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Long-chain N-acylethanolamines inhibit lipid peroxidation in rat liver mitochondria under acute hypoxic hypoxia

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Abstract

Two long-chain N-acylethanolamines (NAEs), N-palmitoyl- (NPE) and N-stearoylethanolamine (NSE), are shown to inhibit an in vitro non-enzymatic Fe^{2+}-induced free radical oxidation of lipids in the liver mitochondria of rats with hypoxic hypoxia. NSE appeared to be more effective than NPE in suppressing some kinetic parameters of the Fe^{2+}-induced chemiluminescence. The inhibitory action of NAEs on non-enzymatic lipid peroxidation supports the idea that they possess membrane protective properties. © 1998 Published by Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Long-chain N-acylethanolamines (NAEs) exhibit a wide range of biological and pharmacological activities (Epps et al., 1982a; Gulaya et al., 1993a). Recently it has been shown that anandamide (N-arachidonoylethanolamine) and some other polyunsaturated NAEs bind to a cannabinoid receptor and serve as lipid agonists in brain and some other organs (Devane et al., 1992; Felder et al., 1993). A very low quantity of saturated NAEs (nanogram per gram of wet tissue) can be generated simultaneously with anandamide (Schmid et al., 1995) by an N-acylation-phosphodiesterase pathways in brain and other organs at normal conditions (for review see Schmid et al., 1996). Dramatically high levels of free saturated NAEs, particularly N-palmitoyl- (NPE) and N-stearoylethanolamines (NSE) approaching 0.5 μmol/g wet tissue, were detected in the infarcted area of canine myocardium (Epps et al., 1979,

Abbreviations: NAE, N-acylethanolamine; NPE, N-palmitoylethanolamine; NSE, N-stearoylethanolamine.

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1980) (see also the review of Schmid et al., 1990). In contrast to polyunsaturated NAEs, the biological role of saturated NAEs is not clear up to now. It was postulated that accumulation of NAEs, particularly 16:0 and 18:0, may imply a response of myocardial tissue to injury directed at minimizing cell damage and promoting survival (Epps et al., 1979). It is well known that one of the main reasons for cell damage and death is the peroxidative deterioration of membrane lipids (Poli et al., 1987). Therefore, it seems probable that the protective effect of NAE might be realized at least partly by the inhibition of free radical-induced lipid peroxidation. Indeed, it was demonstrated that some exogenous NAEs inhibit the formation of thiobarbituric acid reactive substances in isolated mitochondria of intact rat heart (Parinandi and Schmid, 1988) and in different tissues of mice with Lewis carcinoma (Gulaya et al., 1993b). *N*-palmitoylethanolamine was shown to protect neurons against unfavorable conditions in a concentration-dependent manner (Skaper et al., 1996).

However, there are no data concerning the effect of saturated NAEs on free radical-induced lipid peroxidation under acute oxygen deficiency. It is well documented that both hypoxia which is associated with abnormally low O_2 partial pressure and ischemia, which results from the occlusion of blood flow, show an increase in partially-reduced oxygen products, causing a high rate of lipid peroxidation (Emerit and Chaudiere, 1989). It is important to test whether saturated NAEs elicit an antioxidative effect under hypoxic conditions.

We now report that some saturated long-chain NAEs, NPE and NSE, can affect the Fe^{2+}-induced free radical oxidation of lipids in vitro in liver mitochondria of rats with acute hypoxic hypoxia.

2. Materials and methods

The investigation was carried out on 19 male Wistar rats weighing 200–250 g. We used a simple method of acute hypoxic hypoxia which includes a gradual diminution of oxygen concentration in air. Briefly, animals were kept up to 25 min in a hermetically closed tank (with a volume of 3 l) at room temperature. Recently we found that CO_2 may serve as protective agent against the peroxidative damage of polyunsaturated fatty acids (Melnichuk et al., 1998). That is why we used KOH as a CO_2 scavenger to exclude the influence of expired CO_2 on free radical-induced lipid oxidation. To maintain the normal gas pressure in the tank, helium was added instead of scavenged CO_2. The heart rate was recorded at the beginning of the experiment and its progressive fall was established. After 30 min of keeping the tank under experimental conditions all animals were dead. Therefore on the 25 min when the heart rate was 10–30 beats/min we started our investigation.

Experimental rats were divided into four groups. The first group consisted of normal rats. The liver of animals of the first group served as a source of control mitochondria. The second, third and fourth groups included rats with hypoxic hypoxia. The liver of rats of the second, third and fourth groups served as a source of mitochondria of hypoxic rats. Among them, the samples of the last two groups were treated by NPE and NSE, respectively.

Liver mitochondria were prepared by the method of differential centrifugation as indicated in the work of Sottocasa (1976). The final mitochondrial pellet was washed with 150 mmol/l NaCl solution which also served as the incubation medium. The washed pellet was resuspended in a sufficient volume of the incubation medium to give a final protein concentration of 2.5 mg/ml. All isolation procedures were carried out at 1–4°C.

NPE and NSE were added to the suspension of rat mitochondria of the third and fourth groups from ethanolic stock solutions (13 μl) just before the induction of non-enzymatic lipid peroxidation. Simultaneously, the same amount of ethanol was added to the samples of the second group. Then the mitochondrial suspension was incubated at 37°C for 15 min.

Free radical lipid oxidation in liver mitochondria was determined by a chemiluminescent (Chl) method in the presence of 50 μmol/l Fe^{2+} at 37°C (Parinandi and Schmid, 1988). The Chl kinetic

Table 1
The influence of gradual diminution of oxygen concentration in CO_2-free air on blood buffer-base equilibrium and mitochondrial malodialdehyde level in hypoxic rats (M ± S.E.; $n = 3$)

Time (min)	pH	pO_2 (mmHg)	pCO_2 (mmHg)	HCO_3^- (mmol/l)	Malondialdehyde (nmol/mg protein)
0	7.40 ± 0.03	74.6 ± 2.9	34.2 ± 1.9	22.4 ± 1.3	2.39 ± 0.04
25	6.88 ± 0.03[a]	27.3 ± 2.6[a]	37.6 ± 2.8	10.7 ± 0.6[a]	3.84 ± 0.26[a]

[a] $P<0.05$, compared with intact control.

parameters were recorded in absolute units (quant/s·4π) by a PKhL-01 chemiluminometer (Russia) equipped with a FEU 84-3 photomultiplier unit and an AkhLG2-01 computer and calculated by our specially developed computer program. The recorded parameters were: the maximal intensities of the rapid and slow peaks (I_r and I_s, respectively), the induction time (T_{Ind}) and the tangent of the slope of the kinetic curve (Tg α), as was described (Kuzmenko et al., 1997). The NPE and NSE in these experiments were added to the reaction mixture at a concentration of 100 μmol/l.

Lipid peroxidation was assayed as thiobarbituric acid reactive substances formed as measured by the thiobarbituric acid technique. Briefly, 1.0 ml of 0.1 M phosphate buffer (pH 7.35), 0.5 ml of 35% trichloracetic acid, 1.0 ml of 0.75% thiobarbituric acid (in water) were added to 0.2 ml of mitochondrial suspension. The mixture was shaken thoroughly and heated in a water bath for 15 min at 100°C. The tubes were cooled to room temperature, 1 ml of 35% trichloroacetic acid was added to each of them and then they were centrifuged. The absorbance was measured at 532 nm against the appropriate blanks. Lipid peroxidation was expressed as nmol of malondialdehyde per mg protein. Quantification was based upon malondialdehyde molar extinction coefficient (Braughler et al., 1986).

The level of protein was measured by the method of Lowry et al. (1951). Data were analyzed by Student's t-test to determine significant differences between groups. The significance was established at $P < 0.05$.

3. Results and discussion

As was shown in Table 1, pH and pO_2 and HCO_3^- levels in blood drastically decreased without any changes of pCO_2 in experimental animals. Simultaneously, the level of malondialdehyde significantly increased. These data indicate that hypoxic hypoxia with metabolic acidosis and oxidative stress was developed.

We studied the effect of NAEs on non-enzymatic Fe^{2+}-induced lipid peroxidation in liver mitochondria of animals with acute hypoxic hypoxia which is known to be associated with activated lipid peroxidation (Emerit and Chaudiere, 1989). The results of our experiments suggest that NPE and NSE can reduce free radical induced lipid oxidation (see Table 2). Earlier it has been demonstrated that the treatment of heart mitochondria of intact rats by N-oleoylethanolamine in the range of 45–150 μmol/l concentrations caused a considerable reduction of malondialdehyde production induced by 100 μmol/l $FeSO_4$. However, in our experiments with liver mitochondria, we did not observe statistically significant effects of NPE and NSE in mitochondria of intact rats. This can probably be explained by the high amidase activity in liver contrary to the heart where this enzyme is not active under normal conditions (Schmid et al., 1990). To assess the kinetic parameters of non-enzymatic free radical-induced lipid oxidation in liver mitochondria of rats with acute hypoxia, the chemiluminescent method was used.

It is known that the first quickly developing flash (rapid flash I, see Fig. 1) depends on decomposition of hydroperoxides in the system with RO· production. The second slow flash of low level chemiluminescence depends on the initiation of free radical production due to the action of Fe^{2+} ions and O_2 interaction with lipids that reflects the maximal intensity of lipid peroxidative processes after addition of Fe^{2+}. The velocity of lipid peroxidation is characterized by the value of Tg α. T_{Ind}

Table 2
The influence of NAEs (100 μmol/l) on kinetic parameters of chemiluminescence in liver mitochondria of hypoxic rats (M ± S.E.)

Chemiluminescence parameters	Intact rats ($n = 5$)	Hypoxic control ($n = 4$)	Incubation with NPE ($n = 5$)	Incubation with NSE ($n = 5$)
Ir, 10^6 Quant/s·4π	5.59 ± 0.49	24.85 ± 3.82[a]	16.46 ± 2.51[a]	16.89 ± 3.92[a]
Is, 10^6 Quant/s·4π	16.57 ± 1.47	23.33 ± 0.59[a]	21.58 ± 0.27[b,a]	18.20 ± 1.31[b,c]
T, s	147 ± 12	25.7 ± 2.4[a]	23.6 ± 3.6[a]	24.2 ± 1.1[a]
Tg α	0.46 ± 0.013	0.56 ± 0.057	0.58 ± 0.09	0.38 ± 0.042[b]

[a] $P<0.05$, compared with intact control.
[b] $P<0.05$, compared with hypoxic control.
[c] $P<0.05$, compared with the third group (treated with NPE).

depends on the antioxidant potential of the biological system (Kuzmenko et al., 1997).

Table 2 demonstrates the effect of NPE and NSE on kinetic chemiluminescent parameters in liver mitochondria of hypoxic rats. It was found that preliminary addition of 100 μmol/l NPE and NSE into the mitochondrial suspension caused a significant diminution of the I_s value. The effect of NSE was more prominent than that of NPE. NSE caused an increase of T_{Ind} value by 54% and the reduction of Tg α by 32%. These results are in good agreement with earlier data (Parinandi and Schmid, 1988) and indicate that NAEs exert an inhibitory effect on the lipid peroxidation in mitochondria membranes of hypoxic animals. Earlier it was reported about the potential membrane protective properties of NAEs accumulated in the infarcted area of canine heart (Epps et al., 1979, 1980) and antiinflammatory properties of some of them (reviewed in Schmid et al., 1990). We suggest that the beneficial effect of NAEs on cell survival under ischemic and hypoxic conditions as least partly depends on their inhibitory influence on non-enzymatic free radical lipid oxidation.

However, we do not consider that NSE acts as a free radical scavenger. It is well established that NAE can easily penetrate into biological and model membranes (Gulaya et al., 1993a) resulting in modification of the membrane structure and physico-chemical properties (Epps and Cardin, 1987; Ambrosini et al., 1993) that may disturb the activating effect of Fe^{2+} on lipid peroxidation.

The more pronounced inhibitory effect of NSE, as compared with NPE, can probably be explained by the fact that NSE has a longer hydrocarbon chain, which could penetrate deeper into the membrane than the shorter NPE chain. As a consequence, NSE may modify membrane structure and properties more prominently. In any case, the effect of different NAEs on lipid peroxidation seems to be dependent on the length of acyl chain as was found for other effects of NAEs in the cell (Gulaya et al., 1993a).

Our present work compliments earlier results on the membrane protective effects of NAEs, as demonstrated with different models. *N*-oleoylethanolamine protected against increased Ca^{2+} permeability of isolated damaged mitochondria from rat heart (Epps et al., 1982b) and *N*-palmitoylethanolamine was shown to protect neurons against excitotoxic death induced by glutamate (Skaper et al., 1996). The production of saturated NAEs was actually found to be stimulated by glutamate treatment of cortical neurons (Hansen et al., 1995) and current data concerning this topic were reviewed (Hansen et al., 1998). We suggest that antioxidant effects of saturated NAEs may also be involved in their membrane protective properties.

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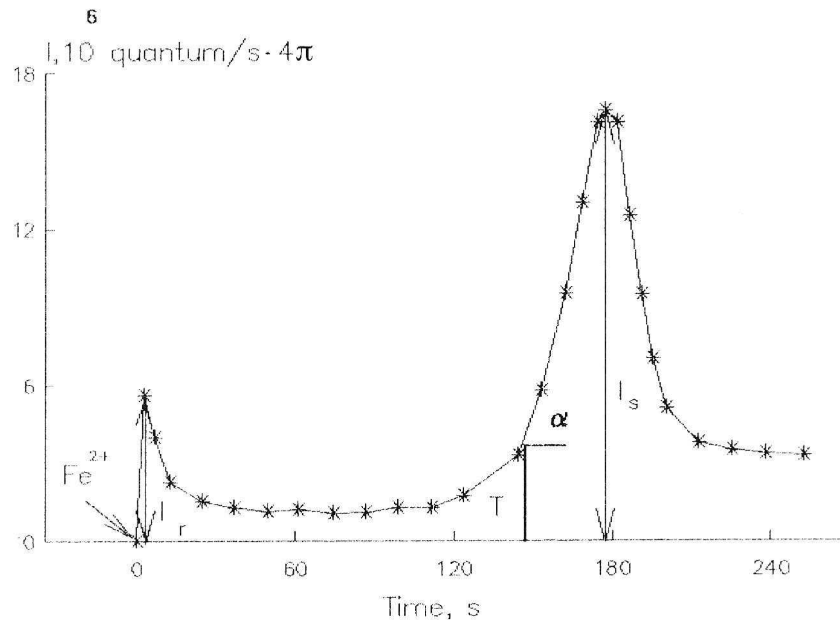

Fig. 1. The chemiluminescence (Chl) curve of the mitochondria of intact rats under the Fe^{2+}-induced (50 μmol/l) free radical oxidation. The following Chl kinetic characteristics were measured: intensity of rapid (I_r) and slow (I_s) flashes; the tangent of the slope of the ascending branch of the low flash (Tg α) and time between I_r and I_s (T_{Ind}).

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