN)_5NO]$, SNP),[13] *S*-nitrosothiols such as *S*-nitroso-*N*-acetylpenicillamine,[14] sydnonimines such as 3-morpholinosydnonimine (SIN-1),[15] and *N*-diazeniumdiolates (NONOates)[16] have received the greatest attention so far. In many cases, the mechanism of NO release is still not clearly understood as it needs the intervention of other chemical or enzymatic species. This is the case, for instance, of the classical organic nitrates and of molsidomine and SIN-1, used in the treatment of angina or acute heart failure.[17–20]

An emerging family of NO-releasing molecules is that of metal nitrosyl complexes, particularly those containing ruthenium.[13,21–24] These complexes evolve from SNP, which is commonly used in experimental and clinical protocols and is recognized as the gold-standard NO-dependent but endothelium-independent vasodilator.[25,26] SNP does not spontaneously release NO and requires reduction or photolytic activation prior to decomposition.[11,27] The decomposition of SNP is accompanied by cyanide and iron(II) release, which are responsible for cellular toxicity.[28] Nevertheless, SNP is such a powerful vasodilator that often it is effective at doses producing very limited amounts of cyanide. Metal nitrosyl complexes are potentially interesting as vasodilators because they release NO in a controlled manner and many of them have low toxicity.[29] In general, ruthenium nitrosyl complexes exhibit vasorelaxation properties similar to those of SNP,[24] but it would be desirable to have NO-releasing compounds with still higher potency and lower toxicity.

In this work we describe representative members of a new family of metal-based NO-releasing compounds, resulting from the association of polydentate *N*-diazeniumdiolate residues derived from piperazine with transition metal ions (Scheme 1). Other metal–NONOate complexes have been reported before, but as they used a simple *N*-dialkylamino diazeniumdiolate, such as $Et_2N(NONO)^-$, as a bidentate metal binding ligand, their NO-releasing properties were not too different from those of the parent free NONOates.[30–33] We thought that anchoring the NONOate group to a much stronger metal binding residue, such as the potentially tridentate *N*-aminoethylpipera-

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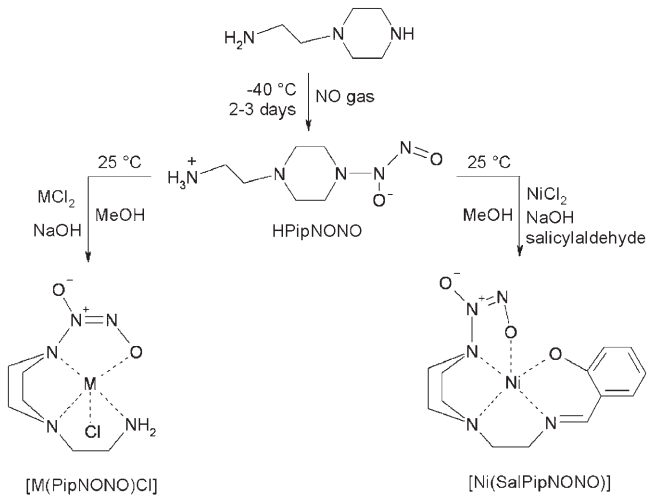


Scheme 1. Preparation of *N*-aminoethylpiperazine-*N*-diazeniumdiolate (HPipNONO), metal complexes of type [M(PipNONO)Cl] (for which M = Cu^{II} or Ni^{II}), and [Ni(SalPipNONO)].

zine (Pip) residue,[34] it would be possible to stabilize the complexes and better modulate their NO-releasing properties. In addition, the *N*-aminoethylpiperazine-*N*-diazeniumdiolate ligand (HPipNONO) can be further modified through reactions at the primary amino group to obtain ligands with still higher denticity, as shown for the salicylaldehyde Schiff base ligand in Scheme 1. Metal–NONOates, like their supporting ligands,[16] are capable of releasing NO upon hydrolysis with water. For the complexes reported herein, the rate of this process changes with the NONOate structure, the pH value of the medium, and the bound metal ion. The biological activity of these NO donor compounds was evaluated by studying their vasorelaxant properties in precontracted vessels. Our data suggest that the biological effect is correlated to the hydrolysis rate, with the slower NO-releasing molecules being most active. In addition we investigated their ability to activate the basic functions of coronary endothelial cells, as the dysfunction of the endothelium is thought to be a common pathogenetic mechanism for many cardiovascular pathologies.[35] The rationale for evaluating the effect of NO donors on the endothelium lies in the evidence which shows that NO has an important role for the maintenance and functional integrity of this tissue.[36–38]

Results and Discussion

Synthesis of metal–NONOates

Metal complexes of type [M(PipNONO)Cl] (M = Ni^{II}, Cu^{II}) were obtained by reaction between HPipNONO and the metal chloride salt, whereas the complex [Ni(SalPipNONO)] was obtained by template synthesis from the metal salt and the in situ prepared Schiff base between HPipNONO and salicylaldehyde[39] (Scheme 1). The Ni^{II} and Cu^{II} ions were chosen for this investigation because they give air-stable divalent complexes among those of the first transition series. The free Schiff base could not be isolated because its preparation requires prolonged heating of the reagent solution, which results in the loss of the thermally unstable NONOate substituent. As is well known[40] and also from our previous experience,[41–43] the metal template effect is extremely efficient in promoting Schiff base formation when the amine and aldehyde residues belong to molecules containing further metal binding groups. Attempts to prepare the complex [Cu(SalPipNONO)] systematically resulted in a material with unsatisfactory analytical data (low N content) and almost complete lack of NO release in solution. As the IR and UV signatures for the salicylaldimine group are present in this material, the problem with isolation is the very fast release of NO from the bound SalPipNONO ligand as soon as it binds to copper(II). As a matter of fact, the copper(II)–salicylaldimine complex derived from Pip, [Cu(SalPip)Cl], was obtained without any problems. Other complexes derived from Pip, that is, [Ni(SalPip)Cl] and $[Cu(Pip)Cl_2]$, were prepared as appropriate reference compounds for characterizing the spectral properties of the metal–NONOate complexes.

In general, the metal–NONOate complexes were isolated with a number of water molecules of hydration, because the samples were simply allowed to dry in air; more severe drying conditions were not applied to prevent the possible release of NO.

Crystal structure of $[Cu(PipNONO)Cl]_2$

Figure 1 shows that the crystal structure of the Cu^{II}–PipNONOate complex contains a dimeric species, $[Cu(PipNONO)Cl]_2$, which appears to contradict the schematic representation given for the complex in Scheme 1. The structure is symmetric, with two PipNONO ligands bridging two nearly identical copper(II) ions in a distorted octahedral arrangement. Thus, the terminal NH_2 group and the tertiary piperazine nitrogen donor of one PipNONOate molecule bind one of the coppers as a bidentate ligand, whereas the oxygen atoms of the NONOate

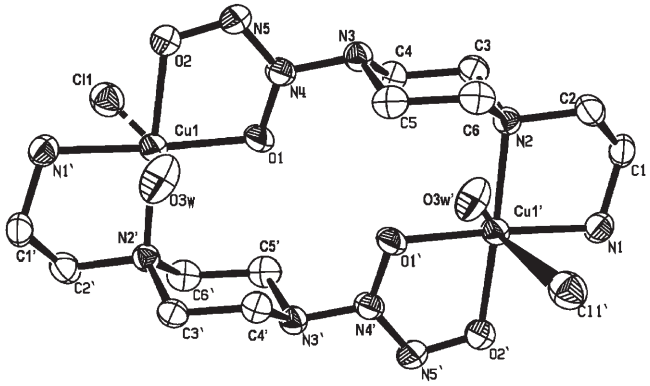


Figure 1. An ORTEP view of the X-ray crystal structure of the complex $[Cu(PipNONO)Cl]_2$ (50% thermal ellipsoids, all hydrogen atoms omitted for clarity). Selected bond distances (Å) and angles (deg) are as follows: Cu1–N1, 1.97; Cu1–N2, 2.104; Cu1–O1, 1.92; Cu1–O2, 1.98; Cu1–Cl1, 3.09; Cu1–O(w), 2.53; N1-Cu1-N2, 87.05; N1-Cu1-O2, 96.17; O1-Cu1-N1, 174.34; O1-Cu1-N2, 97.55; O1-Cu1-N2, 175.99; O1-Cu1-O(w), 92.02; O2-Cu1-O(w), 88.62; N1-Cu1-O(w), 84.37; N2-Cu1-O(w), 94.11; Cl-Cu1-O1, 101.62; Cl-Cu1-O2, 87.21; Cl-Cu1-N1, 81.50; N1-Cu1-N2, 90.89. The structure is perfectly symmetric and bond distances and angles around the other Cu atom are nearly identical.

group bind to the other copper. A chloride ion acts as a fifth axial ligand and the six-coordination of each Cu is completed by a weakly bound water molecule trans to the chloride ligand. The structure also emphasizes the relatively short bond length of the N4–N5 atoms of the NONOate group, which is typical of a nitrogen–nitrogen double bond. This is in accord with previously reported metal–NONOate structures.[30,32,33] The piperazine ring of the PipNONOate residue is in the chair conformation, preventing the coordination to the metal ion of the ring nitrogen atom bonded to the NONOate group. This arrangement is somewhat unexpected, but the whole dimeric structure of the complex in the crystal state may be stabilized by lattice effects. As a matter of fact, in other Cu^{II} complexes with *N*-aminoalkylpiperazine ligands, the piperazine residue is found in the boat conformation, with both the ring nitrogen atoms bound to the Cu^{II} center.[44]

All known structures of metal–NONOate complexes refer to copper(II) species and contain simple *N*-dialkylamino diazeniumdiolate ligands, such as $Et_2N(N_2O_2)^-$, with ancillary ligands ranging from O-donor alkoxides or acetate[30] to N-donor cyclic polyamines or mixed N,O-donor substituted polyamines.[32,33] Thus, the present structure is the first one reporting a metal–NONOate complex derived from a functionalized NONOate ligand. Regarding the binding mode of the NONOate group, it generally acts as a bidentate ligand through the two oxygen atoms, but in one case it has also been found to bind to Cu^{II} as a simple monodentate ligand, through the terminal oxygen atom of $Et_2N(N_2O_2)^-$.[33] The copper(II) centers are systematically five-coordinated in these structures.

Characterization of metal–NONOates

The constitution of the complexes in solution was probed by MS spectroscopy. These experiments showed that the dimeric structure of the Cu^{II}–PipNONOate complex is lost, at least in polar solvents such as methanol and water, and that the chloride ligands are easily exchanged with solvent molecules. Thus, the mass spectrum of a methanolic solution of [Cu(PipNONO)Cl] shows two main peaks, at 284 amu, corresponding to the cation $[Cu(PipNONO)(CH_3OH)]^+$, and at 270 amu, corresponding to $[Cu(PipNONO)(H_2O)]^+$. Both peaks are related to a singly charged ion, indicating the presence of a monomeric species. Fragmentation of the ion at 284 amu occurs with the loss of 30 and 60 amu, corresponding to the release of NO by $[Cu(PipNONO)(CH_3OH)]^+$. A similar behavior is observed for the ion at 270 amu. In the case of the complex [Ni(PipNONO)Cl], a good MS response was obtained from solutions in methanol/acetonitrile 1:1 (*v*/*v*), which showed a singly charged molecular ion at 288 amu, corresponding to the cation $[Ni(PipNONO)(CH_3CN)]^+$. The mass spectrum of a methanolic solution of [Ni(SalPipNONO)] exhibited a molecular peak of 350 amu. The presence of isotopic peaks separated by 1 amu again indicates that the molecular ion observed is singly charged, thus the compound is monomeric in solution. Fragmentation of the parent peak gives rise to peaks with masses of 320 and 290 amu, corresponding to consecutive loss of one and two NO molecules.

In the schematic representation given in Scheme 1 for the structure of the metal–NONOate complexes it is assumed that the NONOate residue is at least weakly coordinating to the metal centers. Although other arrangements are possible, the one indicated has the advantage of allowing the NONOate group to bind through the terminal oxygen atom, which exhibits higher affinity for metal ions.[33] Note that this intramolecular interaction involves the *E* configuration of the NONOate group, which is normally unfavorable for free diazeniumdiolates.[16,45] The most important consequence of such structural arrangement, which will bear on the mode of NO release, is that in complexes of type [M(PipNONO)Cl] coordination of the O-donor of the NONOate group occurs in the equatorial coordination plane, whereas in the imine complex [Ni(SalPipNONO)] binding of this group can only occur in an axial position (Scheme 1).

All the Ni and Cu complexes are paramagnetic and give rise to extremely broad and unresolved NMR spectra. The presence of five-coordinated structures, as it is assumed in Scheme 1, or six-coordinated structures in solution is supported for the copper(II) complexes [Cu(PipNONO)Cl], [Cu(SalPip)Cl], and $[Cu(Pip)Cl_2]$ by the position of the visible absorption band in the range 620–630 nm, which is typical for Cu^{II} complexes with a strong tetragonal ligand field by N-donor ligands and weaker axial interaction.[46] For the nickel(II) complexes the low-energy visible bands associated with d–d transitions are very weak and occur at or near 600 nm; in the case of [Ni(SalPipNONO)] it occurs as a shoulder almost completely buried under the tail of the intense near-UV absorption. We thus assume that the structure of the Ni^{II} complexes in solution is six-coordinate, with one or two solvent molecules occupying the axial coordination positions, as pseudo-octahedral nickel(II) complexes typically exhibit very weak d–d bands.[47]

The near-UV portion of the electronic spectra of the complexes is dominated by ligand-centered electronic transitions. The ligand HPipNONO exhibits the characteristic absorption band of the *N*-diazeniumdiolate group near 250 nm.[16,45] This absorption is present also in the spectra of the [M(PipNONO)Cl] complexes, whereas it overlaps with other bands in the spectrum of [Ni(SalPipNONO)], due to $\pi \rightarrow \pi^*$ transitions within the aromatic imine chromophore, which include the absorption near 360 nm diagnostic of the metal-bound *N*-salicylideneimino chromophore.[41–43] A similar band is present in the spectra of the imine complexes of type [M(SalPip)Cl], where it is slightly red shifted, probably due to the positive charge that these complexes assume in solution upon release of the chloride ligand.

We have tried to unravel the IR characteristic features of the *N*-diazeniumdiolate group in HPipNONO and in the complexes [M(PipNONO)Cl] and [Ni(SalPipNONO)], by comparing their IR spectra with those of *N*-aminoethylpiperazine and the related complexes $[Cu(Pip)Cl_2]$, [Ni(SalPip)Cl], and [Cu(SalPip)Cl], which lack the *N*-diazeniumdiolate group. Comparing the spectra of HPipNONO and *N*-aminoethylpiperazine, there are a number of IR bands that can be attributed to vibrations of the *N*-diazeniumdiolate group.[16,48] Among these, the bands at 1278 and 1180 cm^{-1} can be attributed to N–O stretchings and that at

1155 cm^{-1} to N–N stretching of the NONOate.[16] In the IR spectra of the metal–NONOate complexes, we can attribute the peaks at 1314 cm^{-1} in the spectrum of [Cu(PipNONO)Cl], and at 1301 and 1300 cm^{-1} in those of [Ni(PipNONO)Cl] and [Ni(SalPipNONO)], respectively, to the higher energy N–O stretching, and the peaks at 1120 cm^{-1} for [Cu(PipNONO)Cl], 1126 cm^{-1} for [Ni(SalPipNONO)], and 1172 cm^{-1} for [Ni(PipNONO)Cl] to N–N symmetric stretching of the *N*-diazeniumdiolate.[16] It is noteworthy that the IR spectra of the metal–NONOates exhibit prominent peaks at 997 cm^{-1} ([Cu(PipNONO)Cl]), 996 cm^{-1} ([Ni(SalPipNONO)]), and 961 cm^{-1} ([Ni(PipNONO)Cl]), that may correspond to the strong peak at 953 cm^{-1} for HPipNONO, representing an *N*-diazeniumdiolate vibration perturbed by metal complexation. These IR features are absent in the spectra of the reference compounds lacking the *N*-diazeniumdiolate group.

Table 1. Kinetic data on the release of NO by HPipNONO and the metal–NONOates.[a]

Compound	pH	k [s^{-1}]	$t_{1/2}$ [s]
HPipNONO	7.5	$(2.60\pm0.01)\times10^{-3}$	270
[Ni(PipNONO)Cl]	7.5	$(2.41\pm0.05)\times10^{-3}$	290
[Cu(PipNONO)Cl]	6.8	$(7.29\pm0.05)\times10^{-4}$	950
	7.5	$(1.11\pm0.03)\times10^{-4}$	6200
	8.0	$(8.02\pm0.05)\times10^{-5}$	8600
[Ni(SalPipNONO)]	6.8	$(4.9\pm0.4)\times10^{-3}$	140
		$(9.6\pm0.2)\times10^{-4}$	720
	7.5	$(2.2\pm0.2)\times10^{-3}$	320
		$(4.7\pm0.2)\times10^{-4}$	14800
	8.0	$(9.0\pm0.5)\times10^{-4}$	770
		$<3\times10^{-5}$	>20000

[a] In 50 mM phosphate buffer (pH 7.5) containing 5% methanol (*v/v*), 25 °C, monitored at 252 nm for HPipNONO, [Ni(PipNONO)Cl], and [Cu(PipNONO)Cl], and at 256 nm for [Ni(SalPipNONO)]; for the latter complexes the kinetic data are also given at pH 6.8 and 8.0.

NO release

As for all NONOate derivatives, both HPipNONO and the metal–NONOates containing this ligand moiety, release NO in solution by hydrolysis at a rate that depends on the acidity of the medium. The amount of NO rapidly released by HPipNONO and the metal–NONOates in acidic solution was quantitated by formation of the stable complex [Fe(EDTA)NO][40] and corresponded to: 1.95 mol NO per mol of HPipNONO, 1.90 mol NO per mol of [Cu(PipNONO)Cl], 1.85 mol NO per mol of [Ni(PipNONO)Cl], and 1.92 mol NO per mol of [Ni(SalPipNONO)].

Kinetics of NO release

The kinetics of NO release by HPipNONO and the metal–NONOate complexes were measured in phosphate buffer at pH 7.5 and 25 °C (Table 1). The rate of NO dissociation by HPipNONO in these conditions (2.60×10^{-3} s^{-1}, half-life 270 s) is in perfect agreement with the data reported in the literature for this compound at pH 7.4 and 22 °C (half-life 5.0 min)[34] and at 37 °C (2.58×10^{-3} s^{-1}) in 0.1 M phosphate buffer.[49] Significant differences were instead apparent in the behavior of the metal–NONOates, whereas [Ni(PipNONO)Cl] released NO with a rate similar to that of HPipNONO, for [Cu(PipNONO)Cl] and [Ni(SalPipNONO)] the release was much slower. However, for the metal complexes the profile of the absorbance decay curves was more complicated, and the data could not be fitted considering a single process. For the interpretation of the chemistry involved, it should be considered that the kinetic experiments are initiated by diluting into the proper buffer a concentrated solution of the complexes in basic methanol. Thus, the process actually occurring in the first few seconds involves a fast change (with k_{obs} on the order of 0.1 s^{-1}) in the primary coordination sphere of the metal ions, where a hydroxo group from the basic solution is replaced by a water molecule, and this is followed by NO release. This hypothesis is consistent with the absence of significant changes in the spectrum of the complexes, as expected by the similarity in ligand field strength between the hydroxo and water ligands. However, the initial ligand exchange does not interfere with the NO-release process provided the first few seconds (routinely 10 s) of the kinetic experiments are neglected in the data analysis. Under these conditions, NO release by the complexes [M(PipNONO)Cl] follows a simple exponential curve, as shown for [Cu(PipNONO)Cl] in Figure 2.

For [Ni(SalPipNONO)], apart from the fast initial rearrangement of the coordination sphere, the plot of the absorption at 256 nm with time could not be fitted with a single exponential and is consistent with a biphasic, stepwise release of the two NO molecules contained in the piperazine–NONOate residue (Figure 3). The fitting of the plot with a double exponential

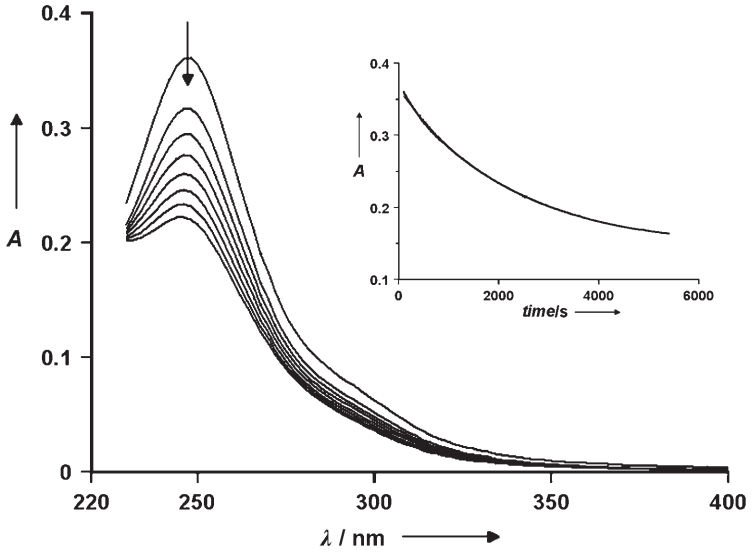


Figure 2. Spectral changes during NO release from [Cu(PipNONO)Cl] in phosphate buffer 50 mM at pH 7.5 and 25 °C. Spectra were recorded every 10 min. In the inset, the fitting of the absorbance data at 252 nm (taken every second) versus time is shown. The first 10 s of reaction were neglected in the fitting.

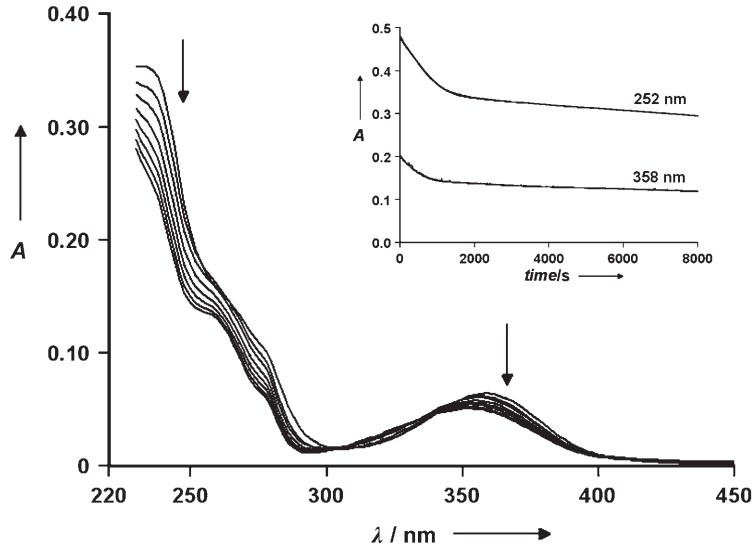
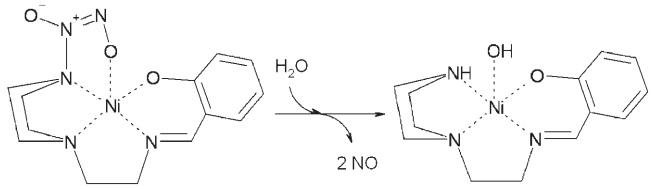


Figure 3. Spectral changes during NO release from [Ni(SalPipNONO)] in phosphate buffer 50 mM at pH 7.5 and 25 °C. Spectra were recorded every 10 min. In the inset, the fitting of absorbance data at 252 and 358 nm (taken every second) versus time is shown.

yielded the rate constants reported in Table 1, that, because of partial overlap between the two processes, are clearly affected by a more significant error than in the previous cases. It is interesting that the release of NO is accompanied by a small hypsochromic shift of the imine band, from 358 to 350 nm. This slight blue shift can be associated with the change in the coordination sphere of the complex occurring upon the loss of the NONOate group. This is shown in Scheme 2, where the global process involved in the reaction is schematically represented.



Scheme 2. Overall process of NO dissociation from the metal–NONOate complex [Ni(SalPipNONO)].

Given the unusual behavior of NO dissociation exhibited by [Ni(SalPipNONO)], we carried out comparative experiments with the complex [Cu(PipNONO)Cl] at pH values of 6.8 and 8.0, to see if such behavior was pH dependent. As expected, in general NO release is faster at pH 6.8 ($1.01\times10^{-2}\,s^{-1}$ for HPipNONO) and slower at pH 8.0 ($1.20\times10^{-3}\,s^{-1}$ for HPipNONO) with respect to pH 7.5, consistent with the hypothesis that the release is promoted by protonation of the nitrogen atom carrying the diazeniumdiolate functionality.[49] However, throughout the investigated pH range, both the biphasic NO release by [Ni(SalPipNONO)] and the monophasic release by [Cu(PipNONO)Cl] were confirmed. We have currently no evidence on the origin of the stepwise transformation of the complex [Ni(SalPipNONO)] upon NO dissociation, and several possibilities exist; for instance, besides a sequential dissociation of two NO molecules from the NONOate complex, the formation of some dinuclear species bonded to one SalPipNONO ligand and one SalPip ligand could be considered. In any case, the mechanistic pathway involved in this process will require separate studies. Regarding the different rate of NO release observed for the [M(PipNONO)Cl] complexes and for [Ni(SalPipNONO)], they can possibly be rationalized by taking into account the different coordination arrangement of the diazeniumdiolate residue in the two types of complexes (Scheme 1). In the complexes of type [M(PipNONO)Cl], the NONOate group binds to the metal ion in an equatorial position, here the slower release exhibited by [Cu(PipNONO)Cl] with respect to [Ni(PipNONO)Cl] likely depends on the stronger binding strength of the Cu^{II} ion with respect to Ni^{II}, as reflected for instance by the higher priority of the former metal ion in the Irving–Williams series.[50] The still slower NO release by [Ni(SalPipNONO)] can be explained by the higher denticity of the salicylaldimine–piperazine ligand, which makes the overall stability of the complex higher. The high lability of NO, which even prevents the possibility to isolate the parent complex [Cu(SalPipNONO)] is consistent with this view because the strong Jahn–Teller effect of the Cu^{II} ion[46] makes the interaction of the ligands in the axial positions extremely weak. Only the complexes [Cu(PipNONO)Cl] and [Ni(SalPipNONO)] were tested in biological assays because of their capacity to strongly reduce the rate of NO release.

Vasorelaxation

The metal–NONOates, but not metal complexes lacking the NO group, elicited concentration dependent relaxation of aorta rings when tested in the concentration range 10 nM–100 μM. The relaxation curves clearly show the relaxant effect exerted by NO donors and indicate their order of potency (Figure 4 A). Both [Cu(PipNONO)Cl] and [Ni(SalPipNONO)] produced 50% relaxation in the nanomolar range, whereas HPipNONO and SNP were effective in the micromolar range. Maximal relaxation of rabbit aorta rings was obtained in the micromolar range (1 to 100 μM). The EC_{50} value (concentration achieving 50% relaxation) for each compound was extrapolated from the relaxation curves and is reported in Table 2. This table also reports the time for achieving maximal relaxation of rings, which was longer for metal–NONOates than for HPipNONO and SNP, suggesting differences in the kinetics of NO release.

The pretreatment of vessel rings with 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), a selective inhibitor of soluble guanylate cyclase, blocked NO donor induced vasorelaxation, demonstrating the involvement of guanylate cyclase pathway (Figure 4 B).

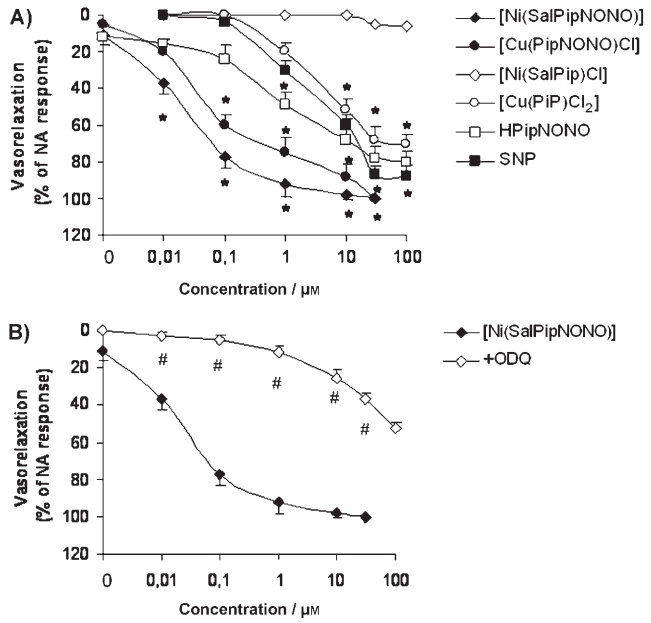


Figure 4. Relaxation of rabbit aortic rings preconstricted with noradrenaline (NA). Increasing concentrations of metal–NONOates and reference compounds were tested on NA-preconstricted vessel rings. A) The effect of metal–NONOates is compared with that of molecules devoid of the NO moiety and SNP. B) The effect of 1 μM ODQ pre-incubation on [Ni(SalPipNONO)]-induced relaxation is shown. Data are reported as percent of NA-induced relaxation (mean ± SEM of at least six rings). *$P < 0.01$ versus NA alone and # versus [Ni(SalPipNONO)] alone (ANOVA test).

Table 2. Pharmacological characterization of metal–NONOates.

Compound	EC_{50} [nM][a]	Relaxation time [min][b]
HPipNONO	1000	8
[Cu(PipNONO)Cl]	50	12
[Ni(SalPipNONO)]	20	12
$[Cu(Pip)Cl_2]$	NE[c]	NE[c]
[Ni(SalPip)Cl]	NE[c]	NE[c]
SNP	3000	7

[a] EC_{50} values calculated on the vasorelaxation response curve obtained with rabbit aorta rings preconstricted with noradrenaline. [b] Relaxation time calculated as the time to reach the plateau of relaxation at the fixed concentration of 1 μM. [c] Not effective up to 100 μM.

Cell proliferation

Preliminary experiments indicate that the metal–NONOates at the concentrations tested (1 nM–1 mM) exhibited neither toxic effects nor changed morphology and viability of cells ($n = 2$, experiments in triplicate). Cell proliferation was stimulated by the metal–NONOates in a concentration dependent manner, to an extent similar to the one elicited by 10 μM SNP and 1 μM NOC-12 (Figure 5 A).

At 10 nM the number of cells increased by 20 to 44% relative to control, the latter value being observed for [Ni(SalPipNONO)], which appeared to exert greater activity. The compounds devoid of the NO moiety were found to be inactive (as shown for [Ni(SalPip)Cl] in Figure 5 B). The proliferative effect exerted by metal–NONOates involved the activation of soluble guanylate cyclase, as it was inhibited by ODQ (10 μM) (Figure 5 B).

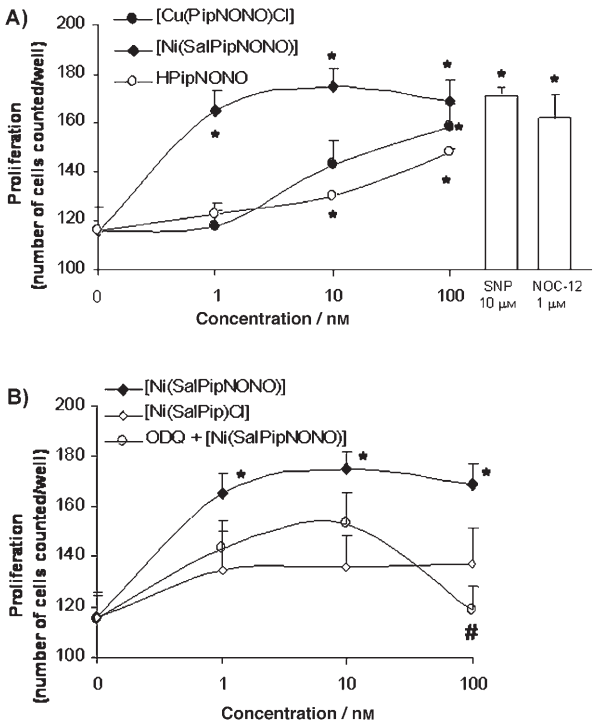


Figure 5. Proliferative effect of metal–NONOates on capillary endothelial cells. Endothelial cell proliferation was measured in synchronized cultures exposed to increasing concentrations of metal–NONOates. A) SNP (10 μM) and NOC-12 (1 μM) were used as control. B) The effect of the piperazine moiety alone and ODQ (10 μM). Data are reported as number of cells counted per well (means ± SEM of four experiments run in triplicate). *$P < 0.01$ versus basal and # versus [(NiSalPipNONO)] alone (ANOVA test).

Conclusions

The metal–NONOates reported in this paper constitute a new family of effective NO-releasing agents. The polydentate nature of the *N*-aminoethylpiperazine ligand conjugated with the *N*-diazeniumdiolate group confers stability to the complexes[50] and in particular contributes to slowing down NO release in solution. In fact, all previously reported metal–NONOates containing simple *N*-dialkylamino diazeniumdiolate ligands, such as $Et_2N(N_2O_2)^-$, rapidly released NO in solution, with an almost negligible effect by the metal ion.[30–33] Although we have no definitive evidence of the structure adopted by the complexes in solution, the slow NO-releasing properties and particularly the biphasic NO release exhibited by [Ni(SalPipNONO)], indicates that a strong interaction of the NONOate group with the metal ion occurs.

The novel metal–NONOates are endowed with interesting pharmacological properties. Complexes [Ni(SalPipNONO)] and [Cu(PipNONO)Cl] clearly emerge as potent compounds, being active in the nanomolar concentration range in all assays performed in this study. The specificity of the aorta ring relaxation effect by [Ni(SalPipNONO)] and [Cu(PipNONO)Cl] is demonstrated by the lack of activity of molecules lacking the NONO

moiety. Inhibition by ODQ of their relaxing effects reinforces the notion that relaxation induced by the above compounds can be attributed to NO release. The relaxation profile exerted by metal–NONOates in aorta rings appears to be longer than that of SNP, indicating a sustained release of NO from the molecules under study. These compounds do not exhibit any toxic effect on cultured cells, indicating a wide safety margin. In addition, [Ni(SalPipNONO)] and [Cu(PipNONO)Cl] exert a significant effect on endothelial cells, promoting proliferation in a concentration related fashion. Also these effects are clearly related to their ability to release NO, as compounds belonging to the same series but without the NONO moiety are not effective. The activity on cell proliferation is completely inhibited by ODQ, suggesting that the intracellular effector mechanism used by these compounds to activate endothelial cells is similar to that of authentic NO, that is, stimulation of cGMP pathway. It is noteworthy that in all comparative assays, the reference agent SNP is 10 to 1000 times less potent than the most effective metal–NONOates. Therefore, metal complexes containing a functionalized piperazineNONOate ligand represent a promising class of NO-releasing agents with potential therapeutic interest.

Experimental Section

Materials and instrumentation. All reagents and solvents used for chemical and biological assays were of the highest purity available. Solutions of methanolic NaOH were prepared by dissolving NaOH pellets in dry methanol. The commercially available NO donors used in the biological assays were sodium nitroprusside (SNP) (form Sigma–Aldrich) and NOC-12 (from Calbiochem). IR and UV/Vis optical spectra were recorded on a Jasco FT-IR-5000 and a HP-8452 A spectrophotometer, respectively. Proton NMR spectra were recorded on a Bruker AVANCE 400 spectrometer. MS spectra were obtained with a LCQ ADV MAX ion trap mass spectrometer equipped with an ESI ion source and controlled by Xcalibur software 1.3 (Thermo-Finnigan, San Jose, CA, USA). ESI experiments were carried out in positive ion mode under the following constant instrumental conditions: source voltage 5.0 kV, capillary voltage 46 V, capillary temperature 150 °C. Note that the capillary temperature was kept relatively low to prevent a fast loss of NO by the samples.

Synthesis of *N*-aminoethylpiperazine-*N*-diazeniumdiolate (HPipNONO). This compound was prepared according to a slight modification of the method reported by Hrabie et al.;[34] the new procedure operates at −40 °C and bears the advantage of using standard glassware and NO gas at atmospheric pressure[39] instead of a reactor under NO pressure.[34] A solution of 1-(2-aminoethyl)piperazine (Pip) (5.0 mL, 38.1 mmol) in acetonitrile (50 mL) was carefully degassed in a Schlenk vessel and cooled to −40 °C in a cryostat. The vessel was then exposed to NO at atmospheric pressure through vacuum/NO cycling, always keeping the solution at low temperature, and the operation was repeated five more times over three days. A white precipitate of the product slowly formed. The precipitate was filtered off anaerobically and washed several times with small amounts of degassed and cooled acetonitrile and then with diethyl ether (yield ~20%). The powder is hygroscopic and was stored in a freezer at −20 °C. Anal. calcd for $C_6H_{15}N_5O_2$: C 38.09, H 7.99, N 37.01, found: C 38.69, H 7.97, N 37.41; ^{1}H NMR (400 MHz, D_2O): $\delta = 2.55$ (t, 2H, CH_2-NH_3^+), 2.65 (broad, 4H, piperazine CH_2-*N*-$NONO^-$), 2.95 (t, 2H, CH_2-piperazine ring), 3.05 ppm (t, 4H, piperazine CH_2-N); IR (Nujol mull): $\tilde{\nu} = 1657$m, 1630 w, 1574 w, 1526m, 1345m, 1325 w, 1278m, 1232m, 1180s, 1155s, 1049m, 1015m, 953s, 884m, 766m, 727m cm^{-1}; UV/Vis λ_{max}, nm (ε, M^{-1}cm^{-1}) in 50 mM phosphate buffer, pH 7.5, containing 5% (*v*/*v*) methanol: 252 (5900).

Synthesis of [Ni(SalPipNONO)]. Salicylaldehyde (22 μL, 2.1×10^{-4} mol) and solid $NiCl_2\cdot6H_2O$ (50 mg, 2.1×10^{-4} mol) was rapidly added to a solution of HPipNONO (40 mg, 2.1×10^{-4} mol) in 3 mL methanolic 0.075 M NaOH. The reaction mixture was stirred at room temperature for several minutes and then diethyl ether was added to allow the formation of a pale-green precipitate, that was filtered off and washed with ether[39] (yield ~70%). The compound was stored at −20 °C prior to use. Anal. calcd for $C_{13}H_{17}N_5O_3Ni\cdot4H_2O$: C 36.99, H 5.97, N 16.59, found: C 37.10, H 5.55, N 17.00; IR (Nujol mull): $\tilde{\nu} = 1650$ s, 1600 m, 1547 w, 1300 m, 1196 m, 1175 w, 1155 w, 1126 m, 1024 w, 996 s, 961 m, 897 s, 766 s, 721 m cm^{-1}; UV/Vis λ_{max}, nm (ε, M^{-1}cm^{-1}) in 50 mM phosphate buffer, pH 7.5, containing 5% (*v*/*v*) methanol: 236 (11 000), 260 (sh, 4700), 276 (sh, 3200), 358 (2000), 600 (sh).

Synthesis of [Cu(PipNONO)Cl]. Solid $CuCl_2\cdot2H_2O$ (36 mg, 2.1×10^{-4} mol) was rapidly added to a solution of HPipNONO (40 mg, 2.1×10^{-4} mol) in 3 mL methanolic 0.075 M NaOH. The precipitate thus formed was collected by filtration after centrifugation of the mixture at room temperature for 10 min and several washings with diethyl ether[39] (yield ~65%). The compound was dried in air and stored at −20 °C prior to use. Anal. calcd for $C_6H_{14}N_5O_2CuCl\cdot5H_2O$: C 19.10, H 6.41, N 18.56, found: C 18.96, H 5.99, N 18.55; IR (Nujol mull): $\tilde{\nu} = 1635$ w, 1576 m, 1314 w, 1287 m, 1251 w, 1169 w, 1120 m, 1086 w, 1069 w, 1035 w, 1016 w, 997 s, 980 w, 922 w, 760 m, 724 w; UV/Vis λ_{max}, nm (ε, M^{-1}cm^{-1}) in 50 mM phosphate buffer, pH 7.5, containing 5% (*v*/*v*) methanol: 252 (9500), 290 sh (2200), 620 (100).

Synthesis of [Ni(PipNONO)Cl]. Solid $NiCl_2\cdot6H_2O$ (62 mg, 2.6×10^{-4} mol) was rapidly added to a solution of HPipNONO (50 mg, 2.6×10^{-4} mol) in 5 mL methanol containing 0.26 mmol NaOH. The solution was stirred for a few minutes and upon addition of diethyl ether a pale-green precipitate formed. This was collected by filtration after centrifugation of the mixture at room temperature for 10 min and several washings with diethyl ether[39] (yield ~70%). The compound was dried in air and stored at −20 °C prior to use. Anal. calcd for $C_6H_{14}N_5O_2NiCl\cdot4H_2O$: C 20.33, H 6.26, N 19.76, found: C 20.29, H 6.18, N 20.22; IR (Nujol mull): $\tilde{\nu} = 1595$ w, 1301 w, 1279 w, 1198 m, 1172 w, 1093 w, 1051 w, 1003 m, 961 s, 918 w, 880 w, 777 w, 721 m cm^{-1}; UV/Vis λ_{max}, nm (ε, M^{-1}cm^{-1}) in 50 mM phosphate buffer, pH 7.5, containing 5% (*v*/*v*) methanol: 252 (9000), 300 sh (1600), 600 (7).

Synthesis of [Ni(SalPip)Cl]. A solution of *N*-aminoethylpiperazine (0.28 mL, 2.13 mmol), salicylaldehyde (0.26 g, 2.13 mmol) and NaOH (85.2 mg, 2.13 mmol) in 20 mL dry ethanol was held at reflux for about 2 h and then rotary evaporated to dryness. The yellow residue was dissolved in methanol (20 mL) and solid $NiCl_2\cdot6H_2O$ (0.51 g, 2.13 mmol) was added under stirring. After addition of diethyl ether (5 mL), the green precipitate was collected by filtration and dried under vacuum (yield ~50%). Anal. calcd for $C_{13}H_{18}N_3ONiCl\cdot H_2O$: C 45.33, H 5.85, N 12.20, found: C 45.12, H 5.97, N 12.43; IR (Nujol mull): 1649 s, 1600 m, 1548 w, 1292 m, 1195 m, 1155 w, 1126 w, 1080 w, 1049 w, 1018 m, 945 w, 897 m, 766 m, 723 w cm^{-1}; UV/Vis λ_{max}, nm (ε, M^{-1}cm^{-1}) in methanol: 282 (2800), 312 (3600), 386 (2500), 660 (80).

Synthesis of [Cu(SalPip)Cl]. This complex was obtained following the same procedure described for [Ni(SalPip)Cl, but using $CuCl_2 \cdot 2H_2O$ (yield ~60%). Anal. calcd for $C_{13}H_{18}N_3OCuCl \cdot H_2O$: C 44.70, H 5.77, N 12.03, found: C 44.82, H 5.85, N 12.13; IR (Nujol mull): $\tilde{\nu} = 1638$ s, 1599 m, 1541 w, 1304 m, 1202 w, 1153 w, 1132 w, 1074 w, 1057 w, 1019 m, 949 w, 905 m, 754 m, 729 w cm^{-1}; UV/Vis λ_{max}, nm (ε, $M^{-1}cm^{-1}$) in methanol: 274 (8500), 310 (3800), 372 (3400), 628 (230).

Synthesis of [Cu(Pip)Cl_2]. A solution of *N*-aminoethylpiperazine (0.28 mL, 2.13 mmol) and $CuCl_2 \cdot 2H_2O$ (0.36 g, 2.13 mmol) in 20 mL methanol was stirred for a few minutes and diethyl ether (5 mL) was added. The blue precipitate was collected by filtration and dried under vacuum (yield ~50%). Anal. calcd for $C_6H_{15}N_3CuCl_2.2H_2O$: C 24.05, H 6.39, N 14.02, found: C 24.26, H 6.68, N 14.25; IR (Nujol mull): $\tilde{\nu} = 1616$ w, 1574 m, 1327 w, 1283 w, 1195 w, 1188 w, 1153 m, 1076 s, 1022 m, 1012 m, 1016 w, 989 m, 835 w, 760 m cm^{-1}; UV/Vis λ_{max}, nm (ε, $M^{-1}cm^{-1}$) in methanol: 276 (3800), 632 (150).

X-ray crystallographic study of [Cu(PipNONO)Cl]$_2$. Blue single crystals of the complex $[Cu(PipNONO)Cl]_2$ were obtained by using the sitting-drop vapor diffusion method, at 4 °C, by mixing equal volumes of a methanolic solution of the complex (1 mg mL^{-1}) containing a stoichiometric amount of NaOH (with respect to the metal–NONOate complex) and 100 mM Tris-HCl buffer, pH 7.5, and equilibrating against a reservoir solution containing the same mixture of solvents. Blue crystals appeared in 1–2 days, reaching an average dimension of $0.5 \times 0.2 \times 0.1$ mm^3. Diffraction data were collected at room temperature on an Enraf Nonius CAD4 diffractometer at the highest resolution of 0.8 Å. The monoclinic crystals belong to the space group *P*21/*n* and have the following cell parameters: $a = 10.31$ Å, $b = 9.44$ Å, $c = 13.77$ Å, $\alpha = 90.00°$, $\beta = 100.49°$, $\gamma = 90.00°$. The structure was solved by direct methods using the program SIR-97[51] and refinement was carried out with SHELXL-97.[52] Calculations were performed with the WinGX package.[53] All non-hydrogen atoms were refined with anisotropic temperature factors.

CCDC 681810 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Measurement of NO released. The amount of NO released by HPipNONO and the metal–NONOate complexes was determined using a method previously described,[54] according to which the released NO is trapped by formation of the stable complex [Fe(EDTA)NO]. The experiment required strictly anaerobic conditions to prevent Fe^{II} oxidation. A degassed 10 mM solution of [Fe(EDTA)] in 10 mM citrate buffer pH 5.0 (2.0 mL) was introduced into an optical cell of 1 cm path length equipped with Schlenk connections. Then, a degassed 1 mM solution of the NO donor (0.5 mL) prepared in methanol and containing an equimolar amount of triethylamine was added by syringe, while maintaining an argon atmosphere in the optical cell. The characteristic absorption band at 432 nm (ε, 780 $M^{-1}cm^{-1}$) of [Fe(EDTA)NO] gradually develops and the absorbance was recorded when the band achieved the maximum intensity.

Kinetics of NO release. The kinetic experiments of NO release were performed in aqueous 0.05 M phosphate buffer, at various pH values, containing a small amount of methanol (about 2% *v/v*), necessary to prepare the mother solutions of the complexes, at 25.0 ± 0.1 °C. Typically, a mother solution was prepared by dissolving 5–10 mg of the metal complex in 5 mL dry methanol containing an equimolar amount of dry triethylamine or NaOH. When kept cold, this solution was sufficiently stable to enable handling of the complex prior to the kinetic experiment. The kinetics were started by adding 50 μL of the mother solution of the metal complex to 2.45 mL aqueous buffer in a thermostatic and magnetically stirred optical cell of 1 cm path length. The progress of the reaction was followed by monitoring the spectral variations with time near 250 nm, where the *N*-diazeniumdiolate residue absorbs, and in the case of the Schiff base complex also near 360 nm, where the metal-bound *N*-salicylideneimino chromophore absorbs. The reactions were followed for several hours, with readings every second. The rate constants were obtained by using the program FigureP, version 2.2a.

Storage and solubilization of metal–NONOates. For biological assays, the compounds were dissolved in DMSO at 10 mM concentration. Further dilution in buffers was made immediately before testing. Compounds were stored at −20 °C as powders or in DMSO solution.

Aorta vessel ring preparations. Experiments have been performed in accordance with the guidelines of the European Economic Community for animal care and welfare (EEC Law No. 86/609). The thoracic segment of the aorta was obtained from male New Zealand rabbits (2.5–3.0 kg), cut into rings of 3–4 mm width (4–6 rings for each aorta), and bathed in Krebs solution as described before.[55] After 60–90 min equilibration, concentration-response curves for noradrenaline (NA) (0.1–1 mM) were performed to determine the concentration able to induce 50% of the maximum effect. The relaxant effects of the NO-releasing agents were tested in the preparation preconstricted by this concentration of the amine. The value of tension developed was taken as 100% and the effects of the agents were referred to this value. The effects of the metal–NONOates were tested 10 min after the contractile response to NA was fully developed. After the concentration-relaxant curve for SNP (range: from 10 nM to 100 μM), considered as the reference NO donor, was obtained, the preparation was washed, and a second concentration-relaxant effect curve to the agents under study was obtained in the same preparation preconstricted by NA. The metal–NONOates explored (10 nM to 100 μM concentration range) were added in a cumulative fashion. As the compounds under study are direct NO-releasing agents, rabbit aortic rings without endothelium were used for this study. Endothelium was removed by carefully scraping the intimal surface by polyethylene tubing, and testing its presence through the relaxant response to 1 mM acetylcholine.

As vasodilation exerted by NO-releasing agents is sensitive to blockade of cGMP production, the intracellular effector molecule in smooth muscle cells, an inhibitor of soluble guanylate cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μM),[37] was employed to assess the direct effect of these compounds.

Cell culture conditions. Coronary venular endothelial cells (CVEC) were obtained and cultured as described.[56] Cells between passages 15 and 25 were used.

Cell toxicity. Endothelial cells (200 000 cells/mL) were suspended in medium with 0.1% bovine calf serum (BCS) and metal–NONOates (1 nM to 1 μM) and incubated at 37 °C for 4 h. Trypan blue exclusion was used to determine cell death. Toxicity was also determined in adherent cells incubated with metal–NONOates for 48 h. Cells were then stained and fixed and their viability and morphology were evaluated by microscopic examination.

Proliferation studies. Cell proliferation was quantified as total cell number.[37,51] Briefly, 1.5×10^3 cells resuspended in 10% BCS were seeded in each well of 96-well plates. After adherence (3–4 h) the medium was replaced with medium containing test substances (1 to 100 nM range) and incubated for 48 h. Data are reported as total cell number counted well^{-1}. Where indicated, cells were pre-treated 15 min with ODQ (10 μM).

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