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The action of n-propyl gallate on gluconeogenesis and oxygen uptake in the rat liver

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ABSTRACT

In the present study the metabolic actions of n-propyl gallate on hepatic gluconeogenesis, oxygen uptake and related parameters were investigated. Experiments were done in the isolated perfused rat liver. n-Propyl gallate inhibited gluconeogenesis and stimulated oxygen uptake at concentrations up to 200 μM. The inhibitory effects on lactate gluconeogenesis (ED_{50} 86.4 μM) and alanine gluconeogenesis were considerably more pronounced than those on glycerol and fructose gluconeogenesis. n-Propyl gallate also stimulated oxygen uptake in both the mitochondrial (63%) and microsomal (37%) electron transport chains. The first one is due mainly to the oxidation of n-propanol, as a metabolite of the first step of n-propyl gallate transformation. The second one results from a direct stimulation of the microsomal electron transport chain. n-Propyl gallate inhibited pyruvate carboxylation (ED_{50} 142.2 μM) in consequence of an inhibition of pyruvate transport into the mitochondria an effect not found for gallic acid. This is probably the main cause for glucose output inhibition. Secondary causes are (1) deviation of intermediates for the production of NADPH to be used in microsomal electron transport; (2) deviation of glucose 6-phosphate for glucuronidation reactions; (3) gluconeogenesis inhibition by n-propanol, produced intracellularly from n-propyl gallate. Inhibition of mitochondrial energy metabolism is not significant in the range up to 200 μM, as indicated by the very small effect on the cellular ATP levels (5% decreased). n-Propyl gallate can be considered a kind of metabolic effector, whose actions on the liver metabolism are relatively mild although they can become harmful for the organ and the whole organism at high doses and concentrations.

1. Introduction

n-Propyl gallate (n-propyl 3,4,5-trihydroxybenzoate) is an ester formed by the condensation of gallic acid with n-propanol. It has been added to foods (especially oils and fats), cosmetics, hair products, adhesives and lubricants [1] to prevent oxidation. n-Propyl gallate also occurs in many products of plant origin. In green tea, for example, the gallic acid moiety is one of the most common structures among its phenolic compounds, free gallic acid and n-propyl gallate being particularly abundant [2]. Hepatotoxic actions of green tea have been partly attributed to its n-propyl gallate and free gallic acid contents. In mice n-propyl gallate doses of 100 and 200 mg/kg, can increase plasma levels of alanine aminotransferase by 57% and 400%, respectively [2]. In earlier studies with isolated rat liver hepatocytes and mitochondria the actions of high n-propyl gallate concentrations were investigated [1,3]. It was found that n-propyl gallate, at concentrations of 1–2 mM, causes cellular ATP depletion and cell death. These effects can be attenuated by fructose. The authors of these studies [1,3] concluded that the mitochondria are an important target of the toxic action of n-propyl gallate and that this action can be influenced by the cellular energy status. An action on mitochondrial energy metabolism has been reiterated recently in a study in which the actions of several alkyl esters were investigated in mouse hepatocytes, mouse sarcoma 786A and mouse mammary carcinoma TA3 cell lines and its multiresistant variant TA3-MTX-R [4]. The alkyl esters of gallic acid, including n-propyl gallate, inhibit respiration of those cells more effectively than free gallic acid. The order of decreasing potency is n-octyl- ≈ iso-amyl- ≈ n-amyl- ≈ iso-butyl- > n-butyl- > iso-propyl- > n-propyl gallate ≫ gallic acid. According to the study [4], the alkyl gallates act by blocking the mitochondrial electron flow, especially at the segment NADH-coenzyme Q, impairing ATP synthesis, an event that could lead to cell death. All tumor cells are more sensitive to the alkyl gallates than the mouse hepatocytes. In the various cancer cells n-propyl gallate produces 50% inhibition of respiration at concentrations between 575 and 800 μM; in mouse hepatocytes 50% inhibition can be expected to occur at concentrations above 1.3 mM.

Based on the n-propyl gallate concentrations that are active on mitochondrial respiration of hepatocytes, above 1 mM [2,4], in vivo

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metabolic effects of the compound can only be expected at very high doses. Nevertheless, it has been recently reported that n-propyl gallate and gallic acid act as free-radical scavengers at concentrations up to 200 μM [5]. Furthermore, gallic acid inhibits gap-junctional intercellular communication in the same concentration range [5]. It must also be added that hepatic transformation of n-propyl gallate generates n-propanol [3], a compound whose metabolic transformation is similar to that of ethanol [6,7], an inhibitor of hepatic gluconeogenesis in consequence of its transformation reactions [7]. All these facts prompted us to investigate possible metabolic actions of n-propyl gallate at concentrations well below 1–2 mM. This purpose can be best achieved by measuring metabolic pathways, such as gluconeogenesis, that are highly sensitive to changes in cell integrity or to relatively small changes in the intracellular concentration of key metabolic intermediates. For measuring gluconeogenesis and associated pathways the isolated perfused rat liver was used. Additionally, sub-cellular fractions, such as microsomes, were used for complementary measurements of several enzymatic activities. The latter should allow to suggest possible mechanisms for the observed effects.

2. Material and methods

2.1. Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. n-Propyl gallate and gallic acid and all enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium [^{14}C]bicarbonate (specific activity of 58 Ci/mmol) and labeled octanoate ([1-^{14}C]octanoate), were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals were from the best available grade (98–99.8% purity).

2.2. Animals

Male albino rats (Wistar), weighing 180–220 g, were fed ad libitum with a standard laboratory diet (Nuvilab®, Colombo, Brazil). In most experimental protocols, the rats were starved for 24 h before the surgical removal of the liver. All experiments were done in accordance with the internationally accepted recommendations in the care and use of animals.

2.3. Liver perfusion and analytics

For the surgical procedure, the rats were anesthetized by intraperitoneal injection of thiopental (50 mg/kg). Hemoglobin-free, non-recirculating perfusion was performed [7]. After cannulation of the portal and cava veins, the liver was positioned in a plexiglass chamber. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37 °C. The flow, provided by a peristaltic pump, was between 30 and 33 mL/min. When perfused with substrate-free perfusion medium livers from 24-h fasted rats respire mainly at the expense of endogenous fatty acids [9]. In the present work, gluconeogenesis from four different substrates was measured: lactate, alanine, fructose and glycerol.

Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their metabolite contents. The following compounds were assayed by means of standard enzymatic procedures: glucose, lactate, pyruvate and n-propanol [10]. The oxygen concentration in the outflowing perfusate was monitored continuously, employing a Teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate [9]. Metabolic rates were calculated from input–output differences and the total flow rates and were referred to the wet weight of the liver.

In those experiments in which tracer [1-^{14}C]octanoate was infused for $^{14}CO_2$ production the outflowing perfusate was collected in Erlenmeyer flasks in 2 min fractions. The Erlenmeyer flasks were rapidly and tightly closed with rubber stoppers to which scintillation vials containing phenylethylamine were fastened by means of stainless steel wires. Trapping of the $^{14}CO_2$ in the phenylethylamine was achieved by acidification of the perfusate with a HCl solution which was injected into the flasks through the rubber stoppers [11]. Radioactivity was measured by liquid scintillation spectroscopy. The scintillation solution was: toluene/ethanol (2/1) containing 5 g/L 2,5-diphenyloxazole and 0.15 g/L 2,2-p-phenylene-bis(5-phenyl-oxazole).

The hepatic contents of adenine nucleotides were measured after freeze-clamping the perfused liver with liquid nitrogen. The freeze-clamped livers were extracted with perchloric acid. The extract was neutralized with K_2CO_3 and AMP, ADP, and ATP were assayed by means of standard enzymatic procedures [8].

2.4. Cell fractionation procedures

Microsomes were isolated by differential centrifugation [12–14]. Rats were decapitated and their livers excised, cut into small pieces with scissors and washed with a cold (4 °C) aqueous solution (isolation medium) containing 150 mM KCl, 0.1 mM phenylmethanesulfonylfluoride (PMSF) and 10 mM Tris–HCl (pH 7.4). After suspension in 10 volumes of the isolation medium, the tissue was homogenized with a Dounce homogenizer. The homogenate was filtered through gauze and centrifuged at 2550 × *g* for 10 min in a refrigerated centrifuge. The supernatant was again centrifuged in two steps of 7100 and 12,400 × *g* for 10 min. Finally, the supernatant of the last centrifugation was collected and centrifuged at 105,000 × *g* for 1 h. The pellet containing the microsomal fraction was suspended in cold isolation medium at a final protein concentration of 20 mg protein/mL.

For mitochondria isolation rats were decapitated, their livers removed immediately and cut into small pieces. The fragments were suspended in a medium containing 0.2 M mannitol, 75 mM sucrose, 2.0 mM Tris–HCl (pH 7.4), 0.2 mM EGTA, and 50 mg% fatty acid-free bovine-serum albumin. Homogenization was carried out in the same medium by means of a van Potter–Elvehjem homogenizer. After homogenization, the mitochondria were isolated by differential centrifugation [15,16] and suspended in the same medium, which was kept at 0–4 °C.

Protein content of the microsomal and mitochondrial suspensions was measured using the Folin–phenol reagent and bovine-serum albumin as a standard [17].

2.5. Enzyme assays

Glucose 6-phosphatase was assayed using the microsomal suspension prepared as described above. The incubation medium contained 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.2), 15 mM glucose 6-phosphate, and 0.1–0.2 mg microsomal protein [18,19]. After 20 min incubation at 37 °C, the reaction was stopped by the addition of one volume of 5% trichloroacetic acid and phosphate release was measured [20].

The D-fructose 1,6-bisphosphatase activity was assayed using the supernatant of the 105,000 × *g* centrifugation obtained during the microsome isolation procedure [19,21]. The reaction mixture contained 0.4–0.8 mg protein/mL, 100 mM Tris–HCl (pH 8), 12 mM $MgCl_2$, 1 mM D-fructose 1,6-bisphosphate, and 5 mM cysteine. After 20 min incubation at 38 °C, the reaction was interrupted by the addition of one volume of 5% trichloroacetic acid and phosphate release was measured [20].