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The antioxidant tempol decreases acute pulmonary thromboembolism-induced hemolysis and nitric oxide consumption

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ABSTRACT

Introduction: Acute pulmonary thromboembolism (APT) is a critical condition associated with acute pulmonary hypertension. Recent studies suggest that oxidative stress and hemolysis contribute to APT-induced pulmonary hypertension, possibly as a result of increased nitric oxide (NO) consumption. We hypothesized that the antioxidant tempol could attenuate APT-induced hemolysis, and therefore attenuate APT-induced increases in plasma NO consumption.

Materials and Methods: APT was induced in anesthetized sheep with autologous blood clots. The hemodynamic effects of tempol infused at 1.0 mg/kg/min 30 min after APT were determined. Hemodynamic measurements were carried out every 15 min. To assess oxidative stress, serum 8-isoprostanes levels were measured by ELISA. Plasma cell-free hemoglobin concentrations and NO consumption by plasma samples were determined. An *in vitro* oxidative AAPH-induced hemolysis assay was used to further validate the *in vivo* effects of tempol.

Results: APT caused pulmonary hypertension, and increased pulmonary vascular resistance in proportion with the increases in 8-isoprostanes, plasma cell-free hemoglobin concentrations, and NO consumption by plasma (all $P < 0.05$). Tempol attenuated the hemodynamic alterations by approximately 15-20% and blunted APT-induced increases in 8-isoprostanes, in cell-free hemoglobin concentrations, and the increases in NO consumption by plasma ($P < 0.05$). Tempol dose-dependently attenuated AAPH-induced *in vitro* hemolysis ($P < 0.05$).

Conclusions: Our findings are consistent with the idea that antioxidant properties of tempol decrease APT-induced hemolysis and nitric oxide consumption, thus attenuating APT-induced pulmonary hypertension.

Introduction

Acute pulmonary thromboembolism (APT) is a critical condition associated with acute pulmonary hypertension secondary to obstruction of pulmonary vessels and aggravated by active vasoconstriction of the pulmonary vascular bed [1]. While it is widely acknowledged that a variety of vasoactive mediators are involved in APT-induced pulmonary hypertension [2,3], recent studies have implicated a new mechanism involving increased hemolysis as an important factor contributing to the hemodynamic derangement during APT [4–6]. It is now becoming clear that hemolysis promotes the release of arginase with subsequent depletion of l-arginine [4,5], the substrate for nitric oxide (NO) synthesis, and increases NO consumption by cell-free hemoglobin [6], thus reducing NO bioavailability.

The identification of key mechanisms contributing to pulmonary vasoconstriction during APT may lead to improved pharmacological management [2]. In this respect, increased oxidative stress during APT [7–9] may impair NO availability during APT and contribute to pulmonary hypertension. In line with this suggestion, we have recently shown that the antioxidant drug tempol attenuated APT-induced pulmonary hypertension [10], and this effect may be due to improved NO bioavailability resulting of less NO inactivation by reactive oxygen species (ROS) [7]. However, oxidative stress may directly induce erythrocyte lysis [11–13], enhance cell-free hemoglobin concentrations, and impair NO bioavailability as a result of NO scavenging (consumption) by decompartmentalized hemoglobin, as previously shown in APT [6] and in other clinical conditions [14–19].

In the present study, we hypothesized that the antioxidant tempol could attenuate APT-induced hemolysis and therefore lower APT-induced increases in plasma NO consumption. Indeed, tempol is a nitroxide that has shown antioxidant effects in a variety of disease models involving oxidative stress [20,21]. This drug interfered with key pathogenetic mechanisms of diseases affecting the kidneys and

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the cardiovascular, nervous, and gastrointestinal systems [20,21]. To validate our experimental findings in animals, we have also studied the effects of tempol on *in vitro* hemolysis induced by increased oxidative stress [22].

Materials and Methods

Animal Model and Hemodynamic Measurements

This study complied with guidelines of Faculty of Medicine of Ribeirão Preto, University of São Paulo. Animals were handled according to guiding principles published by the National Institutes of Health (NIH). Twenty one male lambs weighing 18 ± 3 kg were used in this study. The animals were anesthetized with intramuscular ketamine and xylazine (15 and 0.1 mg/kg, respectively), relaxed with pancuronium (0.1 mg/kg bolus and 0.5 mg/h; i.v.), tracheally intubated, and their lungs were mechanically ventilated with room air using a volume-cycled respirator (C.F.Palmer, London, UK). The tidal volume was 15 ml/kg and the respiratory rate was adjusted to maintain a baseline physiologic arterial carbon dioxide tension. Anesthesia was maintained with intramuscular injections of ketamine (5–7 mg/kg) and midazolam (0.5-1 mg/kg) every 30 min.

A saline-filled catheter was placed into the left femoral artery and left jugular vein for mean systemic arterial pressure monitoring via a pressure transducer and fluid or drug administration, respectively. A 7.5F balloon-tipped Swan–Ganz thermodilution catheter was placed into the pulmonary artery via the left femoral vein, its correct location being confirmed by detection of the typical pressure wave of this artery. The catheter was connected to pressure transducers to allow the monitoring of mean pulmonary artery pressure (MPAP), central venous pressure, and pulmonary artery occlusion pressure. The transducers were zeroed at the level of the right heart and recalibrated before each set of measurements. Thermodilution cardiac output was determined in triplicate by injecting 3 ml of saline and the results were recorded on a computerized system (Monitor DC Baxter, Edwards Critical Care Vigilance, Irvine, CA). The heart rate (HR) was measured using a surface electrocardiogram (lead I). The cardiac index (CI), systemic vascular resistance index (SVRI), and pulmonary vascular resistance index (PVRI) were calculated by standard formulae [23].

A polyethylene catheter was inserted into the right ventricle (RV) via the right jugular vein to monitor right ventricular pressures, which were recorded using a data acquisition system (MP150CE; Biopac Systems Inc. CA, USA) connected to a computer (Acknowledge 3.2, for Windows). The first derivative of right ventricular pressure (dP/dt) was calculated and the maximum rate of isovolumic pressure development (dP/dt max) was used as an index of contractility.

Venous blood samples (2.5 ml/kg) were collected and allowed to clot for at least 60 min, and then cut into 2- to 3-mm cubes. In the present study, APT was induced by infusing autologous clots (250 mg/kg) for 5–10 min via a large-bore cannula placed in the right atrium. This model of APT is very similar to a previously reported [24,25]. After at least 20 min for stabilization, baseline (BL) hemodynamic measurements were carried out.

Experimental Protocols

Complete hemodynamic evaluation was performed 15 and 30 min (E15 and E30 time-points, respectively) after APT was induced and the animals were randomly assigned to one of three experimental groups: (1) Sham group (n = 7), non-embolized animals which received only saline infusions for 120 min; (2) APT group (n = 7), in which the animals were embolized and received only saline infusions; (3) APT + tempol group (n = 7), in which animals were embolized and received an infusion of tempol (St. Louis, MO, USA) at 1.0 mg/kg/min throughout the study (for 90 min) starting immediately after E30 time-point. The dose of tempol used in this study was chosen with basis on previous studies showing beneficial hemodynamic effects of this drug during APT [10]. Additional hemodynamic evaluations were performed 45, 60, 75, 90, 105, and 120 min after embolization (E45, E60, E75, E90, E105, and E120 time points, respectively).

Arterial blood samples were collected into standard Vacutainer tubes (Becton-Dickinson, Brazil) containing heparin at baseline and 120 min after lung embolization for plasma free hemoglobin and plasma NO consumption measurements. The tubes were either centrifuged at 900 ×*g* immediately blood drawing, and plasma and serum samples were stored at −80 °C until used for oxidative stress determination (as described below).

Assessment of 8-Isoprostane Levels in Serum

To assess oxidative stress, serum isoprostanes (8-isoPGF2α) concentrations were measured with commercially available enzyme-linked immunosorbent assay kits (Cayman Chemical Co., Ann Arbor,MI, USA), according to the manufacturer's instructions [26].

NO Consumption Assay

We used a previously described NO consumption assay [14,27] to assess NO consumption by plasma samples. Briefly, a solution of 40 μM DETA NONOate (Cayman Chemical, Ann Arbor, MI) in PBS (pH 7.4) was prepared in a glass vessel purged with nitrogen inline with an NO chemiluminescence analyzer (Sievers Model 280 NO Analyzer, Boulder, CO, USA) to produce a steady-state NO signal of about 40–60 mV. This signal is generated by the decay of DETA-NONOate and the release of NO from DETA NONOate, thus producing a stable baseline signal. Thereafter, we injected 50 μL of plasma samples in triplicate, which produced decreases in baseline NO signal (NO consumption, mV).

The data were analyzed with the software program ORIGIN Version 6.1 (OriginLab, Northampton, MA) and the area under the curve of decreasing NO signal over time was measured [14,27] for each plasma sample. The interassay coefficient of variation was below 6%.

Measurement of Hemoglobin Concentrations

The plasma hemoglobin concentrations were measured with commercially available hemoglobin detection kit (Cat.#K013-H1, Arbor Assays, Ann Arbor, MI, USA) according to manufacturer's instructions.

Oxidative Hemolysis Assay

To further validate our *in vivo* results, we studied the effects of tempol on *in vitro* hemolysis induced by increased oxidative stress generated by a water-soluble radical initiator, 2,2'-azobis-(amidinopropane) dihydrochloride (AAPH) [22] using previously described methodologies [11–13]. Briefly, sheep erythrocytes at a 5% suspension in PBS (150 mM NaCl, 8.1 mM Na_2HPO_4, and 1.9 mM NaH_2PO_4, pH 7.4) were incubated with 50 mM AAPH (Sigma; St. Louis, MO, USA) at 37 °C for 5 min. The reaction mixture was shaken gently during incubation. The extent of hemolysis was determined spectrophotometrically as previously described [28]. Briefly, aliquots of the reaction mixture were diluted with 0.15 mM NaCl and centrifuged at 2500 rpm for 8 min to separate the red blood cells (RBCs). The % hemolysis was determined by measuring the absorbance of the supernatant at 540 nm and compared with that obtained after complete hemolysis induced by mixing the same RBCs suspension with distilled water. To examine the effects of tempol on *in vitro* hemolysis induced by AAPH, tempol (0, 0.1, 0.25, 0.5, 1.0, 5.0 mM) was added 5 min before adding AAPH.