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N-ACYLETHANOLAMINE ACCUMULATION IN INFARCTED MYOCARDIUM

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SUMMARY

Long-chain *N*-acylethanolamines were found at levels of 400-500 nmol per g tissue in the infarcted areas of canine myocardium 24 hours after coronary artery ligation. Peripheral infarct areas also contained substantial amounts (200 nmol/g) while apparently normal heart muscle contained very little (< 10 nmol/g). The amide linked fatty acids were mainly 16:0, 18:0, 18:1 and 18:2. Because of its anti-inflammatory activity, *N*-acylethanolamine may exert beneficial effects in the infarcted area and may be produced as a response to ischemic injury.

Myocardial infarction due to coronary occlusion is a complex process which permits therapeutic intervention aimed at minimizing irreversible tissue damage (1). In this respect, the biochemical events induced by ischemia and the production of physiologically active metabolites in the affected areas of the heart are of major importance. We have recently found substantial amounts of *N*-acylethanolamine phosphoglycerides in the infarcted areas of canine myocardium 24 h after coronary artery ligation (2,3). We now report the identification of long-chain *N*-acylethanolamines (NAE) as constituents of the infarcted tissue.

Small amounts of NAE have previously been detected in mammalian brain and liver, traces in muscle but none in heart (4). The biosynthesis of NAE from ethanolamine and fatty acid catalyzed by a microsomal system from rat liver has also been described (5,6). The accumulation of NAE in infarcted myocardium may be of physiological importance because of its reported pharmacological activities of which the anti-inflammatory (7,8) activity could be of special significance.

Abbreviations used: NAE, *N*-acylethanolamine; TMS, trimethylsilyl; TLC, thin-layer chromatography; GLC, gas-liquid chromatography.

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METHODS

Experimental infarcts and lipid extraction: Three adult mongrel dogs, each weighing about 15 kg, were subjected to ligation of the left descending branch of the coronary artery. After periods of 24 hours (dogs 1 and 3) and 27 hours (dog 2), the animals were heparinized and exsanguinated. All surgical procedures were performed by Dr. David E. Donald and his associates at the Department of Physiology, Mayo Graduate School of Medicine, Rochester, MN.

Hearts from dogs 1 and 2 were dissected into five portions: (a) center of infarct as judged by discoloration, (b) normal left ventricular septum, (c) normal right ventricle, (d) normal posterior right ventricle, and (e) apex of peripheral infarct. Tissue samples (a) and (b) from each heart were homogenized immediately with chloroform-methanol (2:1), and the lipids were extracted according to Folch et al. (9); lipid P was determined according to Bartlett (10). The other samples were frozen in dry ice and stored at -40°C until extraction.

The heart of dog 3 was used specifically for the detection and quantification of NAE. It was dissected into three portions of center infarct, one of peripheral infarct, and one each of the normal left and right ventricles. All samples were extracted immediately as described above.

Chromatography: Thin-layer chromatography was done on layers of Silica Gel H (Merck), 0.5 mm thick, in tanks lined with filter paper. Solvent systems are given by volume. In analytical TLC, fractions were made visible by charring after spraying with 50% sulfuric acid. In preparative TLC, fractions were made visible under UV light after spraying with a 0.1% solution of 2',7'-dichlorofluorescein in absolute ethanol, the adsorbent was removed and extracted with diethyl ether or chloroform-methanol-water (10:10:0.1).

Gas-liquid chromatography was performed with a Packard 428 gas chromatograph equipped with a flame ionization detector and a Spectra-Physics System 1 computing integrator. An aluminum column packed with 10% Alltech CS-10 on 100/120 Chrom W-AW (Alltech Assoc.) was programmed from 185 to 235°C at 3°C/min and a glass column packed with 10% SE-30 was operated isothermally at 210°C.

Mass spectrometry: Mass spectra were taken with an LKB gas chromatograph-mass spectrometer, Type 9000 at 20 eV. The GLC column (3% OV-1) was operated isothermally at 230°C.

Lipid standards and derivatives: Fatty acids and glycerolipids were from Nu-Chek Prep, Elysian, MN. *N*-Hexadecanoyl, *N*-heptadecanoyl, *N*-octadecanoyl, and *N*-octadecenoylethanolamines were prepared by the method of Roe et al. (11). The acetates were prepared by reaction with acetic anhydride-pyridine (2:1) at 80°C for 2 h and the TMS derivatives were prepared by reaction with hexamethyldisilazane and trimethylchlorosilane in dry pyridine (12). The constituent fatty acids of NAE were converted to methyl esters by reaction with 5% (w/v) HCl in methanol at 80°C for 2 hours (4).

RESULTS

Examination of lipid extracts from infarcted canine myocardium by TLC using Solvent A, chloroform-methanol-ammonia (85:15:1.5) revealed that certain lipid classes were present only in the infarcted areas of the heart. A phospho-

lipid fraction was isolated and identified (2,3) as *N*-acylethanolamine phosphoglyceride. A less polar lipid fraction could be further separated by TLC using Solvent B, diethyl ether-acetone-hexane-acetic acid (70:20:10:1), into two fractions of approximately equal amounts. Their migration rates corresponded to 1-monoacylglycerol and *N*-acylethanolamine, respectively. The latter fraction was isolated by preparative TLC using Solvent B from the total lipids of the frozen tissue portion (e), the apex of the peripheral infarct of dog 2 (see above).

GLC-mass spectrometry of its TMS-derivative confirmed the structure of this fraction as *N*-acylethanolamine. The mass spectrum of the TMS-derivative of synthetic *N*-octadecanoylethanolamine showed a molecular ion of low intensity at *m/e* 399 (4%), an intense M-15 ion at *m/e* 384 (60%) and other major ions at *m/e* 309 (48%), 175 (78%) and 116 (100%). TMS-derivatives of the myocardial NAE mixtures from peripheral infarct were fractionated by GLC and peaks were identified by selective ion monitoring of diagnostic ions *m/e* 116 and 175.

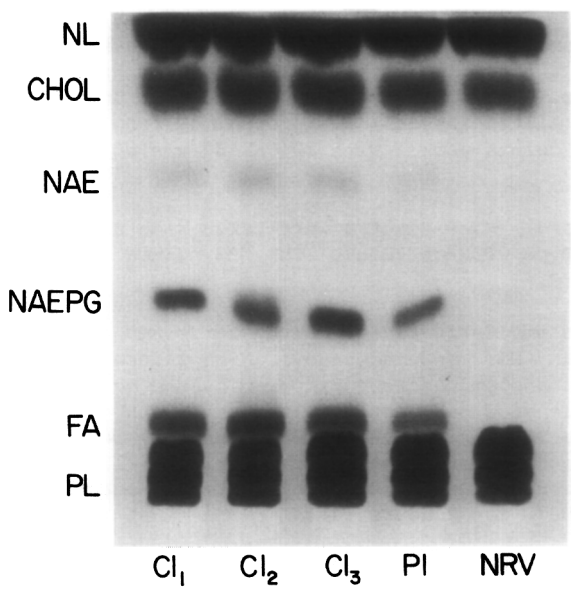

FIG. 1. *Thin-layer chromatogram of total lipids from center infarct (CI_1-CI_3), peripheral infarct (PI) and normal right ventricle (NRV). NL, nonpolar lipids; CHOL, cholesterol; NAE, N-acylethanolamines; NAEPG, N-acylethanolamine phosphoglycerides; FA, fatty acids; PL, polar lipids. Silica Gel H, chloroform-methanol-ammonia (85:15:1.5), charring after spraying with 50% H_2SO_4.*

TABLE I. Amount and fatty acid composition of *N*-acylethanolamine (NAE) in center infarct (CI_1-CI_3), peripheral infarct (PI), and in normal right (NRV) and left (NLV) ventricles.

NAE*	CI_1	CI_2	CI_3	PI	NRV†	NLV†
µg/g tissue	128; 133	168; 158	152; 131	66; 62	3; 4	2; 2
nmol/g tissue	400	498	434	196	11	6
nmol/µmol lipid P	28	30	22	12	0.5	0.3
Constituent fatty acids (mol %)						
16:0	34.8	33.5	33.2	36.0	50.7	56.1
18:0	45.4	43.5	42.7	45.2	36.6	22.1
18:1	7.3	7.1	9.4	5.5	7.5	7.2
18:2	10.4	14.1	13.1	11.5	tr	1.1
others	2.1	1.8	1.6	1.8	5.2	13.5

*µg values were obtained by GLC analysis of fatty acid methyl esters derived (a) from NAE and (b) from NAE acetates using *N*-heptadecanoylethanolamine as internal standard, nmol values were calculated from averages.

†Values were near the limits of detection, NAE was not positively identified in unaffected tissue.

Analysis of the amide linked fatty acids by GLC of their methyl esters revealed mainly 16:0 (37%), 18:0 (39%), 18:1 (5%) and 18:2 (18%). The amount of NAE was estimated as 150 µg per g tissue.

In order to exclude possible artifact formation during the freezing and thawing prior to extraction, the third infarct was produced specifically for the analysis and quantification of NAE. The lipids were extracted immediately after dissection and checked by TLC (Fig. 1). *N*-Heptadecanoylethanolamine (60 µg) was added to an aliquot of each lipid extract, the appropriate fractions were isolated by TLC and quantified by GLC by fatty acid analysis after methanolysis. Aliquots of the NAE fractions were acetylated, repurified by TLC using hexane-diethyl ether-acetone-acetic acid (50:40:10:1), checked for purity by TLC and GLC (SE-30) and subjected to methanolysis for quantification as described above. Results are listed in Table I.

The data clearly show NAE accumulation in the infarcted areas of the myocardium. Trace amounts, near the limits of detection, were also present

in unaffected areas of the tissue but were not positively identified. Furthermore, it is not clear whether these are the normal levels of NAE in canine myocardium or whether the presence of NAE in apparently normal portions of the tissue is also due to NAE biosynthesis induced by ischemia. Further work will clarify this question.

DISCUSSION

Interest in the pharmacological effects of *N*-acylethanolamine began with early observations of antiallergic activities in arachis oil (13), egg yolk (14), and an anti-inflammatory factor in soybean lecithin (8) which were all ascribed to *N*-(2-hydroxyethyl)-palmitamide (8). It has also been shown that this compound can decrease the intensity of several inflammatory and immunological processes (8,15,16), increase the nonspecific tolerance to bacterial toxins (17) and influence tuberculin hypersensitivity (18). Neither the catabolism in mammalian systems nor the nature of the pharmacological activity of NAE has been established.

Although long-chain *N*-acylethanolamines can be formed as artifacts through base-catalyzed aminolysis of esters (19), they were shown to be genuine rather than artifactual trace constituents of mammalian tissue (4). Due to the acidic milieu prevalent in ischemic myocardium and the rapid extraction, artifactual NAE formation by aminolysis in our experiments can also be excluded. However, the similarity of the amide linked fatty acids of NAE and *N*-acylethanolamine phosphoglycerides (2,3) indicates a possible metabolic relationship between these lipid classes.

Our findings establish for the first time the accumulation of NAE under pathological conditions. This accumulation may be a side effect of the degenerative changes induced by ischemia or it may signify a response of myocardial tissue to injury directed at minimizing damage and promoting survival.

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