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Effects of long-chain *N*-acylethanolamines on lipid peroxidation in cardiac mitochondria

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A long-chain *N*-acylethanolamine (*N*-oleoyl-2-aminoethanol) is shown to inhibit the production of thiobarbituric acid-reactive substances in rat heart mitochondria treated with Fe^{2+} or Fe^{3+}/ADP. The inhibition is concentration-dependent in the range 50–150 μM of the agent and can be nearly complete depending on the type and amount of the free radical-generating system. Structural analogues of *N*-acylethanolamine are inhibitory as well, but neither oleic acid nor ethanolamine has measurable effects. *N*-Oleoyl-2-aminoethanol affects peroxidation of linoleic acid micelles only minimally and has no effect on deoxyribose peroxidation.

N-Oleoyl-2-aminoethanol; Oxygen free radical; (Cardiac mitochondria)

1. INTRODUCTION

Long-chain *N*-acylethanolamines have been reported to occur in trace amounts in mammalian tissues [1] and to have anti-inflammatory [2,3] and other [4] activities of pharmacological interest. They were also shown to accumulate to levels of 500 nmol/g tissue in the experimental myocardial infarct in the dog [5,6]. In animal tissues they are generated by *N*-acylation of ethanolamine phospholipids catalyzed by a calcium-requiring transacylase [7–9] followed by phosphodiesterase activity [10]. Because *N*-acylethanolamines can stabilize mitochondrial membranes against a nonspecific permeability increase brought about by Ca^{2+}-releasing agents [11], we postulated that they may be produced as components of a potential defense mechanism against the progressive cell and tissue damage which occurs as the result of irreversible ischemic injury. We now report that *N*-acylethanolamines can also affect free radical-induced lipid peroxidation in cardiac mitochondria.

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Abbreviations: MDA, malondialdehyde; TBA, thiobarbituric acid; NOE, *N*-oleoyl-2-aminoethanol; NOP, *N*-oleoyl-3-aminopropanol; NOPD, *N*-oleoyl-3-aminopropane-1,2-diol

2. MATERIALS AND METHODS

Heart mitochondria were prepared [12] from male Sprague-Dawley rats weighing 170–250 g. The mitochondrial isolation buffer contained 220 mM mannitol, 70 mM sucrose, 5 mM Hepes (pH 7.4), 2 mM EGTA and 0.2% BSA. The final mitochondrial pellet was washed twice with Krebs-Ringer Hepes buffer (pH 7.4) [13], 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 3–5 mg/ml.

Lipid peroxidation in the mitochondria was induced at 37°C by the addition of $FeSO_4$ or $FeCl_3$/ADP (1 : 10) in a final volume of 0.1 ml which contained 0.5 ml of the mitochondrial preparation. *N*-Acylethanolamine (as *N*-oleoyl-2-aminoethanol, NOE), its analogues, and oleic acid were added to the incubation medium from ethanolic stock solutions (15 μl), and the same amounts of ethanol were added to controls. At the end of incubation, lipid peroxidation was assayed as malondialdehyde (MDA) formed, measured by the thiobarbituric acid (TBA) technique [14,15]: to 1.0 ml of the incubation medium, 1.0 ml of 10% trichloroacetic acid, 2.0 ml of 0.67% TBA (in 0.25 N HCl) and 0.01 ml of 2% butylated hydroxytoluene (in ethanol) were added. The mixture was vortex-mixed thoroughly and heated in a water bath at 80°C for 15 min. The tubes were cooled to room temperature, centrifuged and the absorbance

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was measured at 532 nm against appropriate blanks. A standard curve was prepared with MDA bis-methyl acetal and lipid peroxidation was expressed as nmol MDA/time per mg protein. Because the TBA-reactivity assay is not specific for MDA, this method may also include certain other oxidation products [16].

Micelles of linoleic acid were prepared by dispersing 20 μl of the acid in 10 ml of 15 mM Hepes buffer (pH 7.4) which also contained 140 mM NaCl and 3.6 mM KCl, and the effect of various concentrations of NOE on Fe^{2+}-induced peroxidation was determined. After 60 min incubation, TBA-reactive substances were measured according to Gutteridge [17] and expressed as absorbance units at 532 nm. Similarly, deoxyribose (2 mM) was treated with $FeSO_4$ in the presence of different concentrations of NOE and the extent of deoxyribose degradation was expressed as absorbance units at 532 nm at the end of 60 min incubation [17].

The effect of NOE on the MDA-TBA chromophore formation was studied by incubating MDA bis-methyl acetal in 1.0 ml of the incubation medium at a final concentration of 5 nmol/ml for 30 min with the desired concentration of NOE, followed by TBA assay as described above.

3. RESULTS AND DISCUSSION

The formation of MDA in rat heart mitochondria induced by either $FeSO_4$ or $FeCl_3$/ADP was markedly inhibited by NOE in a concentration-dependent manner (fig.1). The inhibitory effect was more pronounced in the Fe^{3+}/ADP system, especially at lower concentrations of the inducing agent (fig.1B) where it approached and even exceeded control values. In this system, maximal effects were observed at NOE concentrations between 45 and 150 μM. In both systems, the effects of NOE leveled off at concentrations exceeding 150 μM.

Structural analogs of NOE, namely *N*-oleoyl-3-aminopropanol (NOP) and *N*-oleoyl-3-aminopropane-1,2-diol (NOPD), were slightly less effective as inhibitors of MDA formation compared to NOE (table 1). Oleic acid and ethanolamine or their equimolar mixtures at 90 and 150 μM showed no effect on MDA formation induced by Fe^{3+}/ADP (50/500 μM), i.e. MDA values were within $\pm$ 1% of those obtained by incubation in the absence of oleic acid and/or ethanolamine (not shown).

When NOE was tested for its effects on peroxidation of linoleic acid micelles induced by 50 μM Fe^{2+}, very little inhibition was observed. NOE at 90 and 150 μM resulted in 97 and 89%, respectively, of TBA-reactive substances formed in its absence, and even 300 μM NOE reduced this production no further than to 81%. Similarly, NOE at 45–300 μM had no effect on Fe^{2+}-induced deoxyribose

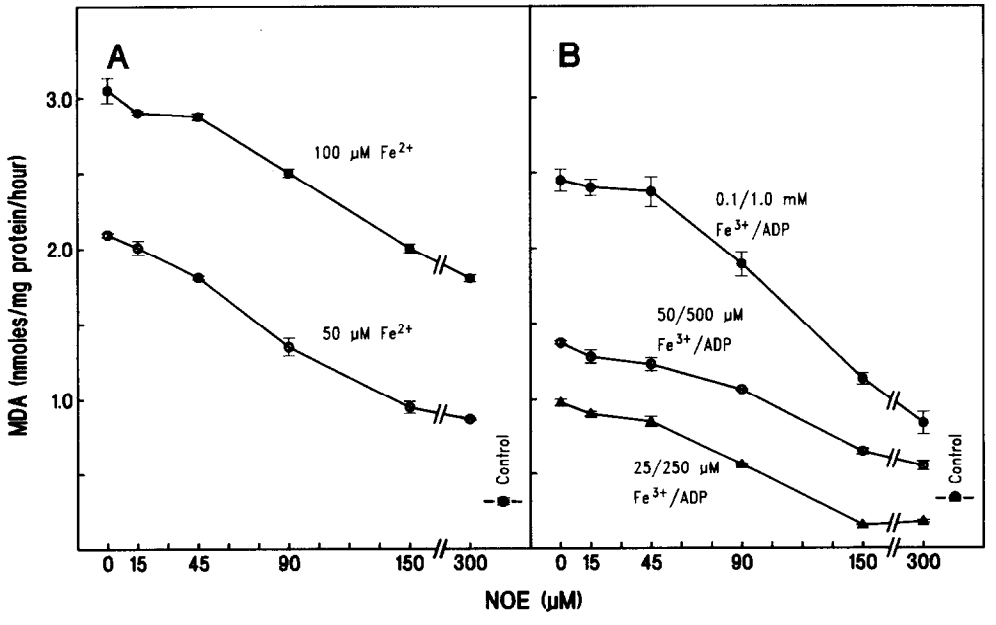

Fig.1. Effect of NOE on MDA formation induced by Fe^{2+} (A) and Fe^{3+}/ADP (B) in rat heart mitochondria for 60 min. Control values were obtained in the absence of NOE and inducing agents. Incubations were as described in section 2. The MDA assay may include other TBA-reactive substances. Values represent averages of three independent assays $\pm$ SD.

Table 1

Effects of NOE and its structural analogues on MDA formation in rat heart mitochondria

Additions	Inducing agent	
	Fe^{2+} (50 μM)	Fe^{3+}/ADP (50/500 μM)
None	100	100
NOE (90 μM)	61	58
(150 μM)	42	42
NOP (90 μM)	68	71
(150 μM)	55	58
NOPD (90 μM)	74	75
(150 μM)	68	63

Results are expressed as % MDA formed. Mitochondrial preparations were incubated for 60 min at 37°C with the appropriate agents as described in section 2. MDA (including other oxidation products) was measured by TBA assay

degradation, at either 50 or 100 μM $FeSO_4$ (fig.2).

We interpret these data to indicate that NOE inhibits MDA production by iron-induced lipid peroxidation primarily or only if the lipid substrate is the component of a membrane. It is also possible that free radical-induced peroxidation of cardiac mitochondria yields some nonlipid-derived TBA-reactive substances other than MDA, as was recently demonstrated with rat heart membranes [16]. Our present data do not indicate whether NOE acts as a free radical scavenger, stabilizes mitochondrial membranes against free radical attack, or inhibits the chain reaction leading to MDA. We have, however, considered a potential effect of NOE on the MDA assay per se, i.e. on MDA-TBA chromophore formation. We therefore tested the effect of 45, 90 and 150 μM NOE directly with MDA over a 60 min incubation but found a slight enhancement (up to 8% over control values of the MDA-TBA chromophore as measured at 532 nm). We therefore conclude that the observed effects are not due to procedural artifacts but could occur in vivo and could be linked to some of the biological effects of long-chain *N*-acylethanolamines, especially their anti-inflammatory activity [2,3].

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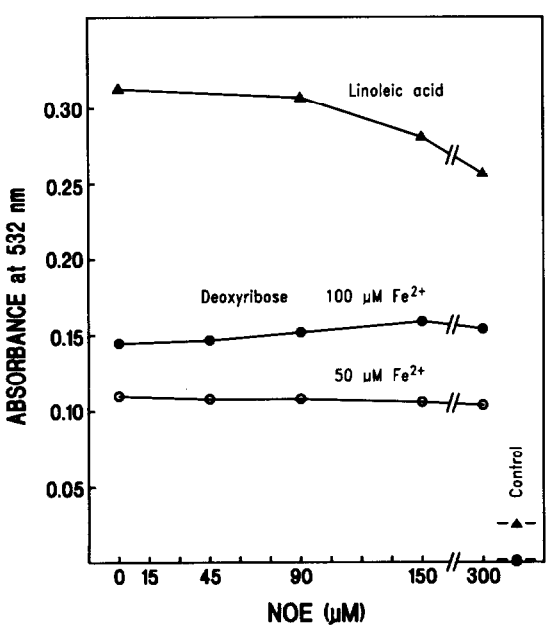

Fig.2. Effect of NOE on Fe^{2+}-induced peroxidation of linoleic acid and deoxyribose. Assay conditions were the same as those used in fig.1.

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