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Propofol affinity to mitochondrial membranes does not alter mitochondrial function

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ABSTRACT

The molecular mechanisms of hepatotoxicity after propofol anaesthesia have not been fully elucidated, although there is a relation with mitochondrial dysfunction. The action of propofol on mitochondrial hepatic functions in a rat model was evaluated by infusion for 4 h with 25 and 62.5 mg/kg/h propofol or 3.125 ml/kg/h (vehicle). Liver mitochondrial respiratory rates were evaluated as well as mitochondrial transmembrane potential (ΔΨ), calcium fluxes, mitochondrial enzymatic activities (Complex I–V) and oxidative stress biomarkers (superoxide dismutase, catalase, glutathione reductase, glutathione S-transferase, lipid peroxidation and the oxidised/reduced glutathione ratio). Biophysical interactions with membrane models were also performed. The mitochondrial transmembrane potential was decreased and the opening time of the mitochondrial permeability transition pore was slightly reduced for the highest dose. The activity of complex II was stimulated by propofol, which also causes fluctuations on some respiratory parameters, whereas the antioxidant system was affected in a nonspecific manner. Fluorescence quenching studies suggested that propofol is preferably located in deeper regions of the bilayer and has a high affinity to mitochondrial membranes. It is suggested that propofol interacts with liver mitochondrial membranes with mild modification in mitochondrial function.

1. Introduction

Propofol, a substituted isopropylphenol medication with both sedative and hypnotic characteristics promoting type A gamma-aminobutyric acid ($GABA_A$) receptor and blocking N-Methyl-D-aspartate (NMDA) receptors (Irifune et al., 2003), is frequently used for sedation, induction and/or maintenance of anaesthesia in common anaesthesia and intensive care medicine (Fagerlund et al., 2010). Its hepatic metabolism occurs by conjugation to inactive glucuronide or sulphated metabolites to produce inactive water-soluble compounds that are excreted in urine (Guitton et al., 1998).

Despite being considered a safe anaesthetic, cases of hepatotoxicity after propofol anaesthesia have been reported (Anand et al., 2001; Asai et al., 2013; Nguyen and Borurn, 2009; Polo-Romero et al., 2008) which can be manifested during short or long term sedation (Kneiseler et al., 2010). Notwithstanding, it is known from the literature that propofol interferes with levels of hepatic enzymes (Granados Llamas et al., 1999; Tiainen et al., 1995) and with cytochrome P450-dependent monooxygenases system (Baker et al., 1993; Chen et al., 1995), which is responsible for the oxidation-reduction reactions of xenobiotics in the liver (Ueng et al., 1997). This system is also responsible, in part, for the fatty acid oxidation that occurs mainly in mitochondria, peroxisomes or microsomes (Reddy and Hashimoto, 2001).

Additionally, reports have surfaced that the occurrence of microvesicular steatosis is associated with propofol infusions (Bray, 1995; Parke et al., 1992) which is related with severe impairment of mitochondrial fatty acid β-oxidation (Fromenty and Pessayre, 1995; Vickers, 2009). Suggestions imply this condition as a result of direct inhibition of mitochondrial functions such as inhibition of respiratory chain complexes, decreased ATP production and oxidative stress

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(Vickers, 2009). Still, the causative factors leading to the hepatotoxicity of propofol have not been completely clarified despite mitochondrial dysfunction being recommended as the principal intervener (Niezgoda and Morgan, 2013) owing to a decrease in the energy supply inside the cell (Vasile et al., 2003). However, the propofol effects at the mitochondrial level remain controversial as a recent study showed that propofol has hepatoprotective effects preserving mitochondrial function and energy homeostasis (Bellanti et al., 2016). In this sense, further studies are required to clarify this issue.

Therefore, this study aimed to identify the *in vivo* effects perfusion rates of propofol on rat mitochondrial hepatic functions focusing on the respiratory chain parameters and oxidative stress biomarkers. It also aimed to analyse the various aspects of propofol interactions with model lipid membranes as well as with mitochondrial membranes.

2. Material and methods

2.1. Chemicals

Propofol (Diprivan 2%) was obtained from AstraZeneca, Barcarena, Portugal. Intralipid 20% was used as a control vehicle and acquired from Sigma-Aldrich, Deisenhofen, Germany. The fluorescent indicator, Calcium Green 5-N, hexapotassium salt, was obtained from Molecular Probes (Invitrogen, Carlsbad, CA, USA). The fluorescent probes 2-AS and 16-AP were acquired from Molecular Probes (Eugene, USA). The phospholipid DPPC (dipalmitoylphosphatidylcholine) was obtained from Avanti Polar Lipids (Alabaster, USA**).** The remaining chemicals used were of the highest quality commercially available.

2.2. Animals

Eighteen male Wistar rats (2–3 months), acquired from Charles River, (Barcelona, Spain) were acclimatized for 7 days to standard laboratory conditions (12-h photoperiod cycle, temperature 21 ± 2 °C and relative humidity 60 ± 5%), following environmental requirements and fed with a standard commercial rodent chow in addition to tap water *ad libitum*. After acclimation, the animals were randomized to 3 groups (n=6 animals/group) in polycarbonate cages. Animal handlers and investigators were trained for animal experimentation accordingly to European requirements and the experimental protocols were previously evaluated and agreed by the Direcção Geral de Veterinária (Portuguese Veterinary Office) under the license number 0420/000/000/2009.

2.3. Anaesthesia induction and maintenance

The experimental animals were first anesthetised with 7–8% sevoflurane (2 l/min) until postural reflexes were lost, after which a gas scavenging system was used to maintain anaesthesia (2.5% sevoflurane at rate of 1 l/min). Meanwhile, a bedding from VetBed (Kennel Needs and Feeds, Morpeth, United Kingdom) and a heating blanket from Harvard Apparatus (Edenbridge, Kent, United Kingdom) were used to place animals in lateral recumbency and to maintain body temperature. After cannulation of lateral tail vein, inhalatory anaesthesia was ended and propofol infusion was started with an i.v. bolus of 10 mg/kg, with a syringe pump (Alaris Medical Systems, San Diego, USA) and continued for 4 h with infusions of 25 or 62.5 mg/kg/h. The doses were selected based on a previous work by our group in which they were defined as effective for maintaining light and deep anaesthesia, respectively, in this species (Antunes et al., 2003). Animals from control group were also subjected to the same protocol using the same conditions and infused with placebo (vehicle of propofol) at a rate of 3.125 ml/kg/h. Following terminal anaesthesia, euthanasia by cervical dislocation was performed and one half of the liver collected for mitochondria studies and another half immediately frozen at −80 °C for biochemical analysis. Responsible investigators were unaware of the treatment groups of animals.

2.4. Mitochondria isolation and mitochondrial respiration

Differential centrifugation was used to extract mitochondria from the liver (Gazzotti et al., 1997) with minor modifications. Briefly, the whole liver was removed and homogenised in 10 ml of isolation medium (250 mM Mannitol, 75 mM sucrose, 10 mM HEPES, 0.1 mM EDTA, 0.2 mM EGTA, and 0.1% bovine serum albumin (BSA), pH 7.4) at 4 °C. Following homogenisation, homogenates were centrifuged at 800×*g* for 10 min at 4 °C (Sigma 3K-16). A new centrifugation was performed at 10,000×*g* for 10 min to recover mitochondria from the supernatant. The brown pellet was suspended in medium (without EDTA, EGTA and BSA) and centrifuged at 12,000×*g* for 10 min. The resulting pellet (mitochondria) was suspended in washing medium, divided in two aliquots and used immediately or frozen at −80 °C. The Biuret method (Gornall et al., 1949) using BSA as standard was used to determine total protein content of samples. Mitochondrial respiration was monitored polarographically using a Clark-type oxygen electrode with constant stirring at 25 °C (Estabrook, 1967) in an incubation chamber (CB1-D Hansatech). The assay consisted of 1 ml buffer (130 mM sucrose, 50 mM KCl, 5 mM KH_2PO_4, 5 mM HEPES and 5 mM $MgCl_2$, at pH 7.2) complemented with 1 mg mitochondrial sample. Following an equilibration period (2 min), mitochondria were energised with pyruvate–malate (5 mM, complex I substrate) or succinate (5 mM, complex II substrate). When analysing complex II, rotenone (3 μM) was added. Adenosine diphosphate (ADP) was added (100 nmol/mg protein) to start State 3. State 4 respiration was obtained when all ADP was phosphorylated. To uncouple respiration, an ionophore was used (Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) at 1 μg/ml). Subsequently, the respiratory acceptor control ratio (RCR) and ADP/O ratios were calculated (Chance and Williams, 1956).

2.5. Mitochondrial membrane potential

According to the Kamo instructions and as previously outlined, the mitochondrial membrane potential (ΔΨ) was assessed with a TPP^+(tetraphenylphosphonium cation) electrode (Kamo et al., 1979). The basal line was adjusted with FCCP and a 1.1 μl/mg protein was implicit for the matrix volume. The assay was performed in 1.0 ml of medium (as described for mitochondrial respiration) with 3 μM TPP^+ and 1 mg of mitochondrial protein. Mitochondria were energised with 5 mM succinate. After the energisation of mitochondria, and the steady-state of TPP^+ occurred, the state 3 respiration was initiated by ADP (100 nmol/mg protein). All reactions were performed at 25 °C.

2.6. Calcium fluxes

The membrane calcium fluxes by mitochondria were assessed using Calcium Green 5-N (Rajdev and Reynolds, 1993). Liver mitochondria (0.6 mg) were suspended in 2 ml of buffer containing 200 mM sucrose, 10 mM Tris (pH 7.4), 1 mM KH_2PO_4 and 10 μM EGTA (in order to chelate residual calcium present outside), to which it was added 3 μM rotenone and 0.1 μg oligomycin. Levels of free Ca^{2+} were examined with 100 nM Calcium Green 5-N and the signal was continuously recorded at 25 °C, using a Varian Eclipse spectrofluorometer (Varian Inc, Palo Alto, CA, USA) operated at excitation and emission wavelengths of 505 and 531 nm, respectively. After a 3 min equilibration period, 10 μM of calcium ($CaCl_2$) were added, and energisation was obtained with 6 mM succinate. A control plus cyclosporine A (CyA, 0.2 μM) was performed to inhibit the mitochondrial permeability transition pore (MPTP) opening. EGTA was added at the end of each experiment to obtain the basal level.

2.7. Mitochondrial respiratory chain enzymatic activities

Before analysis, cycles of freezing–thawing were applied to the