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Effects of nitrosopropofol on mitochondrial energy-converting system

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Abstract

Nitrosopropofol (NOPR) is a relatively stable compound obtained from the reaction between the general anesthetic 2,6 diisopropylphenol (propofol) and nitrosoglutathione (GSNO) and bearing a more acidic phenol group than propofol. It interfered with mitochondrial energetic metabolism in a concentration-dependent manner. Concentrations as high as 100 or 200 μM disrupted both oxidative phosphorylation and electron transport. Low concentrations of NOPR (50 μM) markedly slowed down the electron transport rate which was insensitive both to ADP and uncoupler stimulation and spontaneously gradually stopped. Consequently, both the transmembrane potential production and the ATP synthesis system were affected. In the presence of 10 or 20 μM NOPR, mitochondria respired but showed a worsening of the respiratory control and produced a transmembrane potential useful to respond to a phosphorylation pulse, but were not able to restore it. These results were consistent with ATP synthesis and swelling experiments. NOPR was effective at concentrations lower than those required by the combination of propofol and GSNO, suggesting that mitochondria might be able to catalyze the reaction between GSNO and propofol and that the resulting metabolite was more active on mitochondrial membrane structure than the parent compounds. Although the details of the process are yet unknown, the mechanism presented may be of potential relevance to rationalize the pathophysiological effects of propofol.

Keywords: Anesthetics; Nitrosopropofol; Rat liver mitochondria; Respiration; Transmembrane potential; ATP synthesis

1. Introduction

Propofol is an alkyl-substituted phenol widely used as an intravenous general anesthetic [1]. Recently, it has been suggested that the marked vasodilation observed during propofol treatment might be mediated by the production of NO by the endothelial cells [2,3]. Also the negative chronotropic effects induced by propofol in cultured rat ventricular myocytes, which is in part mediated by M-2 acetylcholine receptors activation, involves an enhancement of NO production [4].

We have previously reported that propofol may interfere with cellular energy availability by affecting mitochondrial oxidative phosphorylation [5–7]. In addition, an NO synthase activity has been recently characterized also in mitochondria [8] where NO appears to modulate the energetic metabolism [8–13]. Following the hypothesis that, in mitochondria, propofol might interfere with the regulatory function of NO, we studied the combined action of the anesthetic and the physiological NO donor GSNO, on the mitochondrial energy metabolism. We have demonstrated that an evident synergistic effect occurs, leading, in the presence of propofol, to a GSNO concentration-dependent restraint of the energy production [14].

It has been reported by Mouithys-Mickalad *et al*. [15] that, in suitable conditions, propofol is able to react with peroxynitrite to form a phenoxyl radical and, more recently, by Cudic and Ducrocq [16], with NO or its derivatives to give addition products. Considering this possibility, we have synthesized and characterized the 2,6-diisopropyl-4-nitrosophenol. On the assumption that

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Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; RLM, rat liver mitochondria; NOPR, nitrosopropofol; GSNO, nitrosoglutathione.

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such a compound might be produced also in the mitochondrial matrix and be active there, we have studied the effects of NOPR on the mitochondrial energy metabolism.

2. Materials and methods

Mitochondria were isolated from liver of albino Wistar rats in 0.25 M sucrose, 10 mM Na-HEPES (pH 7.4), and 0.25 mM EGTA by differential centrifugation [17], omitting EGTA in the final washing. Protein content of the mitochondrial suspension was assayed by the biuret method [18]. Experiments on respiration, membrane potential, and ATP synthesis were carried out at 20° using 1 mg/mL of mitochondrial protein in a basal medium containing 0.25 M sucrose, 15 mM 3-*N*-morpholinopropanesulfonic acid, 1.5 μM rotenone, 1.6 mM $MgSO_4$, 2 mM KH_2PO_4, 6 mM NaCl, 1 mg/mL BSA, and 5 mM Na-succinate, pH 7.0.

Swelling experiments were followed spectrophotometrically by the decrease in absorbance at 540 nm in the presence of 0.25 mg/mL mitochondrial protein in 213 mM mannitol, 71 mM sucrose, 5 mM HEPES–Tris (pH 7.4), 5 mM succinate, 3 μg/mL rotenone, and 3 μM oligomycin. Oxygen consumption was followed using a Clark oxygen electrode (Yellow Springs Instruments Co) in the basal medium supplemented with 5 mM K-succinate and 1.5 μM rotenone.

Transmembrane electrical potential ($\Delta\Psi$) was measured by monitoring the distribution of tetraphenylphosphonium cation across the inner mitochondrial membrane with a tetraphenylphosphonium-selective electrode in the basal medium supplemented with 5 mM K-succinate and 1.5 μM rotenone [19].

ATP synthesis was evaluated by using a semi-micro combined pH electrode (Ingold Messtechnik AG) to monitor the rate of proton concentration decrease due to the shift of ATP, ADP, and phosphate anion dissociation equilibria [20]. The rate of ATP synthesis was taken as the difference between the rates obtained in the absence and presence of 1 μg oligomycin/mg of mitochondrial proteins. The experiments were performed in the basal medium (from which 3-*N*-morpholinopropanesulfonic acid was omitted) supplemented with 5 mM K-succinate, 1.25 μM rotenone, and 240 μM ADP.

NOPR was synthesized following the procedure utilized for the preparation of nitrosophenol [21]. Two milliliters of concentrated hydrochloric acid were slowly added to 3 g of 2,6-diisopropylphenol dissolved in 25 mL ethanol maintained at −5°. A solution of 1.1 g sodium nitrite in 5 mL water was gradually added to the mixture under vigorous stirring and maintaining the temperature below 0°. After 15 min, the yellow product was stirred for 30 min and then poured into iced water. The yellow precipitate was crystallized from toluene and characterized by GC/MS analysis and UV–Vis. The latter spectrum shows a characteristic absorption band at 310 nm [22]. Interestingly, NOPR prepared with the earlier reported procedure or after reaction of propofol with GSNO in the presence of copper(II) gives rise to superimposable GC/MS and UV–Vis spectra [22]. In Fig. 1, the proton NMR spectrum of NOPR is reported and the chemical shifts are consistent with the structure of the compounds. The spectrum presents distinct resonances for the aromatic protons 3 and 5 as well as for the methynic and methylic protons of the isopropyl groups. This is attributed to the hindered rotation of the nitroso group, due to the order (between 1 and 2) of the C–N bond [23]. A purity grade higher than 95% was confirmed by thin layer chromatography. NOPR was used as an ethanol solution and appropriate blanks were run to exclude possible ethanol effects.

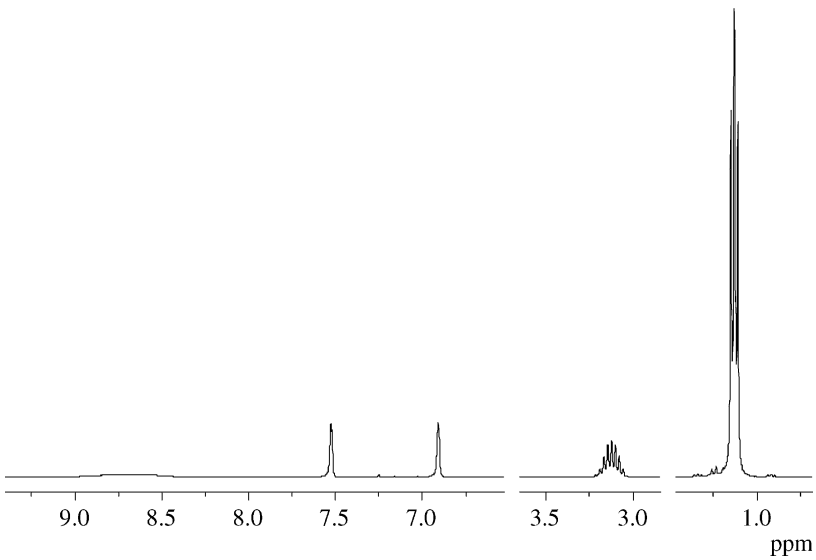

Fig. 1. Proton NMR spectrum of NOPR. ^{1}H NMR ($CDCl_3$, 300 MHz), δ: 8.7 (1H, OH, broad singlet), 7.52 and 6.91 (2H, 3H, and 5H, singlets), 3.06 and 3.1 (2H, methynic, septets, $J = 8.5$ Hz), 1.14 and 1.12 (12H, methyls, doublets, $J = 8.5$ Hz).

Traces reported in the figure correspond to the most representative experiment among those performed on 15 different mitochondrial preparations. It was chosen considering the closest to the mean of the experiments showing the maximal values of $\Delta\Psi$, state 3 respiration, and ATP synthesis.

The values were compared to each other by the paired Student's *t*-test. and *P*-values resulted <0.001 except for those obtained in the presence of 100 or 200 μM NOPR and compared between themselves ($P < 0.01$).

3. Results

Fig. 2 shows the time course of mitochondrial oxygen consumption during resting respiration and ADP- and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP)-stimulated respiration in the presence of increasing concentrations of NOPR in the range 2–200 μM.

Control mitochondria exhibit a state 3 rate of 102 ngat oxygen/min/mg prot, a state 4 rate of 13.8 ngat oxygen/min/mg prot, and an uncoupled respiration of 104.3 ngat Oxygen/min/mg prot (Fig. 2a). Consequently, a respiratory control ratio of 7.3 and an ADP/O ratio of 1.5 may be calculated. In the presence of 10 μM NOPR (Fig. 2b), ADP addition stimulates respiration, although to a lower rate (74.1 ngat oxygen/min/mg prot) with respect to the control. In the same conditions, the resting respiration is faster (51 ngat oxygen/min/mg prot), the restoration of state 4 is delayed, and the addition of the strong uncoupler CCCP evokes a transient increase of the respiration that, afterwards, gradually stops.

In the presence of 20 or 50 μM NOPR (Fig. 2c and d) the respiration, although still slightly sensitive to the ADP addition, is strongly inhibited (57.1 and 52.6 ngat oxygen/min/mg prot, respectively), a true state 4 cannot be restored and CCCP induces a limited stimulation of respiration which spontaneously stops. The respiration inhibition is almost complete when concentrations as high as 100 or 200 μM of NOPR are used (31.1 and 19.4 ngat oxygen/min/mg prot, respectively) and the system appears insensitive to any kind of stimulation.

Control mitochondria produce and maintain an electrical transmembrane potential of about 180 mV (Fig. 3). The addition of a limited amount of ADP induces a phosphorylation pulse observed as a transient protonic gradient utilization that, however, is rapidly rebuilt at the same level present before ADP addition (Fig. 3a). In the presence of 10 or 20 μM NOPR, mitochondria still establish a transmembrane potential, although lower of the control of about 10 or 20 mV, respectively. In addition, they are not able to restore it after the phosphorylation pulse (Fig. 3b and c). NOPR, at concentrations of 50 μM or higher completely prevents mitochondria from producing any significant transmembrane potential (Fig. 3d–f).

The rate and extent of ATP synthesis are consistent with those of the transmembrane potential (Fig. 4). ATP synthesis of control mitochondria is of 42 nmoles/min/mg prot. In the presence of 10 μM NOPR the synthesis rate is about halved (19 nmoles/min/mg prot), with respect to the control, while it is reduced to about a seventh (6.2 nmoles/min/mg prot) with 20 μM NOPR. The synthesis of ATP is undetectable with 50 μM NOPR, while the results obtained with 100 and 200 μM NOPR were not reported since ATP formation is completely inhibited.

Fig. 5 shows the mitochondrial swelling induced by the addition of 10 μM Ca^{2+} in the presence of NOPR in the range 10–200 μM. It appears that the mitochondrial swelling induced by calcium ions uptake is completely abolished by propofol concentrations as high as 100 μM and

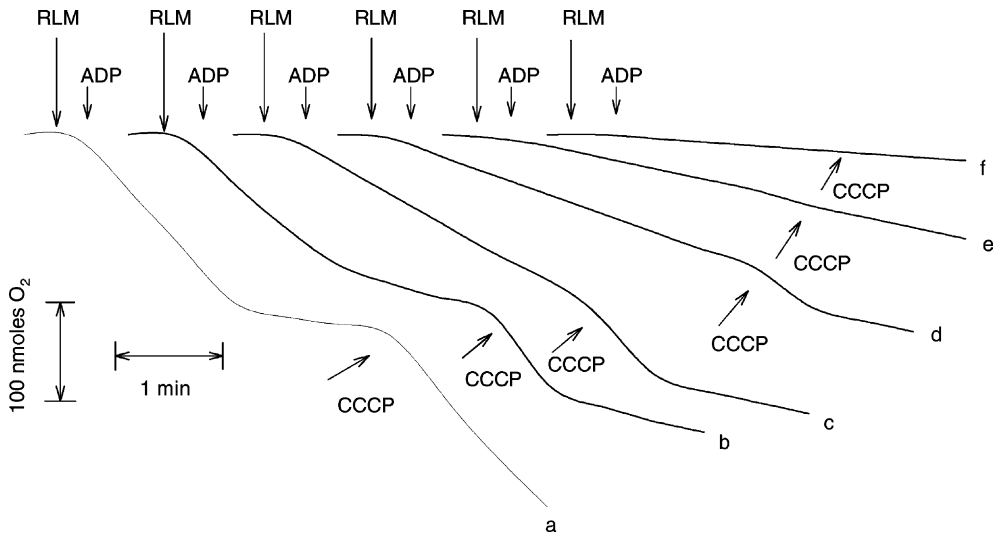

Fig. 2. Effect of NOPR on resting and ADP- or CCCP-stimulated respiration of isolated rat liver mitochondria. Additions (indicated by the arrows): rat liver mitochondria (RLM), 0.3 mM ADP, 1.6 μM CCCP. NOPR was present in the incubation medium before mitochondria addition. NOPR concentrations in (μM): (a) none; (b) 10; (c) 20; (d) 50; (e) 100; and (f) 200.

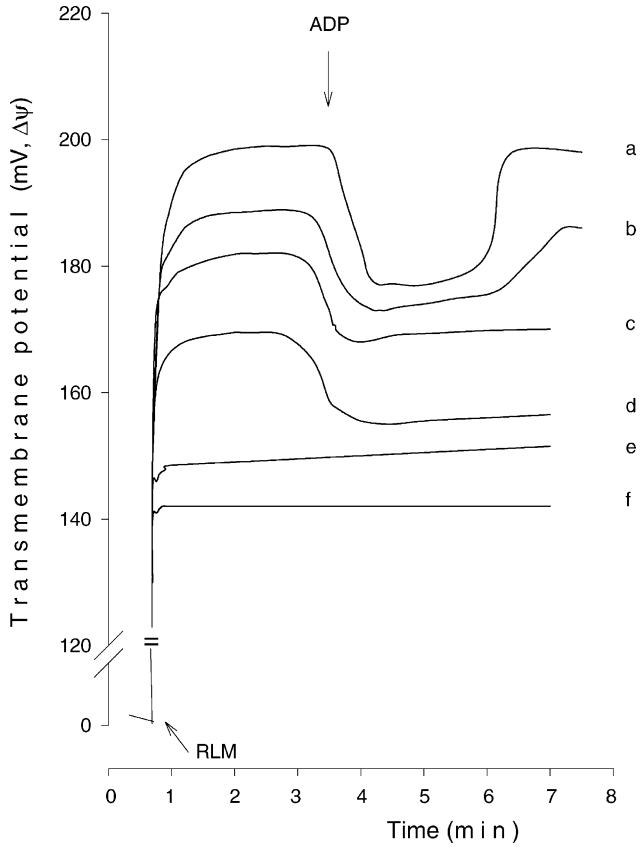

Fig. 3. Effect of NOPR on mitochondrial $\Delta\Psi$ formation. NOPR was present in the medium before mitochondria. At the arrows, 0.3 mM ADP was added. The curves have been redrawn using a linear millivolt scale. NOPR concentrations in (μM): (a) none; (b) 10; (c) 20; (d) 50; (e) 100; and (f) 200.

that the effect is concentration dependent, being the swelling in the presence of 10, 20 or 50 μM NOPR proportionally inhibited.

4. Discussion

NOPR is the main product of the nitrosation reaction of the general anesthetic propofol. Its presence in the incubation medium of respiring mitochondria interferes with both the oxidative phosphorylation and the electron transport in a concentration-dependent manner. Concentrations as high as 100 μM or more completely disrupt the mitochondrial aerobic metabolism (Fig. 2). Also 50 μM NOPR has a dramatic effect on respiration since the electron transport rate is strongly slowed down and afterwards spontaneously stops. Besides, it is insensitive to both ADP and CCCP stimulation, indicating that a major structural damage has occurred. In addition, 10 μM NOPR induces a worsening of the mitochondrial energetic system, although maintaining a good efficiency of electron transport and a significant residual functioning of oxidative phosphorylation. Higher concentrations such as 20 μM NOPR evokes a behavior qualitatively similar to that of 50 μM but quantitatively milder suggesting that the effect is concentration dependent.

These experiments indicate that the major effect of NOPR is referable to an alteration of the mitochondrial membrane structures and that, compared to propofol, the molecular modification due to introduction of the NO group might play a critical role. In fact, titration of propofol or NOPR shows that the presence of the NO group shifts

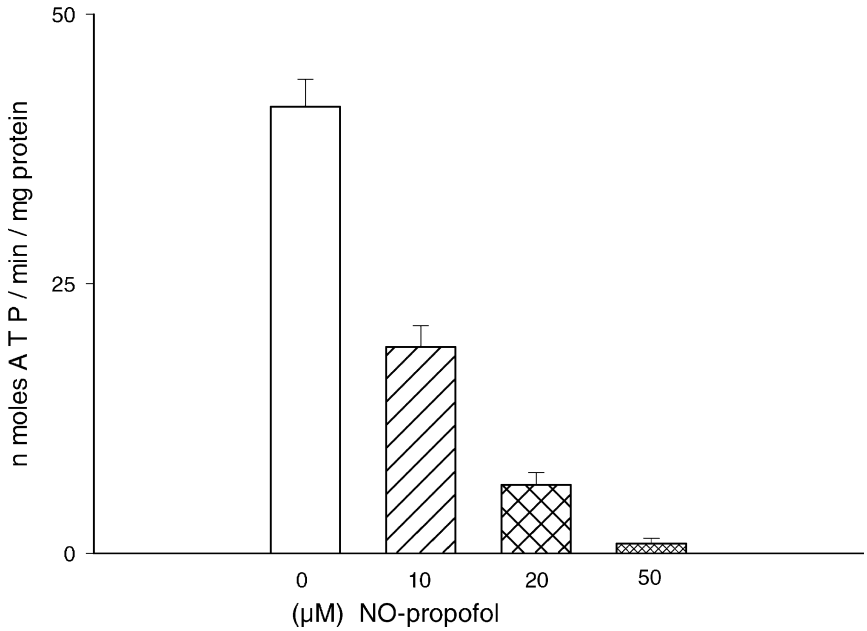

Fig. 4. Effect of NOPR on ATP synthesis by isolated rat liver mitochondria. NOPR, at the concentration indicated, was present in the incubation medium before mitochondria addition. Mitochondria (1 mg protein/mL) and 0.3 mM ADP were also present. The reported rates represent the difference between the rates measured in the absence and in the presence of oligomycin (1 μg/mg protein).

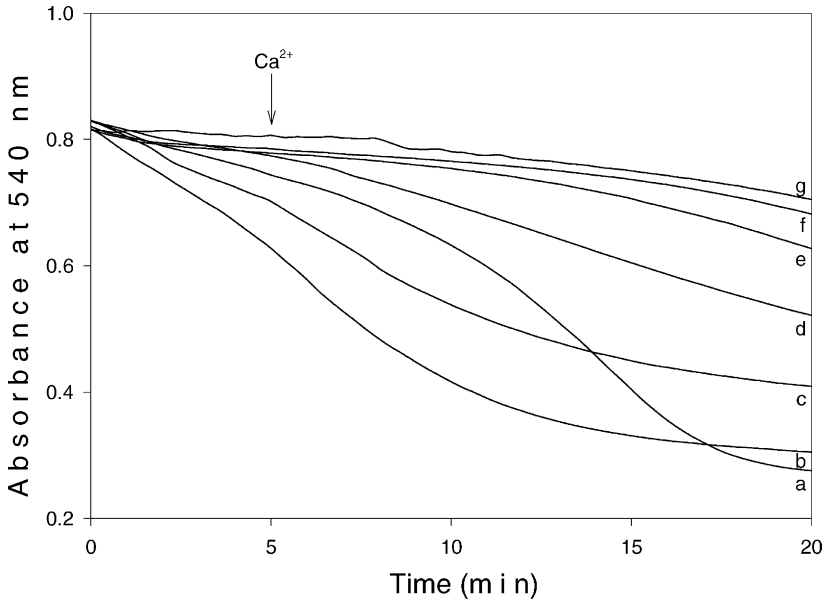

Fig. 5. Mitochondrial swelling induced by calcium addition in the presence of increasing NOPR concentrations. Mitochondria were preincubated for 1 min with NOPR and 10 μM Ca^{2+} was added at the arrow indicated. NOPR concentration in (μM): (a) none; (b) 5; (c) 10; (d) 20; (e) 50; (f) 100; and (g) 200.

the pK_a value of the phenol group from about 11 for propofol to about 7.5 [22], indicating that, at physiological pH, NOPR is more dissociated than propofol, and, consequently, is present mostly in the charged form.

Conceivably, the transmembrane potential formation reflects the respiration behavior and low NOPR concentrations (10 or 20 μM) allow mitochondria to build up a potential lower than that of control, but sufficient to support a phosphorylation pulse. However, after the phosphorylation pulse, mitochondria are unable to restore $\Delta\Psi$. Higher NOPR concentrations (100 or 200 μM) prevent the formation of any significant potential. The rate of ATP synthesis, which utilizes the energy of the transmembrane potential, is consistent with the membrane potential value. Moreover, the addition of NOPR to the incubation medium after the production of a stable potential induces a decrease of the voltage value whose extent appears to be proportional to the NOPR concentration (data not shown).

This indicates that NOPR action might be mainly referable to a derangement of the membrane tightness. However, if the effect would be merely an increase of the proton permeability, respiration should be uncoupled and, consequently, the oxygen consumption rate increased. On the contrary, as apparent in Fig. 2, the respiration is inhibited, i.e. the electron transport seems hindered in a concentration-dependent manner, as confirmed by the uncoupler ineffectiveness.

Calcium ions, taken up by the electrical gradient of metabolically active mitochondria, induce mitochondrial swelling. As apparent in Fig. 5, 100 or 200 μM NOPR completely prevents mitochondrial swelling while lower NOPR concentrations proportionally limit the mitochondrial shape change as may be detected by following the light scattering shift of the suspension. An increase of the matrix volume due to the influx of a solute leads to a proportional decrease of the matrix refractive index, which approaches that of the medium, and results in a decrease of the light scattered. Hence, the reported results may be regarded as a consequence of the NOPR effect on the $\Delta\Psi$ discussed before and confirm those observations. Indeed, in the experimental conditions adopted, the swelling is quantitatively dependent on the calcium entering the matrix and the associated water flux, which, in turn, depends on the membrane potential strength which is decreased by NOPR, according to its concentration.

In a previous paper [14], we have reported that the associated action of relatively high concentrations of propofol and GSNO resulted in an inhibition of the mitochondrial energetic efficiency and suggested that the effects might be ascribed to little amounts of a new molecule possibly derived from the interaction of propofol and GSNO in the mitochondrial environment.

NOPR is the main product of the *in vitro* nitrosation of propofol but may be obtained in the presence of GSNO and of copper ions [16]. It is a stable compound and its phenol group is more dissociable than that of propofol. NOPR is able to impair the mitochondrial energetic system at concentrations far lower than those required, to obtain the same effects, by the combination of GSNO and propofol.

Mitochondria are potentially able to catalyze the reaction between GSNO and propofol and the derived product, NOPR, might be a metabolite more active than the parent compound. Moreover, further work is required to unambiguously detect the presence of NOPR in the matrix of mitochondria supplied with propofol and GSNO.

In the cell, where NO production is relevant and GSH is abundant, the proposed mechanism might be of some importance in rationalizing the way of action of propofol

and in elucidating some clinical effects reported in recent years. Among the latter, bradycardia and hypotension due to the marked vasodilation elicited by propofol and mediated by NO production and release from the endothelial cells should be mentioned [2,3]. In addition, the stimulation of the ciliary beat frequency elicited by propofol in cultured tracheal epithelial cells *via* an NO–cGMP pathway was also observed [24].

Concerning the concentrations tested, propofol is usually administered by the ''Target Controlled Infusion'' which allows to obtain controlled plasma concentrations of propofol (expressed as μg/mL) on the basis of a pharmacokinetic program controlled by a computerized pump. Currently, deep sedation requires a plasmatic concentration level higher than 1.8 μg/mL (i.e. about 10 μM), while maintenance of deep hypnosis needs plasmatic concentrations between 3.6 and 5.3 μg/mL (20–30 μM) and induction of anesthesia about 35–40 μM [25,26]. When the target chosen is not a plasmatic concentration but a ''site effect concentration'', which is another option of the Target Controlled Infusion system, plasmatic levels may reach peaks very high (70–80 μM), even though for a very short time. Therefore, the concentrations of NOPR used in our work (10–200 μM) are in the concentration range clinically employed.

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