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Antinociceptive properties and nitric oxide synthase inhibitory action of new ruthenium complexes

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Abstract

This study evaluates the actions of the new ruthenium complexes *trans*-[$RuCl_2(nic)_4$] (Complex I) and *trans*-[$RuCl_2(i\text{-}nic)_4$] (Complex II) as antinociceptives, and their interaction with nitric oxide isoenzymes and with acetylcholine-induced relaxation of rat and rabbit aorta. Complex II inhibited, in a graded manner, neuronal and inducible nitric oxide (NO) synthase, and was about two fold more effective in inhibiting the neuronal NO synthase than the inducible form of the enzyme. Complex I was inactive. Both complexes failed to interfere with constitutive endothelial nitric oxide synthase because they did not change the mean arterial blood pressure of rats. The vasorelaxant effect of acetylcholine was markedly antagonised by the Complexes I and II in rings of both rat and rabbit aorta. Complexes I and II, given intraperitoneally, like N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{G}-nitro-L-arginine (L-NOARG), inhibited, in a graded manner, both phases of the pain response induced by formalin. The actions of L-NAME, L-NOARG and Complex II, but not that of Complex I, were largely reversed by L-arginine. Both complexes failed to affect the motor response of animals in the rota-rod test and had no effect in the hot-plate assay. Together, these findings provide indications that the new ruthenium complexes, especially Complex II and its derivatives, might be of potential therapeutic benefit in the management of pain disorders.

Keywords: Ruthenium complex; Antinociception; Formalin test; Hot-plate test; Rota-rod test; Blood pressure; Nitric oxide (NO); Nitric oxide (NO) synthase; Pain

1. Introduction

The ruthenium complexes are of great interest to chemists, because the properties of these compounds can be altered by the choice of the ligand, which means that complexes potentially have multiple applications. It has been shown that water-soluble ruthenium complexes are of potential therapeutic value because of their ability to interfere with the nitric oxide (NO) pathway in biological systems (Abrams, 1996; Fricker et al., 1997). In addition, Heijden et al. (1993) observed that ruthenium complexes derived from imidazole bind covalently to molecules such as histidine and DNA.

The involvement of NO in pathological states has provided a new focus for pharmaceutical research. NO is an important second messenger which has the ability to control the environment where it is produced. The physiological effects of NO are attributed to its small size, large diffusion potential, and potent action as a free radical. This molecule participates in numerous events of signal transduction in a individual cell, as well as from one cell to another (for review, see Kerwin et al., 1995; Quinn et al., 1995; Christopherson and Bredt, 1997). It is also known that NO regulates both metabolism and muscle contractility (Nakane et al., 1993; Kobzik et al., 1994).

NO synthase is the enzyme responsible for the synthesis of NO from the nitrogen of the guanidine group of L-arginine and from molecular oxygen. Three isoforms of nitric oxide synthase have been described, which are encoded by a unique gene (Sessa et al., 1993). Two are

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constitutive, neuronal and endothelial, and one is inducible, restricted to pathological states (Marletta, 1994).

In this field, the traditional aim of researchers has been to discover specific inhibitors of the isoenzymes of nitric oxide synthase. Up to the present, non-specific nitric oxide inhibitors have been found: analogues of L-arginine (Fukuto and Chaudhuri, 1995) and other compounds such as aminoguanidine, which displays some degree of selectivity for inducible NO synthase rather than for constitutive NO synthase (Misko et al., 1993), and isothioureas, which display a certain selectivity of action for the nitric oxide synthase isoforms (Southan et al., 1995). While this study was in progress, Fricker et al. (1997) showed the involvement of some ruthenium complexes in the NO pathway.

The aims of the present study were to provide an in vivo and in vitro pharmacological and biochemical characterisation of two new ruthenium complexes, *trans*-$[RuCl_2(nic)_4]$ (Complex I) and *trans*-$[RuCl_2(i\text{-}nic)_4]$ (Complex II), in relation to their ability to produce antinociception in the formalin-induced pain test. We also investigated their direct and indirect inhibition of the three isoforms of NO synthase.

2. Materials and methods

2.1. Animals

Male Swiss mice (25–35 g), male Wistar rats (200–300 g) or New Zealand rabbits (3.0–3.5 kg), housed at 22 ± 2°C under a 12-h light/12-h dark cycle and with food and water ad libitum, were used. Experiments were performed during the light phase of the cycle. The animals were allowed to adapt to the laboratory for at least 2 h before testing and were only used once. Experiments reported in this study were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983).

2.2. In vivo studies

2.2.1. Formalin test

As described previously (Vaz et al., 1996; Beirith et al., 1998), 20 μl of 2.5% formalin solution (0.92% of formaldehyde), made up in phosphate buffer solution (PBS; concentration: NaCl 137 mM, KCl 2.7 mM and phosphate buffer 10 mM), was injected subcutaneously (s.c.) under the surface of the right hindpaw. Two mice (control and treated) were observed simultaneously from 0 to 30 min following formalin injection. The early phase of the formalin-induced nociceptive response normally peaked 0 to 5 min after formalin injection and the late phase 15 to 30 min after formalin injection, representing the neurogenic and inflammatory pain responses, respectively. Animals were treated intraperitoneally (i.p.) with Complex I (45.2–180.7 μmol/kg) or Complex II (0.08–13.6 μmol/kg) 30 min before the formalin injection. Following injection of formalin, the animals were immediately placed in a glass cylinder 20 cm in diameter, and the time they spent licking the injected paw was measured with a chronometer as an indicator of pain. Control animals received, i.p., a similar volume of vehicle (0.9% of NaCl solution with 5% of Tween 80).

The paw oedema associated with the latter phase of the formalin test was measured by comparing the difference in weight of the formalin-treated paw and the weight of the control paw (treated with vehicle). For this purpose, animals were killed 30 min after formalin injection by cervical dislocation, and the paw was cut at the knee joint and weighed on an analytical balance.

To assess the time course of the antinociceptive effect of the complexes, different groups of animals were pretreated with Complex I (135.5 μmol/kg) or Complex II (4.8 μmol/kg) 0.5 to 10 h before intraplantar formalin injection.

In order to compare the antinociceptive potency of the ruthenium complexes with that of known NO synthase inhibitors, other groups of mice were treated with N^G-nitro-L-arginine (L-NOARG, 136.9–684.3 μmol/kg, i.p.) or with N^ω-nitro-L-arginine methyl ester (L-NAME, 37.1–444.9 μmol/kg, i.p.) 30 min prior to formalin injection into the hindpaw.

In order to investigate whether the antinociceptive activity of the complexes against formalin-induced pain was associated with an interaction with the nitric oxide-L-arginine pathway, animals were pretreated with L-arginine (3.4 mmol/kg, i.p.), and after 15 min they received Complex I (90.4 μmol/kg, i.p.), Complex II (4.5 μmol/kg, i.p.), L-NOARG (342.2 μmol/kg, i.p.), L-NAME (278.1 μmol/kg, i.p.) or vehicle (0.9% NaCl solution with 5% of Tween 80), as reported previously (Vaz et al., 1996). The nociception evoked in the first and the second phases of the formalin test was recorded 30 min after the treatments. Other groups of animals received only Complex I, Complex II, L-NOARG, L-NAME, L-arginine or vehicle 30 min before formalin injection and were used as controls.

2.2.2. Hot-plate test

The hot-plate test was used to measure the response latency according to the method described previously by Eddy and Leimbach (1953), with minor modifications. The hot-plate (Ugo Basile, model-DS 37) was maintained at 56 ± 1°C. Animals were placed in a 24-cm diameter glass cylinder on the heated surface, and the time between placement and shaking or licking of the paws or jumping was recorded as the response latency. An automatic 30 s cut-off was used to prevent tissue damage. Each animal was tested before administration of drugs in order to obtain the baseline. Then the response latency of control animals (0.9% NaCl solution with 5% of Tween 80) or mice

pretreated with Complex I (180.7 μmol/kg i.p.) or Complex II (4.5 μmol/kg i.p.) 30 min earlier, was measured.

2.2.3. Measurement of motor performance

In order to evaluate possible non-specific muscle relaxant or sedative effects of the ruthenium complexes, mice were tested on the rota-rod (Rosland et al., 1990). The apparatus consists of a bar with a diameter of 2.5 cm, subdivided into six compartments by disks 25 cm in diameter (Ugo Basile, Model 7600). The bar rotated at a constant speed of 22 revolutions per minute. The animals were selected 24 h previously in order to eliminate those mice that did not remain on the bar for two consecutive periods of 60 s. Animals were treated with Complex I (180.7 μmol/kg i.p.), Complex II (4.5 μmol/kg i.p.) or with the same volume of vehicle (NaCl 0.9% solution with 5% of Tween 80) 30 min before being tested. The results are expressed as the time (s) that the animals remained on the rota-rod. The cut-off time used was 60 s.

2.2.4. Mean arterial blood pressure

Male adult rats were anaesthetised with ketamine/xylazine (100/15 mg/kg, i.m.). The trachea was cannulated to permit spontaneous breathing. Polyethylene catheters (PE 20 tubing) were inserted into the right carotid artery for recording of blood pressure. The mean arterial blood pressure was recorded by use a Digi-Med Blood Pressure Analyser (Model 190, NY, USA) connected to a PC computer in which Digi-Med System Integrator (Model 200) software was used (Gratton et al., 1995). Complex I (180.7 μmol/kg, i.p.), Complex II (13.6 μmol/kg, i.p.) or vehicle (NaCl 0.9% solution with 5% of Tween 80) was administered by the i.p. route and the change in blood pressure was recorded for up to 2.5 h.

2.3. In vitro studies

2.3.1. Rabbit and rat aorta

Male rats and rabbits of both sexes were anaesthetised and killed by cervical dislocation. The aorta were isolated and rings of 2–3 mm in length with an intact endothelium were placed over two steel stirrups and mounted in 5-ml double-jacketed organ baths containing Kreb's solution (mM composition: NaCl 118; KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, $MgSO_4$ 1.2 and glucose 11) pH 7.2 at 37°C bubbled with 95% O_2 and 5% of CO_2, as described previously (Calixto and Medeiros, 1992). The isometric tension was recorded by means of an F-60 force transducer (Narco Biosystem) under a basal tension of 1 g (rat aorta) or 2 g (rabbit aorta). Preparations were allowed to equilibrate for at least 60 min before drug addition. Preparations were contracted by phenylephrine (1 μM), and after the tonic contraction became stable, endothelium-dependent relaxations caused by cumulative addition of acetylcholine (1 nM–10 μM) were obtained in the absence (NaCl 0.9% solution with 5% of Tween 80) and in the presence of Complex I (100 μM) and Complex II (100 μM), added 30 min earlier.

2.3.2. Preparation of macrophages and induction of NO synthase

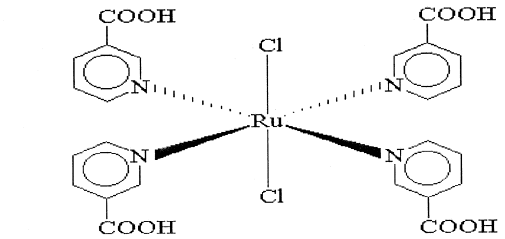
Peritoneal macrophages were harvested from mice that had been injected intraperitoneally 3 to 4 days previously with 2 ml of sterile thioglycollate solution (3% w/v in water). To obtain inducible NO synthase, cells were seeded (5×10^7) in flasks (75 cm^2) in 20 ml RPMI medium (cell culture media, supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, 1 mM glutamine, 20 mM hydroxyethylpiperazine ethanosulfonic acid and 10% fetal calf serum). After 2 h at 37°C, 5% CO_2, non-adherent cells were removed by washing. Fresh medium was added together with *Escherichia coli* endotoxin (LPS, serotype *026:B6*) 100 ng/ml and interferon-γ 20 U/ml, and cells were further incubated for another 8–10 h. The supernatant was assayed for nitrite to determine whether the cells were activated. Cells were scraped off with a rubber policeman, centrifuged at $250 \times g$ for 10 min, the supernatant was discarded, and the pellet was resuspended in 1–2 ml homogenisation buffer (1:5 w/v) containing 50 mM Tris–HCl, pH 7.4, 1 mM ethylenediamine tetraacetic acid, 1 mM dithiothreitol, 10 mg/ml soy bean trypsin inhibitor, 2 mg/ml aprotinin and 320 mM sucrose at 4°C, divided into 200 μl aliquots and frozen at −70°C. When needed, an aliquot was thawed and cells were disrupted by sonication and centrifuged at $10{,}000 \times g$ for 10 min at 4°C. The supernatant was used as a source of inducible NO synthase.

2.3.3. Neuronal constitutive nitric oxide preparation

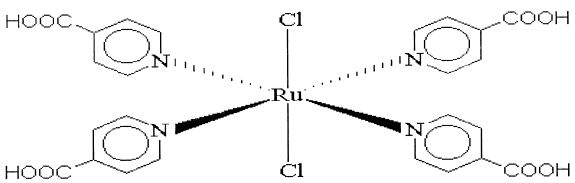
The procedure used was described previously by Knowles et al. (1990). The cerebella of five rats were homogenised with a tissue homogeniser, in a buffer (1:5 w/v) containing 50 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 10 mg/ml soy bean trypsin inhibitor, 2 mg/ml aprotinin and 320 mM sucrose at 4°C. The homogenate was centrifuged at 4°C at $10{,}000 \times g$ for 20 min and the supernatant was divided and frozen at −70°C. Constitutive neuronal NO synthase activity persisted for several weeks after preparation. Protein concentration was determined, in both preparations of NO synthase, by the Lowry method (Lowry et al., 1951).

2.3.4. Inducible and neuronal NO synthase activity assay

NO synthase activity was measured as the ability of tissue homogenates to convert L-[^{3}H]arginine to L-[^{3}H]citrulline. The procedure used was originally described by Bredt and Snyder (1990) and modified by Wolff and Datto (1992). The standard reaction medium contained 50 mM KH_2PO_4, pH 7.2, 1.2 mM $MgCl_2$, 0.25 mM $CaCl_2$, 60 mM L-valine, 1.2 mM L-citrulline, 2.5 μM L-[^{3}H]arginine (1.6 μCi/ml) (not considering the endoge-



(A)



(B)

Fig. 1. Chemical structures of *trans*-[$RuCl_2(nic)_4$] (Complex I) (A) and *trans*-[$RuCl_2$(i-nic)$_4$] (Complex II) (B).

nous concentration of L-arginine), 1 mM dithiothreitol, 4 μM flavine adenine dinucleotide, 4 μM flavine mononucleotide, 10 μM tetrahydrobiopterin and 120 μM nicotinamide adenine dinucleotide phosphate (reduced form), in the presence or in the absence of the compounds. The reactions were started by the addition of enzyme preparation (1.3 mg/ml); no pre-incubation was necessary. After 1 h of incubation at room temperature, the reactions were stopped by the addition of the weak cationic exchange resin AG 50W-X8 (10 g/100 ml in water). The samples were mixed and centrifuged at 10,000 × *g* for 5 min and aliquots of the supernatant were mixed with scintillation liquid and counted. Constitutive neuronal NO synthase activity was obtained by subtraction of Ca^{2+}-independent activity (measured in the presence of 1 mM ethylene glycol tetraacetic acid).

2.4. Drugs

The majority of the substances used in this study were purchased from Sigma (St. Louis, MO, USA), except the salts, formalin and sucrose, which were purchased from Merck, Darmstadt, Germany, and L-[^{3}H]arginine, which was purchased from Amersham International, UK. The complexes *trans*-[$RuCl_2(nic)_4$] (Complex I) and *trans*-[$RuCl_2$(i-nic)$_4$] (Complex II) were synthesised by the group of Dr. Cesar Franco at the Chemistry Department of the Federal University of Santa Catarina (Franco et al., 1998) (see their structures in Fig. 1). Drugs were prepared just before use in 0.9% w/v of NaCl solution, except for the in vivo experiments where the complexes were prepared in Tween 80. The final concentration of Tween did not exceed 5% and did not cause any effect per se. In the neurochemical and in vivo studies, drugs were dissolved in deionized water before testing.

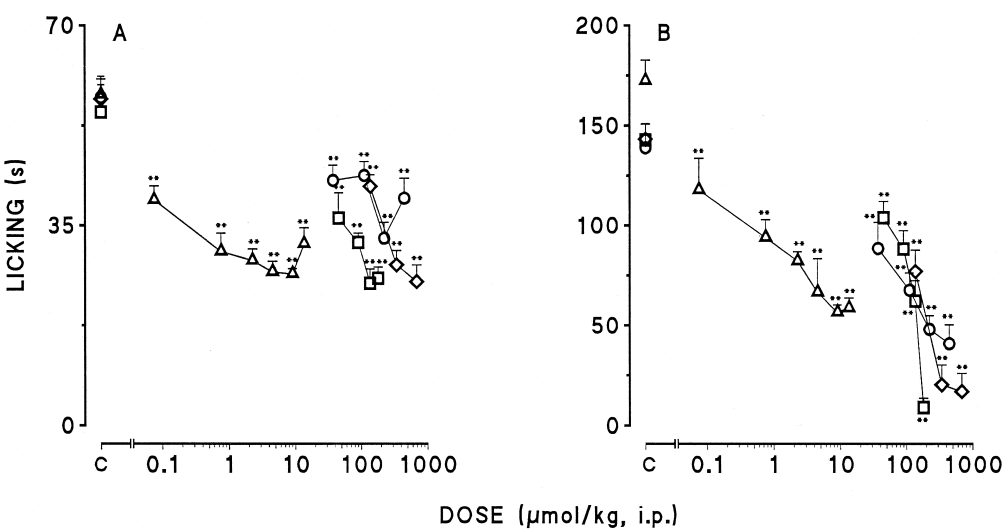


Fig. 2. Effects of Complex I (□, 45.2–180.7 μmol/kg), Complex II (△, 0.08–13.6 μmol/kg), L-NOARG (◇, 136.9–684.3 μmol/kg) or L-NAME (○, 37.1–444.9 μmol/kg) given intraperitoneally 30 min earlier, against the first (A) and the second (B) phase of the formalin-induced pain response in mice. The total time (means ± S.E.M.) spent licking the hindpaw was measured in the early (0–5 min) and the second phase (15–30 min) after subcutaneous injection of formalin into the hindpaw. Each point represents the mean ± S.E.M. for 5–10 animals. The asterisks denote the significance levels, when compared with control groups. Significantly different from controls, $^{*}P < 0.05$ and $^{**}P < 0.01$. In some cases, the S.E.M. are hidden within the symbols.

Table 1
The mean ID_{50}s and maximum inhibition values for the antinociceptive action of Complexes I and II, L-NAME and L-NOARG against the formalin-induced pain response in mice

Compound	Formalin			
	Early phase		Late phase	
	ID_{50}^{a}	MI (%)[b]	ID_{50}^{a}	MI (%)[b]
Complex I (μmol/kg)	121.3 (97.3–151.1)	53 ± 4	90.1 (71.2–114.1)	94 ± 3
Complex II (μmol/kg)	2.8 (2.0–3.8)	54 ± 2	1.5 (0.6–3.7)	67 ± 2
L-NOARG (μmol/kg)	441.3 (348.8–558.4)	56 ± 5	279.4 (157.9–494.4)	88 ± 6
L-NAME (μmol/kg)	> 449.9	43 ± 5	116.0 (81.5–165.2)	71 ± 7

Each group represents the mean from 6 to 10 animals.
[a] ID_{50}s with their respective 95% confidence limits.
[b] Maximal inhibitions.

2.5. Statistical analysis

The results are presented as means ± S.E.M., except the ID_{50} or IC_{50} values (i.e., the dose or concentration of drugs reducing the responses by 50% relative to control value) which are reported as geometric means accompanied by their respective 95% confidence limits. The statistical significance of differences between groups was evalu-

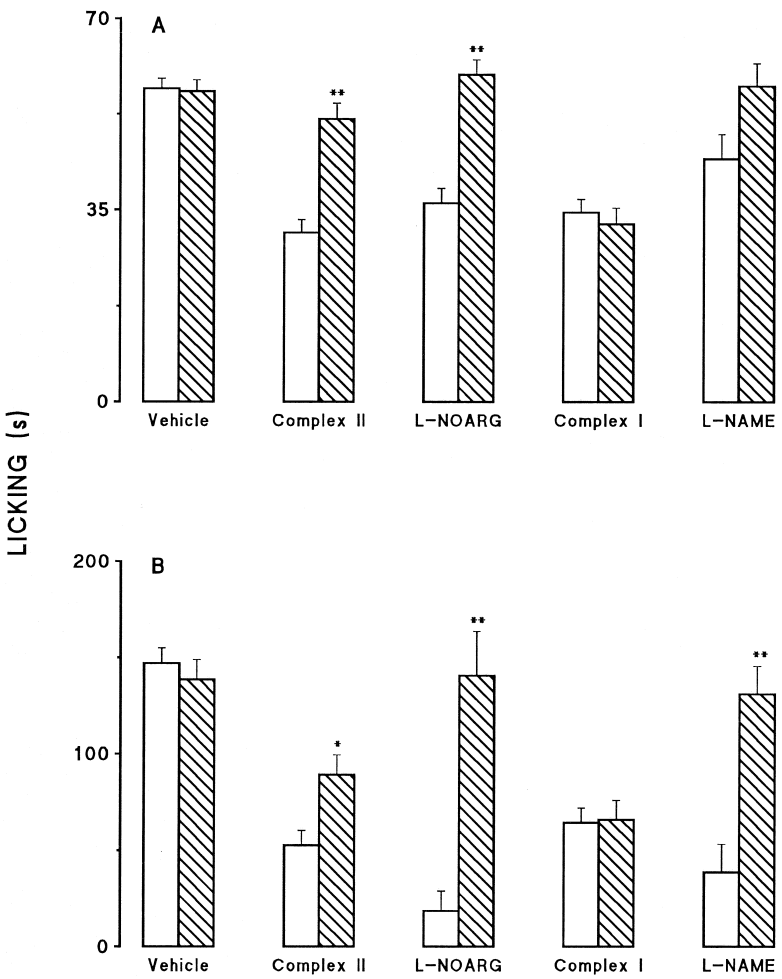


Fig. 3. Effects of pretreatment of animals with L-arginine (3.4 mmol/kg, i.p.) 20 min before injection of Complex I (90.4 μmol/kg, i.p.), Complex II (4.5 μmol/kg, i.p.), L-NOARG (342.2 μmol/kg, i.p.) or L-NAME (278.1 μmol/kg, i.p.) against the first (A) and the second phase (B) of the formalin-induced pain response in mice. The total time (means ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min) and the second phase (15–30 min) after subcutaneous injection of formalin into the hindpaw. Each column represents the mean for 6–8 animals and the vertical lines indicate the S.E.M. The open columns represent the control values and the hatched columns represent the treatment with L-arginine. The asterisks denote the significance levels, when compared with control groups. Significantly different from controls, $^{**}P < 0.01$ and $^{*}P < 0.05$.

ated by means of analysis of variance followed by Dunnett's multiple comparison test or by Newmann–Keuls' test when appropriate. *P*-values less than 0.05 ($P < 0.05$) were considered as indicative of significance. For the inducible and neuronal NO synthase activity assay, the $K_{0.5}$ values were determined by graphical interpolation of data from individual experiments.

3. Results

3.1. In vivo studies

3.1.1. Formalin test

The results of Fig. 2 show that Complex I (45.2–180.7 μmol/kg, i.p.) and Complex II (0.08–13.6 μmol/kg, i.p.), like L-NOARG (136.9–684.3 μmol/kg, i.p.) and L-NAME (37.1–444.9 μmol/kg, i.p.), caused a significant, dose-related inhibition of both phases of the formalin-induced response. The calculated mean ID_{50} (and 95% confidence limits) values (μmol/kg) and the maximal inhibition of these effects are shown in Table 1. Otherwise, none of the treatments had a significant effect on the paw oedema associated with the inflammatory phase of the formalin test (results not shown). The time-course experiments carried out during the first phase of the formalin-induced response revealed that both compounds had a relatively long-lasting antinociceptive effect. The percent inhibition (means ± S.E.M.) for the first phase of the formalin response caused by Complex I (135.5 μmol/kg, i.p.) and Complex II (4.8 μmol/kg, i.p.) was: 50 ± 4, 46 ± 4, 44 ± 5, 40 ± 3, 29 ± 4, 23 ± 3, 0 and 48 ± 5, 48 ± 4, 48 ± 6, 35 ± 6, 24 ± 4, 20 ± 6, 8 ± 8 at 0.5, 1, 2, 4, 6, 8 and 10 h, respectively ($N = 6$–8 in each group).

The antinociception caused by the i.p. injection of L-NOARG (342.2 μmol/kg), L-NAME (278.1 μmol/kg, i.p.) and Complex II (4.5 μmol/kg) given 30 min prior to testing, but not that of Complex I, (90.4 μmol/kg), was almost completely reversed by L-arginine (3.4 mmol/kg, i.p.) given 15 min prior to drug injection (Fig. 3).

3.1.2. Hot-plate test

Neither of the complexes, Complex I (180.7 μmol/kg) and Complex II (4.5 μmol/kg), given i.p. 30 min beforehand, increased the response latency in the hot-plate assay (results not shown).

3.1.3. Measurement of motor performance

Neither of the complexes, Complex I (180.7 μmol/kg) and Complex II (4.5 μmol/kg), given i.p. 30 min beforehand, significantly affected the motor response of animals when assessed in the rota-rod test (results not shown).

3.1.4. Mean arterial blood pressure

The mean arterial blood pressure did not change significantly during the experiment (2.5 h) in animals treated with Complex I (180.7 μmol/kg, i.p.) or with Complex II (13.6 μmol/kg, i.p.) when compared with that of non-treated animals (NaCl 0.9% solution plus 5% of Tween 80, i.p.) ($n = 4$ per group, results not shown).

3.2. In vitro studies

3.2.1. Inducible and neuronal NO synthase activity assay

The effects of Complex I and Complex II on the activity of neuronal and inducible NO synthase was investigated, because the experiments in vivo suggested that these compounds produced antinociception by a mechanism involving the NO pathway. The results of Fig. 4 show the effect of Complex II and Complex I (inset) on the activity of neuronal and inducible NO synthase. Only Complex II produced a significant inhibition of the activity of both enzymes, being about two-fold more selective for neuronal NO synthase ($K_{0.5} = 23.2 \pm 1.4$ μM) than for inducible NO synthase ($K_{0.5} = 54.0 \pm 2.1$ μM).

3.2.2. Rabbit and rat aorta

Cumulative addition of acetylcholine (1 nM to 10 μM) to the bath produced endothelium-dependent and complete relaxation of both rat and rabbit aortic rings precontracted with phenylephrine (1 μM) with a mean IC_{50} of 16 nM and 0.17 μM, respectively. The vasorelaxant effect of acetylcholine was markedly antagonised by both Complex I (100 μM) and Complex II (100 μM) in aortic rings of

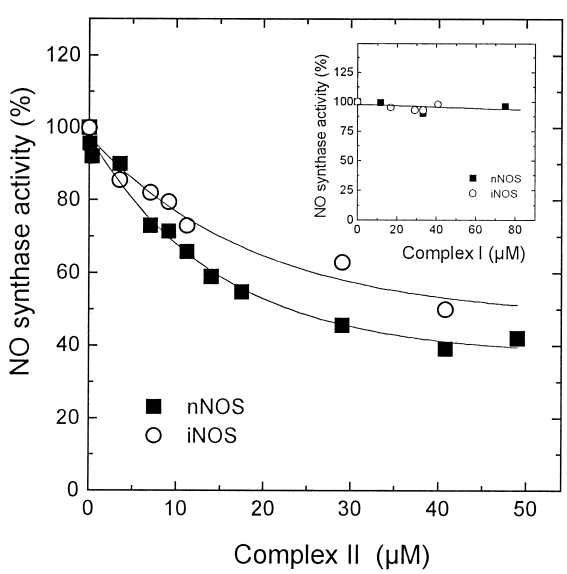


Fig. 4. Effect of Complex II on nitric oxide synthase activity. The enzymes were isolated and the activities were measured by the L-[^{3}H]citrulline method as described in Section 2 (nNOS and iNOS = neuronal and inducible nitric oxide synthase, respectively). For neuronal NO synthase, $K_{0.5} = 23.2 \pm 1.4$, and for inducible NO synthase, $K_{0.5} = 54.0 \pm 2.1$ ($n = 4$). Inset shows the effect of Complex I on NO synthase activity. Results are reported as percentage of the maximum activity (100% is 0.5 ± 0.02 pmol/mg/min for neuronal NO synthase and 5.0 ± 0.05 pmol/mg/min for inducible NO synthase ($n = 4$).

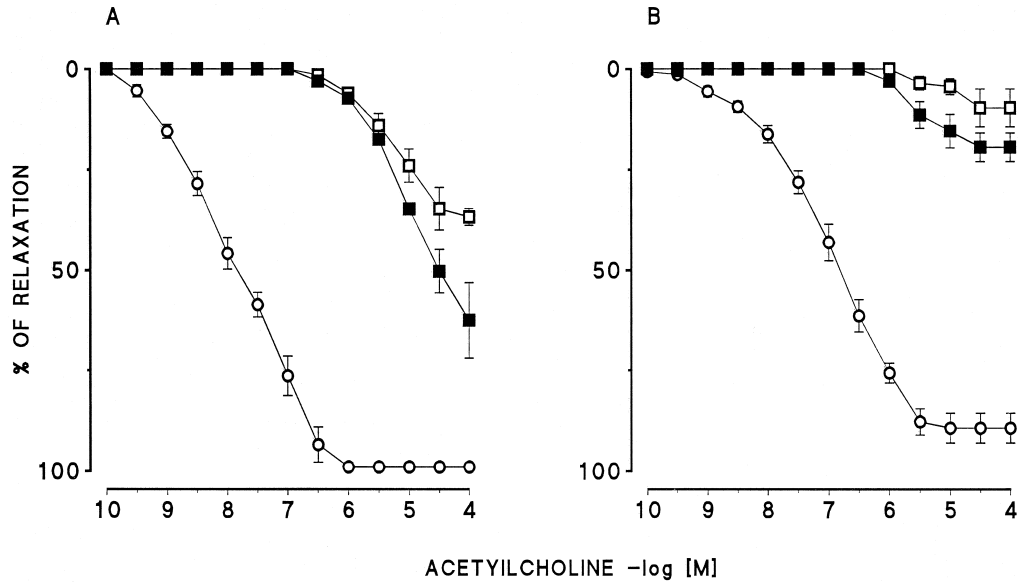


Fig. 5. Effect of Complex I and Complex II on endothelium-dependent relaxation in rat and rabbit aortic rings caused by acetylcholine. Mean vasorelaxant concentration–response curves for acetylcholine (1 nM to 10 μM) in rings of rat (A) and rabbit (B) aortic artery precontracted with phenylephrine (1 μM) in the absence (○) and in the presence of Complex I (□, 100 μM) and Complex II (■, 100 μM). Each point represents the mean of four experiments and the vertical lines indicate the S.E.M. In some cases, the S.E.M. are hidden within the symbols.

rats ($63 \pm 2\%$ and $36 \pm 9\%$) and rabbits ($89 \pm 5\%$ and $79 \pm 4\%$) (Fig. 5).

4. Discussion

There is considerable evidence that the L-arginine nitric oxide pathway plays a pivotal modulatory role in pain transmission, both in the central and in the peripheral nervous systems (Moore et al., 1991; Meller and Gebhart, 1993). It is also reported that L-arginine, a precursor of NO synthesis given systemically, reduces morphine-induced antinociception in both chemical and thermal models of nociception (Brignola et al., 1994). The results presented in this study demonstrate that the new ruthenium complexes, especially the Complex II administered by i.p. route at a dose where no side effects were detectable, elicited a significant and dose-dependent antinociception in mice when assessed against both the neurogenic (first phase) and the inflammatory (late phase) component of the formalin-induced response. When compared with the well-known non-selective NO synthase inhibitors such as L-NAME and L-NOARG, Complex II and, to a lesser degree, Complex I were significantly more potent (about 57 to 186 fold) at the ID_{50} level in inhibiting the pain response elicited by formalin (Moore et al., 1991; results of the present study). When compared with more selective neuronal NO synthase inhibitors such as 7-nitro indazole or 1-(2-trifluoromethylphenyl) imidazole (Moore et al., 1993; Handy et al., 1995), Complex II was significantly (about 30 to 57 fold) more active as an antinociceptive agent, despite it being much less active than 7-nitro indazole (about 20-fold, see below), but Complex II had a potency similar to that of 1-(2-trifluoromethylphenyl) imidazole, as an inhibitor of neuronal NO synthase in the rat cerebellum. Furthermore, unlike 7-nitro indazole and 1-(2-trifluoromethylphenyl) imidazole, which were largely inactive in preventing the early phase (neurogenic component) of the formalin-induced response, both ruthenium complexes, especially Complex II, potently prevented the first phase of the formalin-induced response. However, they were less potent in blocking the neurogenic component of the formalin response. Such results confirm previous evidence suggesting that other mediators besides NO are involved in this response. In contrast with the results reported by Moore et al. (1991), both L-NAME and L-NOARG were found to be effective in preventing the first phase of the formalin-induced response. One possible explanation for such discrepant findings is the difference in the methodology used. While in our study we induced the pain response by intraplantar injection of 2.5% formalin, in Moore's study 5% formalin was used. Furthermore, unlike L-NAME, which causes long-lasting antinociception (> 24 h) (Moore et al., 1991), Complex II causes relatively short-lived antinociception (6 h).

The antinociception caused by Complex II, but not Complex I, like the responses elicited by L-NAME and L-NOARG, was almost completely reversed by the NO precursor L-arginine, suggesting that NO is probably involved in its antinociception. NO synthase inhibitors produce a very similar inhibition of nociception produced by formalin, acetic acid-induced writhing and carrageenan-induced thermal hyperalgesia (Moore et al., 1991; Malmberg and Yaksh, 1993; Meller et al., 1994). The antinociception

produced by ruthenium complexes was not associated with central or peripheral non-specific effects such as sedation or motor ataxia, because even at high doses these agents did not affect the performance of animals in the rota-rod test. Unlike L-NAME (Moore et al., 1991), both ruthenium complexes, at the dose at which they produced significant antinociception in the formalin test, failed to produce antinociception when assessed in the thermal assay of pain, the hot-plate test, which suggests that they do not have an analgesic action per se.

To explore further the mechanisms by which ruthenium complexes interfere with NO, we assessed their in vitro effect against constitutive and inducible isoforms of NO synthase from rat cerebella and peritoneal macrophages, respectively. Complex II but not Complex I concentration dependently inhibited both the constitutive ($K_{0.5} = 23.2 \pm 1.4$ μM) and the inducible ($K_{0.5} = 54.0 \pm 2.1$ μM) isoforms of NO synthase. When compared with other NO synthase inhibitors, Complex II was approximately 6- to 93-fold less potent than L-NAME, L-NMMA, 7-NI or TRIM in inhibiting neuronal constitutive NO synthase and about 10- to 180-fold less potent against the inducible isoform of NO synthase (Moore et al., 1991, 1993; Handy et al., 1995). These results provide consistent evidence that the inhibition of neuronal NO synthase by the ruthenium Complex II certainly accounts for its pronounced antinociceptive property. However, other mechanisms seem likely to contribute to their antinoceptive activity, because Complex I, which displays significant antinociception against both phases of the formalin-induced pain response, had no effect on NO synthase activity.

The results of the present study show indirectly that probably neither of the ruthenium complexes interferes with constitutive endothelial NO synthase. This supposition is supported by the observation that these compounds administered by i.p. route had no significant effect on mean arterial blood pressure in anaesthetised rats, under conditions where non-selective NO synthase inhibitors such as L-NAME and L-NOARG produce marked and sustained hypertension (Rees et al., 1990). Thus, it most likely that their antinociception is not related to local or systemic variations in blood flow. When tested in vitro against acetylcholine-mediated endothelium-dependent relaxation in both rat and rabbit aorta rings, at the same range of doses tested against both neuronal and inducible NO synthase, the ruthenium complexes markedly attenuated the acetylcholine-induced relaxation. Whether these drugs, especially Complex I, which failed to affect the activity of inducible and neuronal NO synthase, have additional mechanisms of action, for instance inhibition of free radicals, are currently being investigated in our laboratory.

In summary, we have shown that the new ruthenium complexes, especially Complex II, produce marked antinociception when assessed against the neurogenic and inflammatory phases of the formalin-induced pain response in mice. The antinociceptive action of Complex II, but not Complex I, was significantly reversed by L-arginine, which strongly suggests the involvement of the nitric oxide L-arginine pathway. The in vivo actions of ruthenium complexes are not associated with central non-specific effects such as sedation and ataxia, nor do these agents interfere with endothelial nitric oxide synthase, the data for which were obtained indirectly by measuring the mean arterial blood pressure. Furthermore, Complex II (but not Complex I), produced graded inhibition of neuronal (rat cerebellar) and inducible (peritoneal macrophage) isoforms of NO synthase. When tested against acetylcholine-mediated relaxation in vitro, both complexes attenuated the endothelium-dependent relaxation. Taken together, Complex II or its derivatives might provide new therapeutic candidates of interest for the development of new drugs to be used in the treatment of pain disorders.

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