Dietary Polyunsaturated Fatty Acids Alter Myocardial Protein Kinase C Expression and Affect Cardioprotection Induced by Chronic Hypoxia

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We examined the influence of dietary fatty acid (FA) classes on the expression of protein kinase C (PKC) δ and ε in relation to the cardioprotective effects of chronic intermittent hypoxia (CIH). Adult male Wistar rats were fed a nonfat diet enriched with 10% lard (saturated FA [SFA]), fish oil (n-3 polyunsaturated FA [n-3 PUFA]), or corn oil (n-6 PUFA) for 10 weeks. After 4 weeks on the diet, each group was divided into two subgroups that were either exposed to CIH in a barochamber (7000 m, 8 hrs/day) or kept at normoxia for an additional 5–6 weeks. A FA phospholipid profile and Western blot analysis of PKC were performed in left ventricles. Infarct size was assessed in anesthetized animals subjected to 20-min coronary artery occlusion and 3-hr reperfusion. CIH decreased the n-6/n-3 PUFA ratio in all groups by 23% independently of the initial value set by various diets. The combination of n-3 diet and CIH had a stronger antiarrhythmic effect during reperfusion than the n-3 diet alone; this effect was less pronounced in rats fed the n-6 diet. The normoxic n-6 group exhibited smaller infarctions (by 22%) than the n-3 group. CIH decreased the infarct size in n-3 and SFA groups (by 20% and 23%, respectively) but not in the n-6 group. Unlike PKCε, the abundance of PKCδ in the myocardial particulate fraction was increased by CIH except for the n-6 group. Myocardial infarct size was negatively correlated ($r = -0.79$) with the abundance of PKCδ in the particulate fraction. We conclude that lipid diets modify the infarct size–limiting effect of CIH by a mechanism that involves the PKCδ-dependent pathway. Exp Biol Med 232:823–832, 2007

Key words: chronic hypoxia; ischemia; infarction; protein kinase C; polyunsaturated fatty acids

Introduction

It is well known that the fatty acid (FA) composition in the diet modulates the tolerance of heart to ischemia/reperfusion injury. Both clinical and experimental studies have demonstrated that saturated FA (SFA) exhibit detrimental effects on ischemic hearts, unlike polyunsaturated FA (PUFA), which are believed to be protective (1, 2). It has been shown in various animal species that long-lasting intake of food enriched with n-3 or n-6 PUFA improves cardiac tolerance to acute ischemia/reperfusion injury (3–9). Beneficial effects of n-3 PUFA have also been reported by some clinical studies that demonstrated reduction of mortality related to coronary heart disease without decreasing the incidence of coronary events in patients consuming increased amounts of n-3 PUFA (10).

Improved ischemic tolerance in the heart can also be achieved by adaptation to chronic intermittent hypoxia (CIH) (11, 12). The cardioprotective effects of CIH persist for several weeks after the termination of adaptation (13–15), which is much longer than the protection induced by n-3 PUFA supplementation (7) or various forms of preconditioning (16). CIH induces a variety of adaptive changes in the myocardium that can be considered beneficial in terms of ischemic tolerance. The molecular mechanism of

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Table 1. FA Composition (Mole Percent) of the Diet Lipids in the SFA, n-3, and n-6 Groups[ab]

	SFA	n-3	n-6
14:0	1.40	5.66	0.58
16:0	24.15	17.86	12.28
16:1n-7	2.48	7.71	0.93
18:0	10.87	2.13	1.81
18:1n-9	35.50	16.48	24.70
18:1n-7	2.49	3.44	0.95
18:2n-6	19.03	15.38	53.16
18:3n-3	1.62	2.61	2.31
20:1n-9	0.96	7.31	1.03
20:5n-3	0.18	9.94	N.D.
22:5n-3	0.07	1.78	N.D.
22:6n-3	0.21	8.04	N.D.
Others	1.04	1.66	2.25
ΣSFA	36.42	25.65	14.67
ΣMUFA	41.43	34.94	27.61
Σn-6 PUFA	19.30	15.38	53.16
Σn-3 PUFA	2.08	22.70	2.31

[a] Values are means of two separate analyses.
[b] N.D., not detected; ΣSFA, total SFA; ΣMUFA, total MUFA; Σn-3 PUFA, total n-3 PUFA; Σn-6 PUFA, total n-6 PUFA.

protection by CIH is poorly understood, although several signaling pathways have been proposed to play a role (11). Our recent studies suggested that the cardioprotection afforded by CIH in rats involves protein kinase C (PKC), in particular isoform δ (17–19). Concerning lipids, Jezkova *et al*. (20) showed that repeated hypoxic exposure altered the FA profile, decreased the n-6:n-3 PUFA ratio, and increased the unsaturation index in rat heart phospholipids (PL). A similar shift of FA composition in myocardial PL occurred in animals fed a diet enriched with n-3 PUFA (6, 9, 21). It was assumed that the changes in myocardial PL FA composition induced by CIH may contribute to improved ischemic tolerance (20). Thus, the primary goal of this study was to find out whether an altered FA profile plays a role in the cardioprotective mechanism of CIH in a PKC-dependent manner. We compared the effects of diets enriched with SFA, n-3 PUFA, or n-6 PUFA on FA composition of myocardial PL, the expression and distribution of PKC isoforms δ and ε between cytosolic and particulate fractions, and the susceptibility of normoxic and chronically hypoxic rat hearts to acute ischemia/reperfusion injury induced by coronary artery occlusion. These experiments revealed that lipid diet composition is important for the manifestation of the infarct size–limiting mechanism of CIH, which involves the PKCδ-dependent pathway while the hypoxia-induced changes in n-3 and n-6 PUFA proportion in membrane PL seem unlikely to play a direct role.

Materials and Methods

Animal Model. Adult male Wistar rats (250–280 g) were fed a nonfat diet based on standard ST1 (Velaz, Prague, Czech Republic) enriched with 10% lard (SFA), fish oil (n-3 PUFA; Lehmann & Voss, Hamburg, Germany) or corn oil (n-6 PUFA; Olmühle GmbH, Bruck, Austria) for 10 weeks. The FA composition of the dietary oils is shown in Table 1. After 4 weeks on the diet, each group was divided into two subgroups that were either exposed to intermittent hypobaric hypoxia of 7000 m for 8 hrs/day 5 days/week or kept at normoxia for an additional 5–6 weeks. Barometric pressure (P_B) was lowered stepwise so that the level equivalent to an altitude of 7000 m ($P_B = 308$ mm Hg, 41 kPa; $P_{O_2} = 65$ mm Hg, 8.6 kPa) was reached after 13 exposures; the total number of exposures was 24–30 (22). The control normoxic subgroups of animals were kept for the same period of time at P_B and P_{O_2} equivalent to an altitude of 200 m ($P_B = 742$ mm Hg, 99 kPa; $P_{O_2} = 155$ mm Hg, 20.7 kPa). All animals had free access to water. The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996).

All animal experiments commenced the day after the last hypoxic exposure. Rats to be used for biochemical analyses were anesthetized with sodium pentobarbital (60 mg/kg body wt ip; Sanofi, Montpellier, France). The right ventricle (RV) was catheterized *via* the jugular vein, and RV pressure was measured with a Gould P23Gb transducer (Statham, Hato Rey, Puerto Rico). Thereafter, hearts were rapidly excised, washed in cold (0°C) saline, and dissected into RV and left ventricle (LV) free walls and the septum. All parts were weighed, and the LVs were frozen and stored in liquid nitrogen. All the chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Analysis of FA Composition. PL and their FA composition were analyzed as described previously (20). Briefly, frozen LV myocardium was pulverized and homogenized. Lipids were extracted in three consecutive steps according to the modified method of Folch *et al*. (23). The first extraction was performed in three portions of chloroform-methanol mixture (1:3, 2:1, and 2:1) in a chilled mortar. Subsequent extractions were performed in the 2:1 mixture. Saline (20% volume of extract) was added, and after vigorous shaking the lower lipid layer was dried at 40°C under a stream of nitrogen. Total PL were separated by one-dimensional thin-layer chromatography (0.5-mm silica gel H; Merck, Darmstadt, Germany) using the solvent mixture hexane-ether-acetic acid (80:20:3). Spots were observed under ultraviolet light after staining with 0.005% 2,7-dichlorofluorescein, scraped out, and stored in a nitrogen atmosphere at –20°C until the next day when methyl esters were prepared. FA methyl esters were separated with a gas chromatograph (CP 438 A; Chrompack, Middelburg, The Netherlands).

PKC Analysis. Myocardial samples were pulverized to a fine powder, followed by Potter-Elvehjem homogenization in eight volumes of ice-cold buffer composed of (in m*M*): 12.5 Tris-HCl (pH 7.4), 250 sucrose, 2.5 EGTA, 1

EDTA, 100 NaF, 5 dithiothreitol, 0.3 phenylmethylsulfonyl fluoride, 0.2 leupeptin, and 0.02 aprotinin. The homogenate was centrifuged at 100,000 *g* for 90 mins. The resulting pellet represented the particulate fraction; the supernatant was the cytosolic fraction. The pellet of particulate fraction was resuspended in homogenization buffer containing 1% Triton X-100, held on ice for 90 mins, and then centrifuged at 100,000 *g* for a further 90 mins. The resulting detergent-treated supernatant was used for immunoblot analyses. Triton X-100 was added to the cytosolic fraction to reach a final concentration of 1%. Protein content was determined according to the Lowry assay modified by Peterson (24). Detergent-treated extracts of the subcellular fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on an 8% bis-acrylamide gel at 20 mA/gel for 90 mins on a Mini-Protean III apparatus (Bio-Rad, Hercules, CA); the resolved proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). Equal protein transfer efficiency was verified by staining with Ponceau S. After blocking with 5% dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 mins at room temperature, membranes were washed and probed with PKCδ- or PKCε-specific polyclonal primary rabbit antisera (1:8000 in TTBS) for 90 mins at room temperature. Anti-PKCδ was elicited against a synthetic peptide corresponding to the C-terminal variable (V5) region (amino acids 662–673) of rat PKCδ, and anti-PKCε was elicited against a synthetic peptide corresponding to the C-terminal variable (V5) region (amino acids 726–737) of PKCε. The membranes were washed again and incubated with secondary swine antirabbit IgG antibody labeled with horseradish peroxidase (1:4000 in TTBS; Sevapharma, Prague, Czech Republic) for 60 mins at room temperature. Before enhanced chemiluminescence (ECL), the membranes were washed and stored in TTBS for at least 2 hrs. For ECL, substrates A (luminol solution) and B (H_2O_2 solution) were prepared, mixed 1:1 just before use, and poured on the membranes. The specific signal was detected on the autoradiographic film (Amersham Biosciences). Scanning (Perfection 1240U Scanner; Epson, Meerbusch, Germany) and ImageQuant software were used for quantification of the relative abundance of individual PKC isoforms. To ensure the specificity of PKCδ- and PKCε-immunoreactive proteins, prestained molar-mass protein standards (Fluka, Buchs, Switzerland), recombinant human PKCδ and PKCε standards, rat brain extract, and the respective blocking immunizing peptides were used (18). From each group, one sample was run on the same gel and quantified on the same membrane. The amounts of protein applied to the gel was 15 μg (cytosolic fraction) and 5 μg (particulate fraction).

Infarct Size Determination and Analysis of Arrhythmias. Animals were subjected to acute myocardial ischemia/reperfusion as described previously (22). Anesthetized rats (as above) were ventilated (Columbus Instruments, Columbus, OH) *via* tracheal cannulas with room air at 68 strokes/min (tidal volume of 1.2 ml/100 g body wt). Both blood pressure in the left carotid artery and a single-lead electrocardiogram (ECG) were continually recorded and subsequently analyzed by our custom-designed software. The animals' temperatures (rectal measurements) were maintained between 36.5°C and 37.5°C by a heated table throughout the experiment. Hematocrit in the tail blood was estimated by using the capillary micromethod.

Left thoracotomy was performed and a polyester suture 6/0 (Ethicon, Edinburgh, UK) was placed around the left anterior descending coronary artery about 1–2 mm distal to its origin. After a 10-min stabilization, regional myocardial ischemia was induced by tightening the suture threaded through a polyethylene tube. Characteristic changes in the configuration of the ECG and a transient decrease in blood pressure verified the coronary artery occlusion. After a 20-min occlusion period, the ligature was released and reperfusion of previously ischemic tissue continued.

After 3 hrs of reperfusion, the hearts were arrested with 0.25 mg of verapamil (Krka, Novo Mesto, Slovenia) administered into the jugular vein. The hearts were excised and washed with 20 ml of saline through the cannulated aorta. The area at risk and the infarct size were determined as described earlier (22) by staining with 5% potassium permanganate (Lachema, Brno, Czech Republic) and 1% 2,3,5-triphenyltetrazolium chloride dissolved in 0.1 *M* phosphate buffer (pH 7.4), respectively. The hearts were cut perpendicularly to the LV long axis into slices 1-mm thick and stored overnight in 10% neutral formaldehyde solution. The day after the infarct size staining, the RV free wall was separated and both sides of the LV slices were photographed. The sizes of the infarct area (IA), area at risk (AR), and LV were determined by a computerized planimetric method. The IA was normalized to the AR (IA/AR), and the AR was normalized to the LV (AR/LV).

Severity of ventricular arrhythmias occurring during prolonged ischemic insult and the first 5 mins of reperfusion were assessed according to the Lambeth Conventions (25). Premature ventricular complexes (PVCs) occurring as singles, salvos, or tachycardia (a run of four or more consecutive PVCs) were counted separately. The incidence of ventricular tachycardia (VT) and fibrillation (VF) was also evaluated. VF lasting more than 2 minutes was considered sustained (VFs). The severity of arrhythmias in each individual heart was evaluated by means of a 5-point arrhythmia score: single PVCs were given a score of 1, salvos 2, VT 3, reversible VF 4, and VFs 5. An assigned number corresponded to the most severe type of arrhythmia observed in that heart. Scores were used for group analysis of severity of arrhythmias.

Statistical Analysis. The results are expressed as means ± SEM. Two-way analysis of variance and subsequent Student-Newman-Keul's test were used for comparison of differences in normally distributed variables

Table 2. Weight Parameters, RV Systolic Pressures, and Hematocrits of Normoxic and Chronically Hypoxic Rats Fed SFA, n-3, or n-6 Diet[a]

	n[b]	Body weight, g	LV, mg	RV, mg	Septum, mg	LV/body weight, mg/g	RV/body weight, mg/g	RV systolic pressure, mm Hg	Hematocrit, %
Normoxic									
SFA	9	414 ± 9	420 ± 17	167 ± 4	208 ± 9	1.02 ± 0.06	0.40 ± 0.01	25.5 ± 0.9	48.2 ± 0.6
n-3	9	434 ± 9	457 ± 16	179 ± 6	205 ± 9	1.05 ± 0.05	0.41 ± 0.01	27.2 ± 1.2	47.7 ± 0.6
n-6	9	430 ± 11	468 ± 18	195 ± 9	217 ± 11	1.09 ± 0.05	0.45 ± 0.02	24.4 ± 1.4	48.0 ± 0.4
Hypoxic									
SFA	10	365 ± 9*	460 ± 13	276 ± 13*	216 ± 8	1.26 ± 0.03*	0.76 ± 0.04*	42.8 ± 1.0*	78.0 ± 0.8*
n-3	7	366 ± 10*	517 ± 42	258 ± 11*	244 ± 18	1.42 ± 0.12*	0.70 ± 0.02*	37.2 ± 1.9*§	77.6 ± 0.6*
n-6	9	359 ± 6*	477 ± 23	250 ± 8*	196 ± 7	1.33 ± 0.09*	0.70 ± 0.02*	44.9 ± 2.4*	76.6 ± 0.9*

[a] Values are means ± SEM.
[b] n, number of animals.
* $P < 0.05$ versus corresponding normoxic group; § $P < 0.05$ versus other corresponding diet groups.

among the groups. Differences were considered statistically significant at $P < 0.05$.

Results

Weight Parameters, Hematocrit, and RV Pressures. Adaptation of rats to CIH led to a marked increase in hematocrit and a significant retardation of body growth compared with age-matched normoxic controls in all diet groups. The heart weights increased mainly due to hypertrophy of the right ventricles. The relative weights of the RV (RV/body weight) and LV (LV/body weight) increased in all chronically hypoxic groups by about 75% and 30%, respectively. Lipid diets had no effect on weight parameters and hematocrit in both normoxic and hypoxic rats. CIH raised the RV systolic pressure in all diet groups, but this effect was significantly less pronounced in the n-3 group (by 36%) compared with the SFA (by 67%) and n-6 (by 84%) groups (Table 2).

Table 3. Proportion of Main FA Classes (Mole Percent) in Total Myocardial PL in Normoxic and Chronically Hypoxic Rats Fed SFA, n-3, or n-6 Diet

	Normoxic		
	SFA	n-3	n-6
Normoxic			
ΣSFA	36.2 ± 1.0	36.6 ± 2.0	35.5 ± 1.9
ΣMUFA	7.0 ± 0.2	7.9 ± 0.2§	6.1 ± 0.2§
Σn-6 PUFA	44.1 ± 1.5	33.1 ± 1.0§	49.8 ± 1.8§
Σn-3 PUFA	12.7 ± 0.6	22.3 ± 1.1§	8.6 ± 0.8§
Hypoxic			
ΣSFA	37.3 ± 1.0	37.9 ± 1.6	37.3 ± 1.7
ΣMUFA	7.3 ± 0.3	8.3 ± 0.2§	5.8 ± 0.2§
Σn-6 PUFA	40.4 ± 0.5	28.9 ± 0.6*§	46.7 ± 1.4§
Σn-3 PUFA	15.0 ± 0.9	24.9 ± 1.1§	10.3 ± 0.5§

[a] Values are mean ± SEM from seven hearts in each group.
* $P < 0.05$ versus corresponding normoxic group; § $P < 0.05$ versus other diet groups.

Myocardial FA Composition. Despite different FA compositions of individual lipid diets (Table 1), the proportions of SFA, monounsaturated FA (MUFA), and total PUFA in the myocardial PL did not essentially differ among all normoxic and hypoxic diet groups (Table 3). However, in normoxic myocardium, the proportion of n-3 and n-6 PUFA substantially differed in individual diet groups. The n-3 diet increased the proportion of n-3 PUFA and decreased the proportion of n-6 PUFA compared with the SFA diet. This effect was mainly due to an accumulation of docosahexaenoic acid (DHA; 22:6 n-3, by 66%) at the expense of arachidonic acid (AA; 20:4 n-6, decreased by 47%). The n-6 diet had an opposite effect: it reduced the proportion of n-3 PUFA in favor of n-6 PUFA compared with the SFA diet. The DHA decrease (by 32%) and linoleic acid (LA; 18:2 n-6) increase (by 28%) substantially contributed to this effect. CIH tended to increase the proportion of n-3 PUFA (mainly due to an accumulation of DHA) and decreased that of n-6 PUFA (by a drop of LA) (Table 3). Consequently, the n-6:n-3 PUFA ratio declined by about 23% independent of the diet groups (Fig. 1).

Infarct Size. Table 4 summarizes the values of heart rate and mean arterial blood pressure in all groups, determined at baseline (before ischemia), at the end of test ischemia, and at the end of the 3-hr reperfusion. No significant differences were found in the baseline values of heart rate among the groups; heart rates were significantly lower at the end of reperfusion in each group compared with corresponding baseline values. CIH tended to increase the mean arterial pressure in all groups; significantly higher values were recorded at the end of reperfusion.

The normalized area at risk (AR/LV) did not significantly differ among the groups: its mean values ranged from 40% to 42%. Normoxic rats fed the n-6 diet exhibited significantly smaller infarct areas (43.6% ± 3.2% of the AR) as compared with the normoxic n-3 group (56.1% ± 3.9%). The IA/AR of the normoxic SFA group (49.3% ±

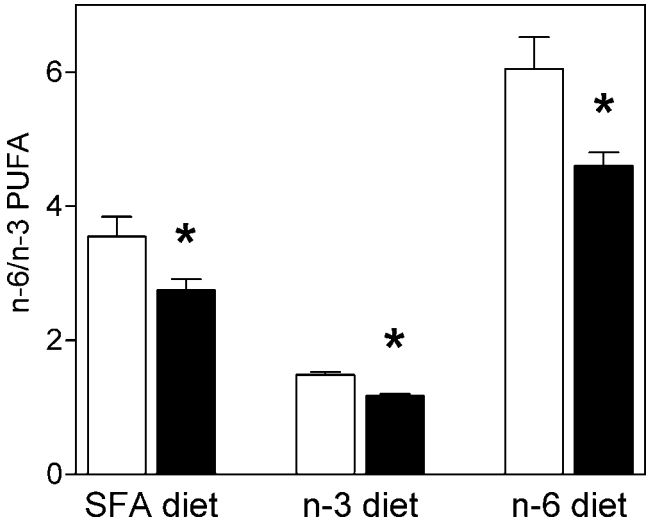

Figure 1. The n-6:n-3 PUFA ratio in total myocardial phospholipids of chronically hypoxic rats (black columns) and normoxic controls (open columns) fed SFA, n-3, or n-6 diet. Values are means ± SEM from seven hearts in each group. *$P < 0.05$ versus corresponding normoxic group.

2.3%) did not significantly differ from that of the other normoxic groups (Fig. 2). Adaptation to CIH had a significant infarct size–limiting effect in the SFA (IA/AR 38.2% ± 2.4%) and n-3 (45.0% ± 1.8%) groups but not in the n-6 group (42.6% ± 2.5%; Fig. 2).

Table 4. Heart Rate and Mean Arterial Blood Pressure After Stabilization (Baseline), at the End of 20-Min Coronary Artery Occlusion and at the End of the 3-Hr Reperfusion in Normoxic and Chronically Hypoxic Rats Fed SFA, n-3, or n-6 Diet[a]

	Baseline	Ischemia	Reperfusion
Heart rate, bpm			
Normoxic			
SFA	456 ± 8	452 ± 9	402 ± 11**
n-3	438 ± 11	439 ± 13	369 ± 11**
n-6	441 ± 12	451 ± 8	389 ± 10**
Hypoxic			
SFA	436 ± 6	431 ± 5	397 ± 8**
n-3	437 ± 6	432 ± 6	398 ± 5**
n-6	420 ± 8	411 ± 9*	384 ± 10**
Blood pressure, mm Hg			
Normoxic			
SFA	103 ± 7.1	104 ± 6.7	97 ± 7.7
n-3	109 ± 7.6	112 ± 7.9	90 ± 7.7
n-6	104 ± 8.4	110 ± 5.1	94 ± 7.2
Hypoxic			
SFA	128 ± 5.5	128 ± 6.1	134 ± 5.6*
n-3	131 ± 2.8	134 ± 3.3	131 ± 4.2*
n-6	121 ± 6.6	128 ± 6.8	127 ± 7.3*

[a] Values are mean ± SEM from 8–12 animals in each group.
* $P < 0.05$ versus corresponding normoxic group; ** $P < 0.05$ versus baseline.

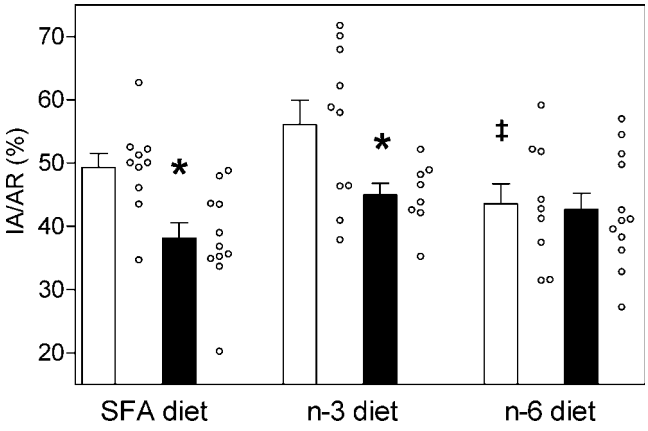

Figure 2. Myocardial IA expressed as a percentage of the AR (IA/AR) in chronically hypoxic rats (black columns) and normoxic controls (open columns) fed SFA, n-3, or n-6 diet. Open circles indicate individual experiments. Values are means ± SEM. *$P < 0.05$ versus corresponding normoxic group; ‡$P < 0.05$ versus corresponding n-3 group.

Ventricular Arrhythmias. Neither lipid diet nor CIH affected the values of ischemic arrhythmia score (AS) (Table 5). Nevertheless, both normoxic and hypoxic groups fed the n-3 diet tended to have decreased severity of ischemic arrhythmias. The value of reperfusion AS in the normoxic n-3 group also tended to exhibit decreases compared with the other diet groups. CIH decreased AS for reperfusion arrhythmias in all groups, but this effect was significant only in rats fed the SFA or n-3 diet. Reperfusion arrhythmias were almost eliminated by a combination of the n-3 diet and CIH (Table 5). In this study, no sustained VF was observed in any group.

Expression and Distribution of PKC Isoforms. As for diet effects, the abundance of PKCδ in the particulate fraction of normoxic animals fed the n-3 diet was lower compared with the corresponding SFA and n-6 groups. CIH increased the relative protein content of PKCδ

Table 5. AS Over 20-Min Coronary Artery Occlusion (Ischemic Arrhythmias) and Over the First 5 Mins of Reperfusion (Reperfusion Arrhythmias) in Normoxic and Chronically Hypoxic Rats Fed SFA, n-3, or n-6 Diet

	n	Ischemic arrhythmias	Reperfusion arrhythmias
Normoxic			
SFA	10	2.80 ± 0.25	2.70 ± 0.21
n-3	10	1.80 ± 0.36	1.80 ± 0.44
n-6	9	2.44 ± 0.29	2.33 ± 0.37
Hypoxic			
SFA	11	2.73 ± 0.45	2.00 ± 0.23*
n-3	8	1.75 ± 0.41	0.13 ± 0.13*§
n-6	12	2.23 ± 0.36	1.38 ± 0.29

[a] Values are mean ± SEM.
* $P < 0.05$ versus corresponding normoxic group; § $P < 0.05$ versus other corresponding diet groups.

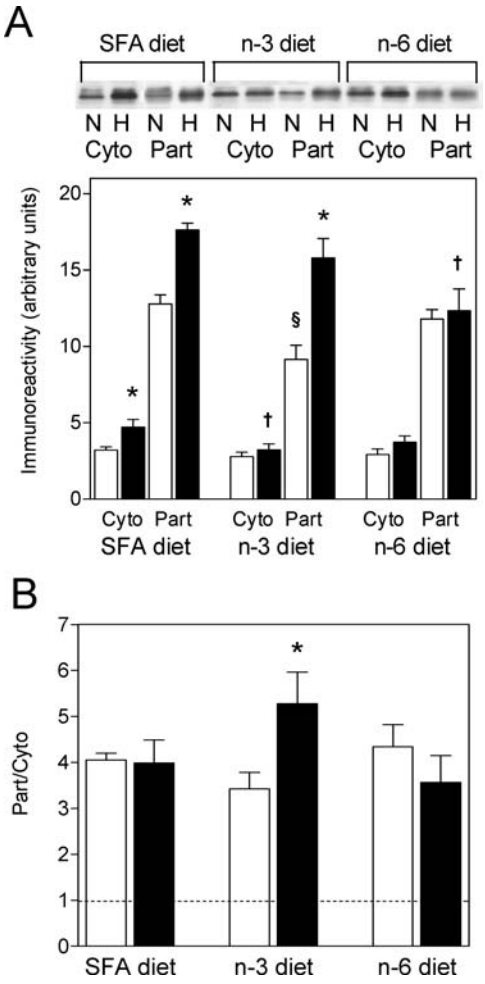

Figure 3. (A) Expression of PKCδ in cytosolic (Cyto) and particulate (Part) fractions and (B) its distribution between the fractions from the myocardium of chronically hypoxic rats (H; black columns) and normoxic controls (N; open columns) fed SFA, n-3, or n-6 diet. All samples compared were electrophoresed on the same gel and quantified on the same membrane. The amount of protein applied to the gel was 15 μg (Cyto) or 5 μg (Part). Values are means ± SEM from six hearts in each group. *$P < 0.05$ versus corresponding normoxic group; †$P < 0.05$ versus corresponding SFA group; §$P < 0.05$ versus other diet groups.

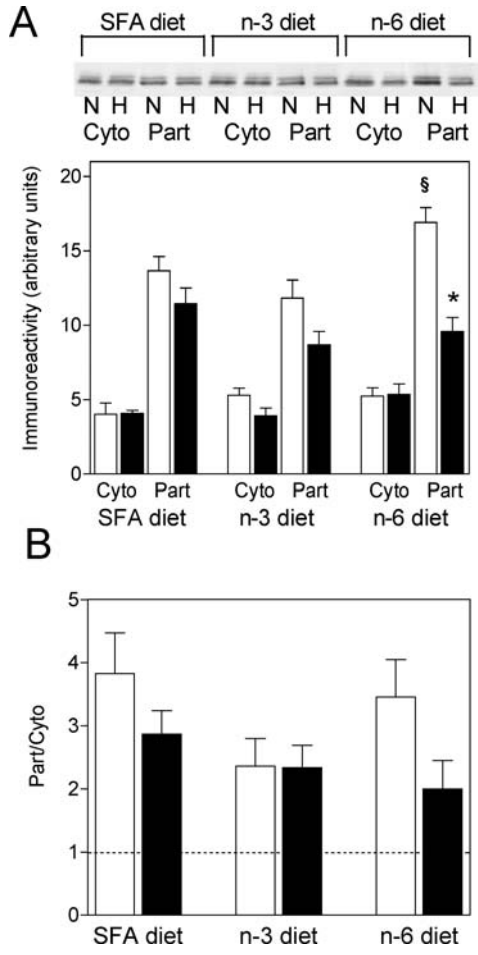

Figure 4. (A) Expression of PKCε in Cyto and Part fractions and (B) its distribution between the fractions from the myocardium of chronically hypoxic rats (H; black columns) and normoxic controls (N; open columns) fed SFA, n-3, or n-6 diet. All samples compared were electrophoresed on the same gel and quantified on the same membrane. The amount of protein applied to the gel was 15 μg (Cyto) or 5 μg (Part). Values are means ± SEM from 5 hearts in each group. *$P < 0.05$ versus corresponding normoxic group; §$P < 0.05$ versus other diet groups.

in the particulate fraction of the SFA and n-3 groups (by 40% and 82%, respectively) but not in the n-6 group. CIH increased the relative protein content of PKCδ in the cytosolic fraction of the SFA group (Fig. 3A) and significantly redistributed this isoform from cytosolic to particulate fractions in the n-3 group only (Fig. 3B).

The abundance of PKCε was higher in the particulate fraction of normoxic rats fed the n-6 diet compared with the SFA and n-3 groups. In contrast with PKCδ up-regulation, CIH did not significantly influence the abundance of PKCε in the particulate fraction of the SFA and n-3 groups, and it even decreased the content of this isoform in the n-6 group (by 41%). Neither lipid diet nor CIH affected the PKCε content in the cytosolic fraction (Fig. 4A). CIH tended to decrease the proportion of PKCε in the particulate fraction in the SFA and n-6 groups (expressed as a ratio of cytosolic to particulate level), but this effect did not reach statistical significance (Fig. 4B).

Figure 5 presents relationships between the mean values of PKCδ (A) or PKCε (B) relative content in the myocardial particulate fraction and the mean infarct size for three normoxic and three hypoxic groups. Regression analysis demonstrated a negative linear relationship between PKCδ abundance and infarct size with the correlation coefficient approaching 0.8 (Fig. 5A). Note that the hypoxic groups were shifted to the lower right portion of the regression line (smaller infarction and higher PKCδ content).

Compared with PKCδ, the relationship between PKCε abundance and infarct size differed considerably. Although the infarct size decreased with increasing relative PKCε content within the normoxic or hypoxic groups, the hypoxic

groups were shifted to the lower left portion of the graph (smaller infarction and lower PKCε content) (Fig. 5B).

Discussion

Myocardial FA Composition and General Effects of CIH and Diets. Dietary supplementation with saturated fats did not significantly affect the FA composition of myocardial PL as compared with the standard ST1 diet (20). It is important to point out that the SFA diet, besides a large amount of SFA, also contained a similarly high proportion of MUFA. Both PUFA diets had no effect on the myocardial content of SFA, only slightly influenced MUFA, and as expected, markedly changed n-3 PUFA and n-6 PUFA in normoxic hearts. This is in agreement with the results of numerous studies showing that supplementation with fish oil (n-3 PUFA) increased the myocardial content of n-3 PUFA at the expense of n-6 PUFA (9, 21, 26), whereas diets containing corn oil (n-6 PUFA) had the opposite effect (27, 28). Surprisingly, CIH decreased the n-6:n-3 PUFA ratio proportionally in all diet groups. This means that different PUFA loads offered by lipid diets that resulted in substantially altered compositions of myocardial PL apparently did not affect the additional PUFA remodeling induced by CIH. The observed decrease in the n-6:n-3 PUFA ratio most likely resulted from the adaptation of the deacylation-reacylation cycle to chronically hypoxic conditions that may be viewed as an important mechanism of membrane protection against oxidative stress (29).

We did not observe any effect of diets on basic heart weight parameters or hemodynamics in normoxic animals. CIH-induced adaptive responses were not affected by diet composition, except for the increase in RV systolic pressure, which was significantly less pronounced in rats fed the n-3 diet. This finding is in agreement with the previous observation of Archer *et al*. (30) and suggests that a fish oil diet can partially protect against the development of hypoxic pulmonary hypertension.

Effects of CIH and Diets on Ischemia/Reperfusion Injury. It is generally accepted that cardiac susceptibility to ischemia/reperfusion injury can be modulated by lipid diets. In particular, numerous experimental studies have demonstrated that long-lasting feeding of various animal species by diets enriched with PUFA protect the heart against ischemic and reperfusion ventricular arrhythmias (3, 5, 9, 31). The antiarrhythmic influence of n-3 PUFA appears to be superior to that of n-6 PUFA (32). Our data support previous findings about the antiarrhythmic effect of a diet enriched with n-3 PUFA against both ischemic and reperfusion arrhythmias (9, 31, 32). CIH decreased the severity of reperfusion arrhythmias as previous reports indicated (13, 22, 33); this effect manifested itself in all diet groups, although with different potencies. A combination of the n-3 diet and CIH had a stronger protective effect on reperfusion arrhythmia severity than n-3 diet alone, suggesting independent additive actions.

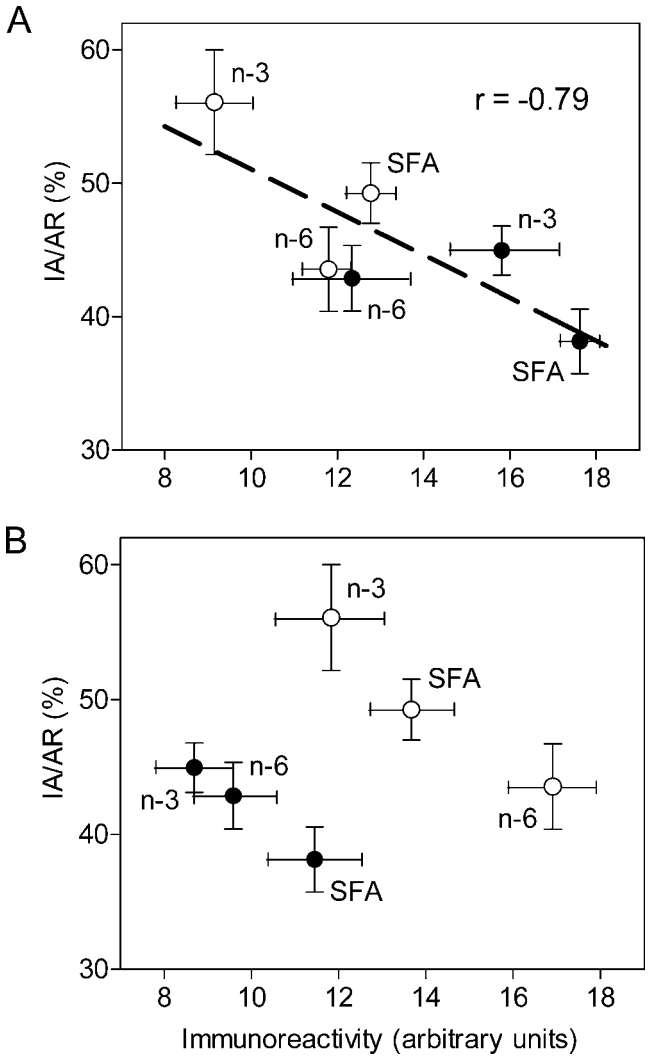

Figure 5. Relationships between the mean values of (A) PKCδ and (B) PKCε relative content in the myocardial Part and the mean IA normalized to the AR (IA/AR) in chronically hypoxic rats (black circles) and normoxic controls (open circles) fed SFA, n-3, or n-6 diet. r indicates correlation coefficient.

However, diets enriched with PUFA seem to have much fewer clear effects when myocardial infarct size is set as the major end point of ischemia/reperfusion injury. Factors that may influence the results of dietary studies with PUFA include animal species (34), age (35), duration of treatment, and relative amounts of n-3 and n-6 PUFA in the diet (36, 37). Against our expectations, we observed significantly smaller infarction in rats fed the n-6 diet compared with the n-3 diet group, which exhibited the largest extent of injury. Of the three previous studies that analyzed infarct size in rats *in vivo*, two demonstrated protective effects of either the n-6 diet (sunflower seed oil; 12%) (4) or the n-3 diet (fish oil; 12%) (7); however, the third one (28) did not find any limitation of infarction after 6–12 weeks on fish oil or corn oil diets (20%). Similarly, several *in vitro* studies failed to detect significant cardioprotection following n-3 or n-6 PUFA supplementation (38,

39). Our observation of weak myocardial ischemic tolerance of rats fed the n-3 diet supports the previous findings of Gudbjarnason and Oskarsdottir (40), who demonstrated that an n-3 diet (cod liver oil; 10%; 12 weeks) aggravated isoproterenol-induced cardiac necrosis and mortality. It seems, therefore, that the impact of diets enriched with PUFA (particularly n-3 PUFA) on cardiac susceptibility to injury is not always favorable as previously thought. Interestingly, a recent thorough systematic review on cardiovascular events and total mortality found no evidence of a clear benefit of n-3 PUFA on health (41).

The cause of aggravated injury due to an n-3 diet is unclear. One potential explanation is that highly polyunsaturated long-chain FA, in particular n-3 PUFA from fish oil, are extremely susceptible to peroxidation compared with n-6 PUFA (36, 42), even in the presence of added dietary antioxidants (43). Thus, we can speculate that the marked enrichment of membranes with n-3 PUFA in rats fed the diet supplemented with 10% fish oil increased myocardial susceptibility to oxidative stress induced by ischemia/reperfusion that resulted in larger infarction compared with the n-6 group.

The major result of this study is that lipid diets had distinct effects on infarct size limitation afforded by CIH. Whereas less tolerant diet groups (SFA and n-3) were protected by CIH, the protective effect of the n-6 diet on myocardial infarction was not further enhanced by adaptation. As the decrease in the n-6:n-3 PUFA ratio in membranes induced by CIH was the same in all groups (i.e., independent of the level set by diets), it seems unlikely that this adaptive response is directly involved in the mechanism of the infarct size–limiting effect in chronically hypoxic hearts.

Effects of CIH and Diets on PKC. We showed previously that PKCδ plays a role in the cardioprotective mechanism of CIH (18), and both PKCδ up-regulation and protection depend on the level of oxidative stress during the adaptation period (17). The present study demonstrated for the first time that lipid diets alone have distinct effects on the expression and subcellular distribution of PKCδ and PKCε and also modulate the hypoxia-induced changes of these isoforms.

Isoforms δ and ε belong to the group of novel PKC. Both PKCδ and PKCε are sensitive to diacylglycerols (DAG) and phosphatidylserine (PS) (44). Several other lipid second messengers have been shown to activate PKC *in vitro* and *in vivo*. *cis*-Unsaturated FA, such as AA, LA, and oleic acid, can activate PKC in the absence of DAG and PS (45, 46). It has been demonstrated that free *cis*-unsaturated FA could affect PKC-dependent signaling pathways in cardiac (47–49) and other cell types (50, 51). Moreover, the quality of the acyl chain of DAG also plays a role in different PKC isoform activation (52, 53). Despite PKCδ and PKCε overall structural similarities, these isoenzymes are differentially regulated (54). Kashiwagi *et al.* (55) showed that AA and ceramide induced different patterns of PKCε translocation compared with PKCδ. The differential sensitivity of PKCδ and PKCε to AA is related to the subtle differences in the conserved domain (C1B). The C1B domain defines the isoform-specific sensitivity of PKC to lipid second messengers. In line with our data, it seems that the higher content of n-6 PUFA (mainly AA) in membranes can be associated with improved cellular signaling mediated by PKC (45, 49, 51). We can speculate that the highest amount of PKCε in the particulate fraction of the normoxic n-6 group is linked to increased ischemic tolerance. This view is supported by the data of Mackay and Mochly-Rosen (56), who demonstrated that AA selectively activates PKCε in neonatal rat cardiac myocytes and this activation could lead to protection.

On the other hand, we observed a lower expression of PKC isoforms in normoxic myocardium of the group fed the n-3 diet, which contained higher amounts of DHA and eicosapentaenoic acid (EPA) in PL compared with the SFA and n-6 groups. This is in agreement with studies showing that n-3 PUFA can decrease the activity of PKC *in vitro* (57, 58). Similarly, a fish oil diet inhibited redistribution of PKCε but not PKCδ from the cytosol to the membrane fraction in hypertrophic mouse hearts (59). EPA and DHA supplementation decreased PKC activity in cardiac cells compared with cells supplemented with AA (60). In addition, we cannot exclude that the decreased expression of PKC isoforms observed in the n-3 diet group was due to the effect of n-3 PUFA on gene expression of these proteins. It has been shown that PUFA could be important regulators of gene expression in various tissue types (61–63), but any data on direct effects of n-3 PUFA on PKC genes have not yet been generated.

In addition to the fact that various lipid diets influence PKC properties through the quality of lipid-signaling molecules, membranes containing different levels of FA, such as SFA, MUFA, and PUFA, differentially affect the propensity of membranes to form nonlamellar phases (64). Nonlamellar phases regulate PKC translocation to membranes (65), and this mechanism should also be taken into account as potentially contributing to the observed effects of lipid diets.

CIH led to the up-regulation of PKCδ in both the SFA and n-3 diet groups but not in the n-6 diet group in compliance with the presence or absence, respectively, of the infarct size–limiting effect. Unlike PKCδ, myocardial PKCε was down-regulated in all three hypoxic groups. Regression analysis of the mean values of the PKCδ relative content in the particulate fraction and infarct size revealed a close negative correlation between these variables; all hypoxic groups were located in the lower right portion of the graph (smaller infarction and higher PKCδ content). These results further support our previous conclusion (17, 18) that PKCδ plays an important role in the infarct size–limiting mechanism of CIH in adult rat hearts. Concerning PKCε, the infarct size also decreased with increasing relative PKCε content within the normoxic or hypoxic

groups, which is in agreement with a generally accepted view that this isoform is cardioprotective. However, all values of the hypoxic groups exhibited a parallel shift to the lower left portion of the graph (smaller infarction and lower PKCε content), suggesting that CIH protects the myocardium by a mechanism independent of PKCε.

In conclusion, our data demonstrated distinct effects of lipid diets on myocardial ischemic tolerance and the expression and subcellular distribution of PKC isoforms δ and ε in normoxic and chronically hypoxic rats. These data support the view that lipid diet composition is important for the manifestation of the infarct size–limiting mechanism of CIH, which involves the PKCδ pathway, while the hypoxia-induced changes in n-3 and n-6 PUFA proportions in membrane PL seem unlikely to play a direct role.

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