Abstract
The ability of three fatty acids and their respective ethyl esters, to promote generation of reactive oxygen species (ROS), was compared in a preparation of rat brain synaptosomes. Arachidonic but not palmitic or linoleic acids promoted ROS generation. Ethyl esterification of each fatty acid significantly enhanced ROS production and also levels of lipid peroxidation. Pro-oxidant activity was enhanced by fatty acids, proportionally to their degree of unsaturation. Since ethanol consumption is known to lead to esterification of membrane lipids, this transformation may in part account for the ROS-promoting potential of alcohol.

Key words: Ethanol; Free radical; Fatty acids

1. Introduction

Ethanol exposure is known to lead to a loss of unsaturated fatty acids within membranes, [1]. Another effect of ethanol consumption on membrane lipids is the formation of ethyl esters of fatty acids. This non-oxidative pathway of ethanol metabolism has been found to be present to a varying degree in a large range of tissues derived from both human and animal studies, and has been proposed to be important in the pathophysiology of ethanol-induced damage [2]. The formation of fatty acid ethyl esters after ethanol consumption occurs predominantly in the pancreas and liver, but also within the brain [3]. These esters have been proposed to produce neurological damage perhaps by causing both membrane disordering and disruption of mitochondrial function [4], and their accumulation may be a factor in the pathogenesis of fetal alcohol syndrome [5].

Some of the harmful effects of ethanol have also been attributed to the induction of metabolic processes which lead to the generation of excessive levels of reactive oxygen species [ROS]. Such changes have been invoked as underlying ethanol-induced liver damage [6-8] but there are also data suggesting a parallel sequence of events within nervous tissue [9].

The purpose of the current study was to inquire as to whether these two putative mechanisms underlying ethanol toxicity might be linked. Therefore, experiments examining the pro-oxidant potential of ethyl esters of fatty acids were conducted.

2. Experimental

2.1. Tissue preparation

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 125-150 g were utilized. These were decapitated and brains excised. The cerebrocortex was dissected out and stored at -70°C. The crude cerebral synaptosomal-mitochondrial fraction was prepared by homogenizing in 10 vols. of 0.32 M sucrose and centrifugation at 1800 x g for 10 min. The resulting supernatant fraction was then centrifuged at 31,500 x g for 10 min to yield the synaptosomal-mitochondrial P2 pellet. This was resuspended in HEPES buffer to a concentration of 0.1 g·eq./ml. The composition of the HEPES buffer was (mM): NaCl 120, KCl 2.5, NaH2PO4 1.2, MgCl2 0.1, NaHCO3 5.0, glucose 6.0, CaCl2 1.0, and HEPES 10, pH 7.4.

2.2. Assay for oxygen reactive species formation

Reactive oxygen species (ROS) were assayed using 2′7′-dichlorofluorescin diacetate (DCFH-DA), which is de-esterified within cells to the ionized free acid, DCFH, where it is accumulated [10]. DCFH is capable of being oxidized to the fluorescent 2′7′-dichlorofluorescein by reactive oxygen. 100 μl P2 suspension was incubated with 5 μM DCFH-DA (added from a stock solution of 1.25 mM in ethanol) in a final volume of 2 ml of 40 mM Tris-HCl, pH 7.4, at 37°C for 15 min. After this loading with DCFH-DA, the fractions were incubated for a further 60 min, sometimes in the presence of 100 μM of a fatty acid or its ethyl ester. At the beginning and at the end of the 60 min incubation, fluorescence was monitored on a Perkin-Elmer Spectrofluorometer, with excitation wavelength at 488 nm, and emission wavelength 525 nm. The DCFH assay method has previously been validated [11], and reflects the balance of production of active oxygen species over their removal by antioxidant defences.

2.3. Lipid peroxidation

The quantitation of lipid hydroperoxide utilized a method which involves the formation of a colored complex between Xylenol orange ([o-cresolsulfophthalein-3,5-bis-methyliminodiacetic acid] sodium salt) and ferric iron [12]. 20 μl P2 suspensions were incubated at 37°C for 1 h in the presence of 10 μl of ethanol containing a fatty acid or its ethyl ester, to give a final concentration of 100 μM. In order to minimize the hazard of direct fatty acid ethylation by ethanol, solutions were prepared immediately prior to use. Control samples received ethanol vehicle. Then, 0.98 ml of a freshly prepared mixture was added to each sample tube. This mixture consisted of 0.2 ml of 25 mM Fe(NH4)2(SO4)2 in 2.5 mM H2SO4 combined with 20 ml of 125 mM Xylenol orange in 100 mM sorbitol solution. After a further 20 min incubation at 21°C, absorption at 600 nm was determined. The presence of fatty acids or their derivatives at the same concentrations as those used in this study but in the absence of tissue, had no effect on the rate of formation of the ferric iron-Xylenol orange complex, implying that these chemicals contained no detectable peroxides.

2.4. Materials

DCFH-DA, (Cbz-Arg-NH2)2 was purchased from Molecular Probes Inc. (Eugene, OR), while DCF required for calibration was obtained from Polysciences Inc. (Warrington, PA). Protein assay dye was from Polysciences Inc. (Warrington, PA). Protein assay dye was from Polysciences Inc. (Warrington, PA).
2.6. Statistical analyses

Differences between groups were assessed by one-way analysis of variance followed by Fisher's Least Significant Difference Test. The acceptance level of significance was \( P < 0.05 \) using a two-tailed distribution. Each value presented was obtained from tissue derived from 5–8 individual rats.

3. Results

The rate of generation of ROS as judged by DCFH oxidation in the P2 fraction was inhibited by palmitate, unaffected by linoleate and enhanced by arachidonate, each at a final concentration of 100 \( \mu \text{M} \). In each case, ROS production was significantly elevated by ethyl esters of these compounds, over the rate achieved by the corresponding free fatty acid (Fig. 1). Thus the effect of fatty acids upon the rate of synaptosomal oxidation of the DCFH probe was strictly governed by two factors; the degree of unsaturation of the fatty acid, and whether or not it was present as the ethyl ester.

A more indirect means of estimation of reactive oxygen species levels, is by assay of the appearance of hydroperoxide products of oxidative degradation of lipids. Using this method, no unesterified fatty acid tested was found to promote lipid peroxidation. Both linoleic and arachidonic esters significantly enhanced production of lipid peroxides, this being most pronounced with the most unsaturated derivative, ethyl arachidonate (Fig. 2).

4. Discussion

The responsiveness of the assay involving quantitation of lipid peroxides, to various fatty acids, was different to that obtained using the rate of DCFH oxidation as an index of pro-oxidant activity. Lipid peroxidation could be stimulated by ethyl linoleate and ethyl arachidonate to a greater proportion than could DCFH oxidation. This may reflect the autocatalytic potential of lipid peroxidation. A recent report [14] also finds a close correlation between the degree of unsaturation of fatty acids and their potential for promotion of lipid peroxidation.

The most precise correlation between pro-oxidant effects and molecular structure was obtained using the DCFH oxidation assay. This may be due to the fact that the rate of DCFH oxidation constitutes a more direct measurement of reactive oxygen species, a opposed to the detection of products of the oxidative degradation of lipids. Nevertheless, using either assay, two factors were clearly related to the ability of fatty acids to promote ROS production; namely the degree of fatty acid unsaturation and whether or not the fatty acid was esterified. Using the DCFH oxidation assay, these two factors appeared to act in concert, in an additive rather than a synergistic manner.

The chemical basis for relation between susceptibility to oxidation and degree of unsaturation of fatty acids is clear. However, in the case of ethyl esters of fatty acids, the basis for an analogous relationship, accounting for an increased capacity to promote ROS formation, is not as obvious. Results could not be attributed to differential detergent-like effects of fatty acids and their derivatives, since basal rates of DCFH oxidation by synaptosomes were not altered in the presence of 1.6% (v/v) Triton X-100, or after repeated freezing and thawing of this fraction.

After ethanol consumption, concentrations of fatty acid ethyl esters can accumulate in concentrations as high as 115 \( \mu \text{M} \) in human myocardium [15]. Concentrations of 25 \( \mu \text{M} \) of ethylated fatty acids have been reported for cerebral cortex of acutely intoxicated humans, the bulk of these being derived from unsaturated fatty acids [3]. The concentrations of fatty acid esters used in the present study are thus physiologically relevant.

Ethanol enhances release of fatty acids from lipids, possibly by activation of phospholipase A2 [1]. This release is partially selective for highly polyunsaturated fatty acids such as arachidonic acid [16], and occurs also in synaptosomal fractions [17]. The subsequent esterification by the brain of such liberated fatty acids, is effected by several glutathione S-transferases...
and these esters are reported to disrupt cerebral mitochondrial function [4]. A relationship between ethanol toxicity and ethyl esters of fatty acids is further suggested by the report that inadequate nutrition, which is known to exacerbate the effects of ethanol, can also activate fatty acid ethyl ester synthase activity [18]. It may be that excessive rates of formation of reactive oxidizing species effected by ethyl esters of unsaturated fatty acids may be the final step in this chain of events set in motion by ethanol.

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References