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Release of NO from a nitrosyl ruthenium complex through oxidation of mitochondrial NADH and effects on mitochondria

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ABSTRACT

Nitrosyl ruthenium complexes are promising NO donor agents with numerous advantages for the biologic applications of NO. We have characterized the NO release from the nitrosyl ruthenium complex $[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$ (**I**) and the reactive oxygen/nitrogen species (ROS/RNS)-mediated NO actions on isolated rat liver mitochondria. The results indicated that oxidation of mitochondrial NADH promotes NO release from (**I**) in a manner mediated by NO_2 formation (at neutral pH) as in mammalian cells, followed by an oxygen atom transfer mechanism (OAT). The NO released from (**I**) uncoupled mitochondria at low concentrations/incubation times and inhibited the respiratory chain at high concentrations/incubation times. In the presence of ROS generated by mitochondria NO gave rise to peroxynitrite, which, in turn, inhibited the respiratory chain and oxidized membrane protein–thiols to elicit a Ca^{2+}-independent mitochondrial permeability transition; this process was only partially inhibited by cyclosporine-A, almost fully inhibited by the thiol reagent *N*-ethylmaleimide (NEM) and fully inhibited by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). These actions correlated with the release of cytochrome *c* from isolated mitochondria as detected by Western blotting analysis. These events, typically involved in cell necrosis and/or apoptosis denote a potential specific action of (**I**) and analogs against tumor cells via mitochondria-mediated processes.

Introduction

Mitochondria are both sources and targets of nitric oxide (NO), derivative reactive nitrogen species (RNS) such as peroxynitrite and reactive oxygen species (ROS) in general [1,2]. Depending on the concentration and exposure time NO can either promote or inhibit cell death; in the latter case, NO may contribute to carcinogenesis [3,4]. At relatively low concentrations (0.1–100 nM), the NO generated by nitric oxide synthases works as key signal in many cellular mechanisms [2,5,6]; however, at relatively high concentrations (above 1 μM), NO is cytotoxic [5]. Therefore, select concentrations of agents that release NO at specific targets may trigger death of specific cells and present potential applications in cancer therapy [7,8].

Classical NO derivatives such as organic nitrites and nitroprusside still present severe restrictions on therapeutic use due to their photochemical instability and toxicity [9,10]. The object of this study was a ruthenium metal complex that is able to capture NO [11,12] to yield the nitrosyl ruthenium complex $[Ru(NO_2)(bpy)_2(4\text{-pic})]^+$ (**I**). (**I**) belongs to a particular class of NO donor compounds whose members have widely studied structures and chemical/photochemical reactivity [13–16]; these compounds release NO via reduction reactions [17] and light irradiation [16]. However the biologic activities and potential applications of members of this class of NO donors have not been reported.

Mitochondria are able to facilitate the release of NO from nitrosyl ruthenium complexes because they are sources of reducing agents such as NADH [17]. The reducing capacity of mitochondria with respect to this type of compounds, as well as some effects of NO release on these organelles was previously reported [17,18]. Moreover, our preliminary study on the stability of (**I**) revealed the presence of nitrite (NO_2) in the structure of the complex at neutral pH, so we hypothesized that NO release occurs via an oxygen atom transfer mechanism (OAT) [19]. Also in mammalian cells NO_2 is an intermediary which is reduced to NO through a reaction that employs electron-donor systems such as NAD(P)H [20,21]. In this context, we characterized the NO release from (**I**) and its

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ROS/RNS-mediated actions on mitochondria, emphasizing the involvement of the mitochondrial NADH oxidation/OAT mechanism in the NO release and the opening of permeability transition pores (PTPs) in the NO action. PTP opening involves the oxidation of mitochondrial membrane protein–thiols by ROS/RNS and is implicated in the release of cytochrome *c* from mitochondria, which is, in turn, associated with cell death induction.

Materials and methods

*Synthesis of [Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$(**I**)*

The nitrosyl ruthenium complex (**I**) was prepared by dissolving [RuNO(bpy)$_2$(4-pic)](PF$_6$)$_3$, synthesized as previously reported [22], in an aqueous solution at pH 7.4. The aqua-bound specie, *cis*-[Ru(H$_2$O)(bpy)$_2$(4-pic)]$^{2+}$ complex, used as control of the complex (without NO), was synthesized in solution by reducing [RuNO(bpy)$_2$(4-pic)]$^{3+}$ through controlled potential electrolysis at −0.6 V *vs* Ag/AgCl, in 0.01 M phosphate buffer solution pH 7.4 [22]. Complexes were characterized by UV–visible spectroscopy and the results were compared to their previously described properties [22].

Isolation of rat-liver mitochondria

Mitochondria were isolated by standard differential centrifugation [23]. Male Wistar rats weighing approximately 200 g were sacrificed by decapitation, according to research protocols approved by the CEUA-USP Rib. Preto; livers (10–15 g) were immediately removed, sliced in medium (50 ml) consisting of 250 mM sucrose, 1 m Methyleneglycol-bis(β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–KOH, pH 7.2, and homogenized three times for 15 s at 1 min intervals using a Potter–Elvehjem homogenizer. Homogenates were centrifuged (580*g*, 5 min), and the resulting supernatant was further centrifuged (10300*g*, 10 min). The pellets were then suspended in medium (10 ml) consisting of 250 mM sucrose, 0.3 mM EGTA and 10 mM HEPES–KOH, pH 7.2, and centrifuged (3400*g*, 15 min). The final mitochondrial pellet was suspended in medium (1 ml) consisting of 250 mM sucrose and 10 mM HEPES–KOH, pH 7.2, and used within 3 h. Mitochondrial protein contents were determined by the Biuret reaction. For assays, mitochondria were energized with 5 mM potassium succinate (plus 2.5 μM rotenone) in a standard medium consisting of 125 mM sucrose, 65 mM KCl and 10 mM HEPES–KOH, pH 7.4, at 30 °C.

*Direct detection of NO release from (**I**)*

NO release from (**I**) (50 μM) after incubation with succinate-energized mitochondria (1 mg protein/ml) in the standard medium was assessed through a Selective Sensor Electrode (AmiNO 700) in the dark. NO release was also assessed in the absence of mitochondria but in the presence of added β-NADH (1 mM), as well as in the presence of $NaNO_2$ (10 μM).

Assessment of superoxide generated by the xanthine/xanthine oxidase (X/XO) reaction

Superoxide generated by the X/XO reaction was assessed by measuring the absorption at 560 nm due to the nitro blue tetrazolium (NBT) reduction in a reaction mixture containing 10–100 μM (**I**), 80 μM sodium pyrophosphate buffer pH 7.5, 120 μM xanthine (X), 0.1 U/ml xanthine oxidase (XO) and 100 μM NBT [24]. The interaction between the NO released from (**I**) and the superoxide generated by the X/XO reaction was assessed both in the presence of mitochondria and under photo-irradiation of the complex.

Assessment of protein–thiols

Mitochondrial membrane protein–thiols remaining after the oxidation were assessed with 5,5-dithiobis(2-nitrobenzoic)acid (DTNB, Ellman's reagent) as previously described [25]. The mitochondrial suspension was incubated briefly in the standard medium, submitted to three subsequent freeze–thawing procedures to release matrix proteins and then centrifuged for 2 min at 6708*g* in a 5415 C Eppendorf Centrifuge (Eppendorf-5 Prime, Inc., Boulder, CO.). The pellet was treated with 200 μl of 6.5% trichloroacetic acid and centrifuged at 6708*g* for 2 min to precipitate the protein. This procedure was repeated twice. The final pellet was suspended in 1 ml of a medium containing 0.5% sodium dodecyl sulfate, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 M tris(hydroxymethyl)aminomethane (Tris) pH 8.3, and 100 μM DTNB. The absorbance was measured at 412 nm; glutathione was used for calibration.

Mitochondrial assays

Oxygen consumption was monitored polarographically in the standard medium in an oxygraph equipped with a Clark-type oxygen electrode (Hansatech instruments, oxytherm electrode unit, UK). The mitochondrial membrane potential was estimated spectrofluorimetrically using 10 μM safranine *O* as a probe at the 495/586 nm excitation/emission wavelength pair [26]. ROS/RNS production was determined by monitoring the oxidation of dichlorodihydrofluoresceindiacetate (H_2DCFDA, 2 μM) (Molecular Probes, Eugene) at the 503/528 nm excitation/emission wavelength pair [27]. All fluorimetric experiments were conducted in a Model F-4500 Hitachi spectrofluorimeter (Tokyo, Japan). Mitochondrial swelling was estimated from the decrease in apparent absorbance at 540 nm using a Model U-2910 Hitachi spectrophotometer [28].

Western blotting for cytochrome c

Cytochrome *c* released from mitochondria was assessed by Western blotting. After incubation, harvested mitochondria were resuspended in CelLytic™M (Sigma) lysis buffer for 2 min on ice and centrifuged at 10000*g* for 5 min. The resulting supernatants were subjected to 12% SDS–PAGE, transferred to a nitrocellulose membrane and probed with antibodies against cytochrome *c* (BD Pharmigen, purified mouse anti-cytochrome *c*) followed by horseradish peroxidase-coupled detection.

Statistical analysis

Statistical analysis was performed using two-way ANOVA and assuming equality of variance with Bonferroni's post hoc test for pair-wise comparisons. Results with $P < 0.05$ were considered statistically significant.

Results

*Chemical aspects of[Ru(NO$_2$)(bpy)$_2$(4-pic)]$^+$(**I**)*

The NO donor property of (**I**) was previously proposed based on its vasodilator activity [29], and the involvement of OAT [19] in NO release was suggested by our preliminary study on its stability. The stability of nitrosyl ruthenium complexes of the type [RuL(bpy)$_2$(NO)]$^{3+}$ (L = ligand) is pH dependent [30], similar to that

demonstrated for (**I**) (Fig. 1A); the hydroxide electrophilic attack of this type of complex yields $[Ru(NO_2)(bpy)_2L]$ in solutions with pH $\geqslant$ 5.0 [29,30] according to the equation bellow. The spectral profile of $[Ru(NO)(bpy)_2(4\text{-pic})]^{3+}$ in an acidic solution (pH = 1.5) shows strong absorption in 290 nm that has been assigned to the intra ligand transition and a shoulder at 330 nm, indicated by the arrow at Fig. 1A, attributed to the metal–ligand charge transfer band due to transition $d\pi_{\{Ru(II)\}}-\pi^*(NO^+)$. This assignment is in conformity with molecular orbital description for similar nitrosyl ruthenium complexes [29,30]. However, in neutral solutions (pH = 7.4), the spectrum revealed the presence of coordinated nitrite, as indicated by the arrow (b). Therefore, we assumed that at pH 7.4, $[Ru(NO)(bpy)_2(4\text{-pic})]^{3+}$ essentially generates (**I**) and that NO_2^- is involved as an intermediate in NO release from this complex (OAT mechanism).

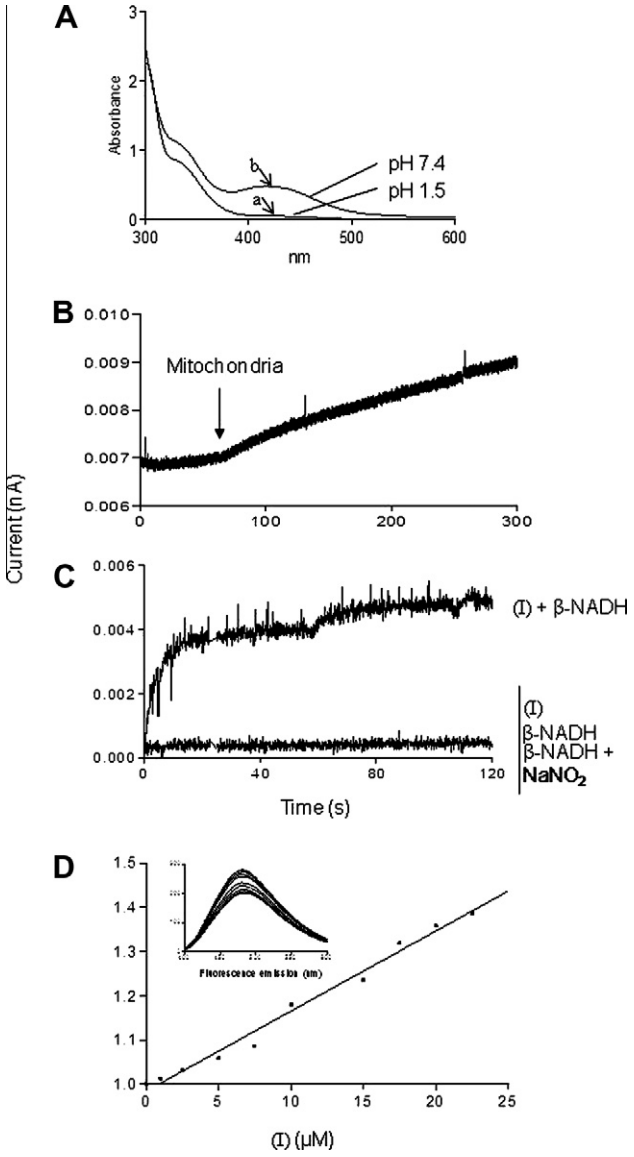

Fig. 1. (A): Absorption spectra of (**I**) solubilized at acidic pH (a) and neutral pH (b) in a standard medium consisting of 125 mM sucrose, 65 mM KCl and 10 mM HEPES–KOH.(B) and (C): Direct determination by a selective electrode of the NO released from 50 μM (**I**) in the presence (B) or absence (C) of succinate-energized mitochondria (1 mg protein/ml) incubated in the standard medium; in (C): 50 μM (**I**), 1 mM β-NADH and 20 μM $NaNO_2$ were added at the beginning. Traces are representative of three experiments. (D): Interaction of NADH with (**I**) assessed by fluorescence quenching and analyzed by a Stern–Volmer plot; insert: β-NADH (1 mM) incubated in the standard medium in the presence of (**I**) at final concentrations of 1–25 μM. Experimental conditions are described in materials and methods.

$$[RuL(bpy)_2(NO)]^{3+} + 2OH^- \overset{K_{eq}}{\rightleftharpoons} [RuL(NO_2)L(bpy)_2]^+ + H_2O$$

*Interaction of (**I**) with mitochondrial NADH induces NO release*

Fig. 1B shows a continuous release of NO from (**I**) incubated with mitochondria that were isolated from rat liver and energized with the site II respiratory substrate succinate (+rotenone); mitochondria were probably involved in the release of NO because in aqueous solution this release only occurs upon light irradiation [30]. Fig. 1C shows the NO release from (**I**) when elicited by NADH in the absence of mitochondria, suggesting that NADH oxidation was involved in the release of NO as well. The $NaNO_2$ was used as a negative control for the OAT mechanism. In fact $NaNO_2$ was unable to produce NO either in the absence or presence of NADH. These results indicate that NO release from (**I**) was mediated by the oxidation of mitochondrial NADH via the OAT mechanism, which renders NO binding to the complex labile. In an attempt to confirm the correlation between NO release from (**I**) and NADH oxidation [18], we measured the intrinsic fluorescence of β-NADH in the presence of (**I**) (Fig. 1D, insert). In fact, the Stern–Volmer analysis [31]resulted in $K_D = 9.0 \times 10^3$ in aqueous solution (Fig. 1D) and, therefore, a 1:1 stoichiometric association between (**I**) and NADH.

*Effects of NO released from (**I**) on mitochondrial respiration*

Fig. 2 shows the effects of (**I**) on the respiration of succinate-energized mitochondria in the presence of ADP (phosphorylating, state 3 respiration). After ADP was consumed, a basal respiration condition was reached (state 4 respiration); in this condition, carbonyl cyanide m-chlorophenylhydrazone (CCCP) induced uncoupled respiration (V_{CCCP}). The presence of (**I**) increased the state 4 respiration rate (V_4) at relatively low concentrations: 20 and 50 μM (Fig. 2A and D); the presence of 20 μM (**I**) for 20 min resulted in the same effect (Fig. 2B and D). Nevertheless, incubation with 50 μM (**I**) for 20 min inhibited mitochondrial respiration, as evidenced by a decrease in the state 3 respiration rate (V_3) and V_{CCCP} (Fig. 2C and D); incubation with 80 and 100 μM (**I**) led to the same effect (Fig. 2A and D). Therefore, (**I**) acted as a mitochondrial uncoupling agent at relatively low concentrations regardless of the incubation time and as a respiratory chain inhibitor at higher concentrations/incubation times. These effects resulted in a concentration-dependent decrease of the mitochondrial respiratory control ratio (RCR) (Fig. 2E).

*Interaction of NO released from (**I**) with superoxide radicals*

It is well established the basal generation of superoxide by mitochondria and its rapid conversion to H_2O_2 by SOD. The interaction of NO released from (**I**) with large amount of superoxide was assessed in presence of the xanthine/xanthine oxidase (X/XO) reaction in order to guarantee an excess of the radical able to react with the NO released. Fig. 3 shows that (**I**) decreased the levels of superoxide generated by the X/XO reaction in the presence of succinate-energized mitochondria, in apparent association with peroxynitrite yielded by the interaction of NO released from (**I**) with this radical. Accordingly, the amount of NO available after photo-irradiation of (**I**), monitored by a selective electrode (Fig. 3, insert) markedly decreased the levels of superoxide generated by the X/XO reaction, indicating that NO, indeed, interacted with superoxide. Since superoxide is generated at the intra-matrix space of mitochondria through respiratory chain complex I and III [32] and NO is released from (**I**) through NADH oxidation, it is probable that this reaction occurs mostly intramitochondrially.

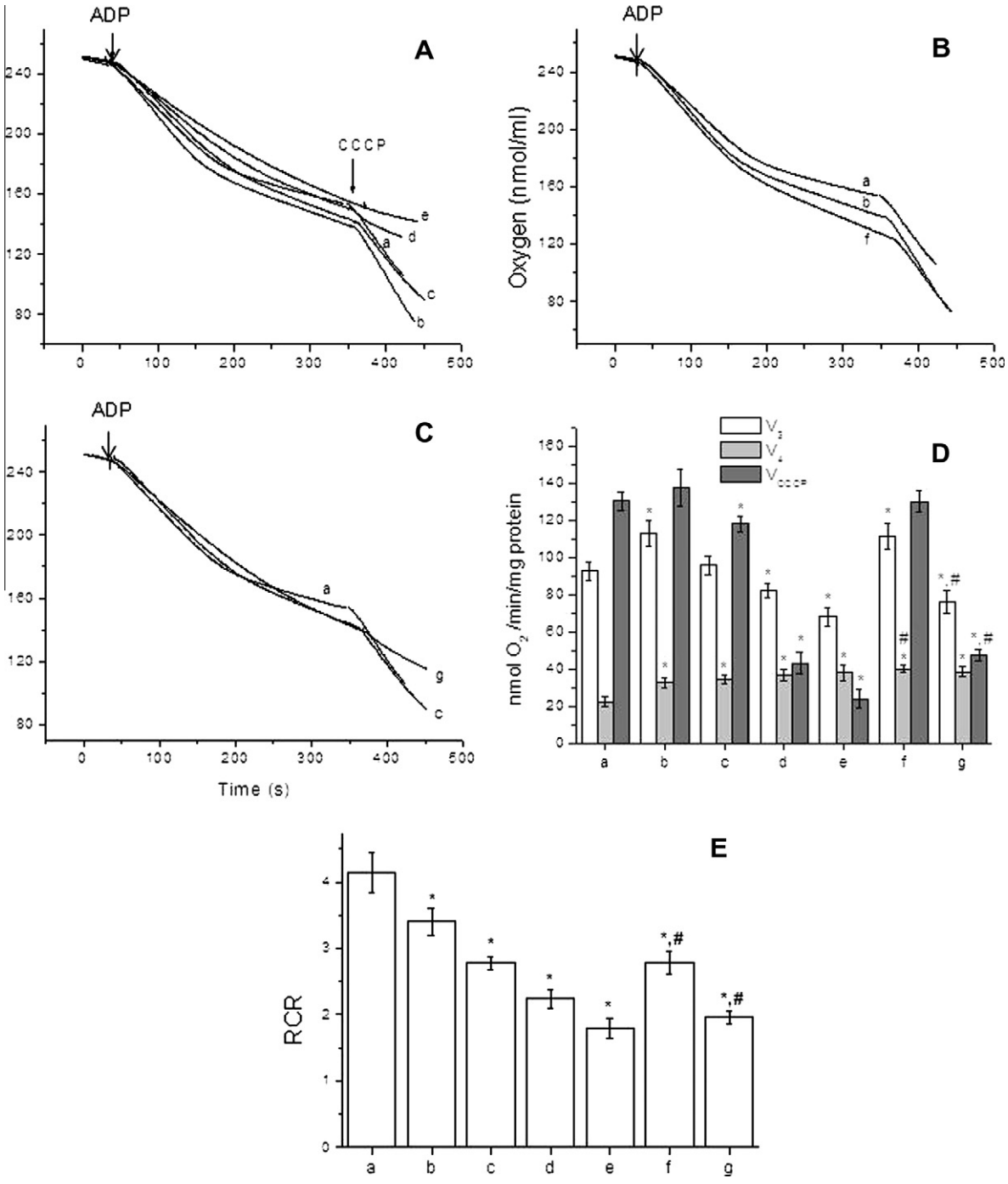

Fig. 2. Effects of NO released from (**I**) on the respiration of succinate-energized mitochondria. Representative traces were monitored immediately after addition of (**I**) to mitochondria (A) and after 20 min incubation (B) and (C). State 3 (V_3), state 4 (V_4) and uncoupled (V_{CCCP}) respirations are shown in (D); respiratory control ratios (RCRs) are shown in (E). Mitochondria (0.5 mg protein/ml) energized with 5 mM succinate (+2.5 μM rotenone) were incubated in the standard medium in the presence of 5 mM phosphate with 0 (a), 20 (b), 50 (c), 80 (d) or 100 μM (e) (**I**) and pre-incubated for 20 min with 20 (f) or 50 (g) μM (**I**). For the V_3 determination, 200 μM ADP was added. V_4 was determined after the ADP was exhausted and V_{CCCP} was determined after addition of the uncoupler CCCP (1 μM). Values are presented as mean ± SEM ($n = 3$), $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$comparing the two incubation times.

*Effects of NO released from (**I**) on mitochondrial ROS/RNS levels and protein–thiol oxidation*

Fig. 4A/4A-1 shows an increase of ROS/RNS levels in succinate-energized mitochondria incubated with(**I**) as indicated by oxidation of the nonspecific probe H_2DCFDA[27]; this increase was potentiated by100 μM Ca^{2+}(Fig. 4B/4B-1), which is in agreement with the established property of this cation in stimulating ROS generation by the respiratory chain [32]. The presence of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), an NO scavenger, prevented this effect under both conditions. However, in the absence of Ca^{2+}, the effect of (**I**) (at 100 μM) was only slightly inhibited by the mitochondrial permeability transition inhibitor cyclosporine A (CsA) (Fig. 4A/4A-1); in the presence of Ca^{2+}, a higher inhibition was observed (Fig. 4B/B-2). At the same concentration range and under the same conditions in which (**I**)

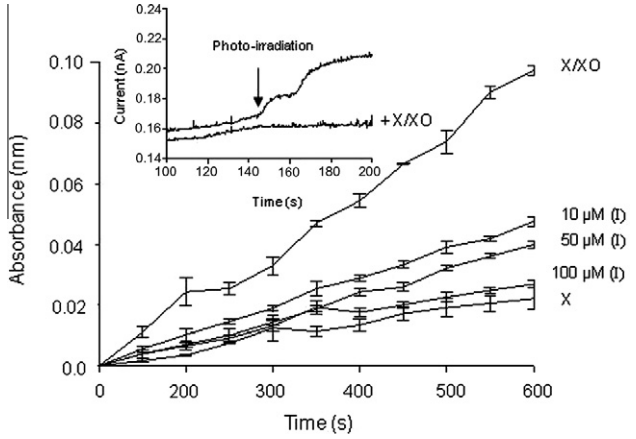

Fig. 3. Interaction of NO released from (**I**) with superoxide radicals generated by the xanthine/xanthine oxidase (X/XO) reaction in the presence of succinate-energized mitochondria, assessed by NBT reduction as described in Materials and methods. Bars represent mean ± SEM ($n = 3$). Insert: direct NO assessment in the presence of 20 μM (**I**) after photo-irradiation in the standard medium, measured using a diode laser operating at 447 nm (Colibri quantum Tec), with 12.4 mW potency and 8.1 J-cm^{-2} dose, in the presence or absence of X/XO.

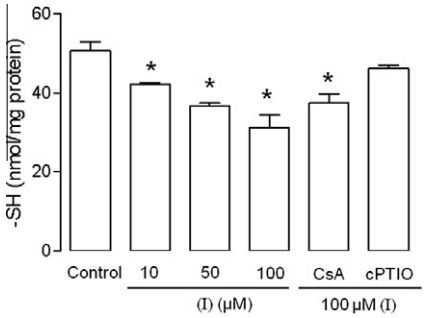

Fig. 5. Effects of NO released from (**I**) on protein–thiol (–SH) oxidation in succinate-energized mitochondria. Mitochondria (0.5 mg protein/ml) were incubated in the standard medium with DTNB, which assesses free thiols, in the presence of (**I**), with or without cPTIO (100 μM) and CsA (1 μM); experimental conditions are described in Materials and methods. Values are presented as the mean ± SEM ($n = 3$) $^*P < 0.05$ *vs.* control.

promoted an increase in the reactive species levels, (**I**) induced the oxidation of mitochondrial membrane protein–thiols in a manner that was inhibited by cPTIO but insensitive to inhibition by CsA (Fig. 5).

*Effects of NO released from (**I**) on mitochondrial swelling, mitochondrial membrane potential and cytochrome c release from mitochondria*

Fig. 6A shows the induction of mitochondrial swelling by (**I**). This swelling was only partially inhibited by CsA almost fully inhibited by the thiol reagent *N*-ethylmaleimide (NEM) and fully inhibited by cPTIO. A very similar pattern was observed for the mitochondrial membrane potential dissipation promoted by (**I**) (Fig. 6B). The cytochrome *c* released from mitochondria incubated with (**I**) was assessed by Western blotting analysis (Fig. 7A). An (**I**)-induced increase of cytochrome *c* content in the mitochondrial supernatant was revealed; this increase was not affected by CsA but significantly inhibited by NEM and cPTIO (Fig. 7A-1). The mitochondrial assays were also performed with the aqua-bound specie *cis*-$[Ru(H_2O)(bpy)_2(4\text{-pic})]^{2+}$as a control (results not shown) and didn't show the effects observed in the presence of (**I**).

Discussion

Nitrosyl ruthenium complexes are promising NO donor agents with numerous advantages for the biologic applications of NO [8,18,33,34]. The present work indicates that mitochondrial NADH oxidation promotes NO release from the nitrosyl ruthenium complex (**I**) in a manner mediated by NO_2 formation (at neutral pH), as in mammalian cells [20,21], followed by an OAT mechanism. The slow rate of NO release from (**I**) observed after incubation with

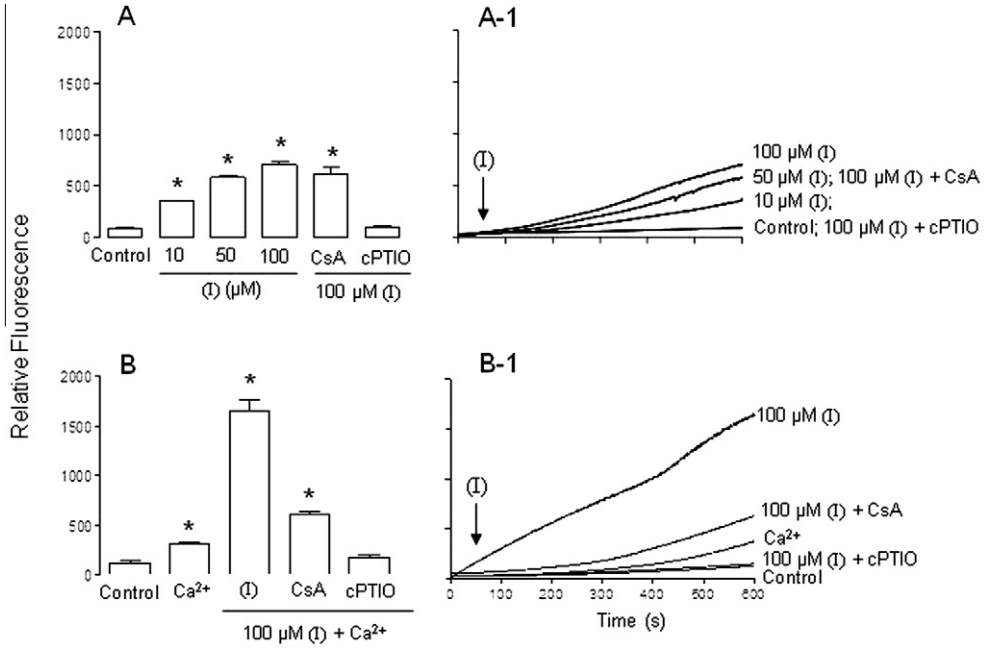

Fig. 4. Effects of NO released from (**I**) on ROS/RNS levels in succinate-energized mitochondria, assessed by H_2DCFDA oxidation as described in Materials and methods. Mitochondria (0.5 mg protein/ml) were incubated with 2 μM H_2DCFDA in the standard medium with 1 mM EGTA (A) or with 50 μM added Ca^{2+} (B); (**I**) additions, as well as cPTIO (100 μM) and CsA (1 μM) pre-incubation, are indicated. Values are presented as the mean ± SEM ($n = 3$) of ROS/RNS levels after 600 s, obtained from traces that are represented in A-1 and B-1. $^*P < 0.05$ *vs.* control.

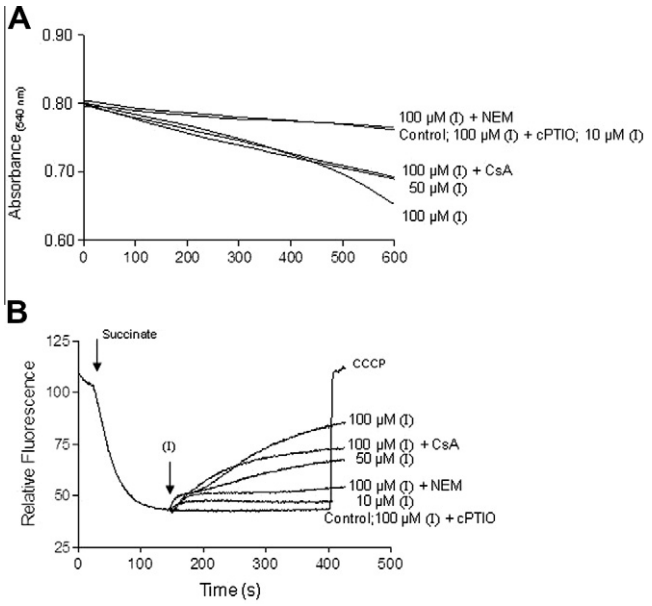

Fig. 6. Effects of NO released from (**I**) on swelling (A) and membrane potential (B) in succinate-energized mitochondria. (A): Mitochondria (0.5 mg protein/ml) were incubated in the standard medium with (**I**), with or without cPTIO (100 μM), CsA (1 μM) or NEM (20 μM) and energized with 5 mM succinate (+2.5 μM rotenone). (B): Mitochondria (1 mg protein/ml) were incubated in the standard medium with 10 μM safranine *O*, 2.5 μM rotenone and (**I**) at the same conditions used for the swelling assay; the reaction was initiated with 5 mM succinate. Traces are representative of at least three experiments from different mitochondrial preparations.

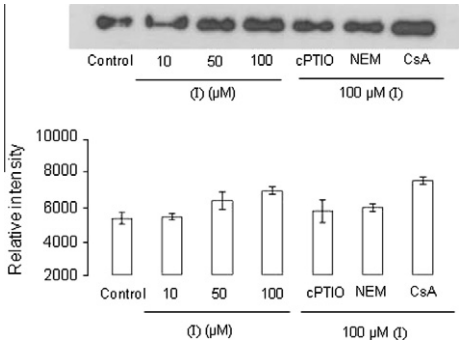

Fig. 7. Effects of NO released from (**I**) on cytochrome *c* release from succinate-energized mitochondria. Mitochondria were incubated as described for the swelling assay (Fig. 5A), centrifuged at 14000 rpm, and the supernatants were analyzed by Western blotting as described in materials and methods.

mitochondria compared to the rapid release observed after photo-irradiation suggests a high stability of (**I**) in a controlled system like these organelles. Although some NO donor agents release this species spontaneously, they can hazardously reach regions that are not necessarily NO targets [30,35,36]. Thus (**I**) may be advantageous as an NO donor agent due to its NO reservoir quality in a reducing environment like that provided by mitochondria through NADH. In this regard, a controlled NO release is beneficial for the organism, as has been observed during the prevention/delay of cerebral vasospasm [37].

NO and its derivatives have multiple effects on mitochondria which ultimately can lead to cell death [1,4,7]. While NO *per se* can inhibit cytochrome *c* oxidase through a reversible process, the rapid reaction between this radical and the superoxide generated by mitochondria yields RNS, mainly peroxynitrite, which can irreversibly inhibit the mitochondrial respiratory chain [38]. At relatively high concentrations of (**I**) or long incubation times with mitochondria, V_3 and V_{CCCP} decreased. Moreover, (**I**) promoted a decrease in the amount of superoxide generated by the xanthine/xanthine oxidase reaction and photo-irradiation of (**I**) in the presence of superoxide decreased the NO levels, indicating that, in fact, the peroxynitrite produced by the interaction between NO and superoxide could mediate the action of (**I**) on mitochondria. In this regard, the oxidation of H_2DCFDA incubated with (**I**) may reflect the presence of peroxynitrite once this nonspecific probe is reactive towards products of this species [27,39].

The mitochondrial permeability transition (MPT) is a well-documented phenomenon [40,41] that is typically associated with the oxidation of mitochondrial membrane protein–thiols [42,43]. The MPT results from the opening of permeability transition pores (PTPs), which causes mitochondrial swelling *in vitro*; the process is usually dependent on Ca^{2+} and sensitive to inhibition by CsA. [44–48]. We demonstrated that (**I**) induces a Ca^{2+}-independent mitochondrial swelling that is only partially inhibited by CsA but completely prevented by the thiol reagent NEM; this observation is consistent with the ability of (**I**) to induce the oxidation of protein–thiol groups. Indeed peroxynitrite can react directly with protein–thiols to form sulfonic acids, which are readily transformed to disulfides (cross-linking) or higher thiol oxidation products [19,49]. A consequence of the oxidation of mitochondrial membrane protein–thiols is a shift in the voltage-dependence of PTPs, allowing them to open in association with conformational protein changes; PTP opening, in turn, may be accompanied by the release of cytochrome *c* from mitochondria, which has been associated with cell death by necrosis or intrinsic pathway of apoptosis [40]. Although the proteins that must be oxidized/cross-linked to open PTPs are still unknown, the ADP/ATP translocator is probably involved [50]. In fact, it was previously reported that both NO and peroxynitrite react with the ADP/ATP translocator to induce mitochondrial membrane permeabilization [51].

The present results indicate that (**I**) release NO in a manner that is mediated by mitochondrial NADH via an OAT mechanism. The NO released from (**I**) uncoupled mitochondria at low concentrations/incubation times and inhibited the respiratory chain at high concentrations/incubation times. In the presence of ROS generated by mitochondria, which increases in the presence of Ca^{2+}, NO gave rise to peroxynitrite, which, in turn, inhibited the respiratory chain and oxidized membrane protein–thiols to elicit a Ca^{2+}-independent mitochondrial permeability transition that was only partially inhibited by CsA; these actions correlated with the release of cytochrome *c* from mitochondria. These mitochondrial events are typically involved in cell necrosis and/or apoptosis [52,53], and consequently present the potential to kill tumor cells. Changes in the membrane potential of mitochondria in tumor cells due to low oxygen availability and low Complex I activity could increase the organelle's levels of NADH [54,55]. This metabolic peculiarity of these cells may render them preferential targets for NO release from (**I**)/analogs and cause mitochondria-mediated cell death. In fact, a ruthenium complex analog of (**I**) was toxic to the C6 astrocytoma cell line but not to primary rat astrocytes [56]. In relation to our [18] and other previous studies the present data adds the following new information on the biologic action of the nitrosyl ruthenium complexes: (**I**) potential release of NO in a manner that is mediated by mitochondrial NADH via an OAT mechanism; (ii) characterization of a Ca^{2+}-independent mitochondrial permeability transition process in association with oxidized membrane protein–thiols; and (iii) correlation with the release of cytochrome *c* from isolated mitochondria and so, potential action regarding the intrinsic apoptotic pathway. Although these studies were performed in an extreme *in vitro* system, they denote a potential specific action of (**I**) and analogs against tumor cells via mitochondria-mediated processes. Studies of these processes in tumor cell lineage exposed

to (**I**) are being conducted both to highlight this potential and to characterize its action in this setting.

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