PUFA and aging modulate cardiac mitochondrial membrane lipid composition and Ca\(^{2+}\) activation of PDH

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Pepe, Salvatore, Naotaka Tsuchiya, Edward G. Lakatta, and Richard G. Hansford. PUFA and aging modulate cardiac mitochondrial membrane lipid composition and Ca\(^{2+}\) activation of PDH. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H149–H158, 1999.—Aberrations in cell Ca\(^{2+}\) homeostasis have been known to parallel both changes in membrane lipid composition and aging. Previous work has shown that the lowered efficiency of work performance, which occurs in isolated hearts from rats fed a diet rich in n-6 polyunsaturated fatty acids (PUFA), relative to those fed n-3 PUFA, could be raised by mitochondrial (Mito) Ca\(^{2+}\) transport inhibition. We tested whether, after Ca\(^{2+}\)-dependent stress, the Ca\(^{2+}\)-dependent activation of pyruvate dehydrogenase (PDH\(_A\)/PDH\(_{Total}\)) and Mito Ca\(^{2+}\) cycling could be manipulated by varying the ratio of n-3 to n-6 PUFA in Mito membranes in young (6 mo) and aged (24 mo) isolated rat hearts treated to n-3 or n-6 PUFA-rich diet. Inotropic stimulation by 1 \(\mu\)M norepinephrine (NE) of 24-mo n-6 PUFA-rich hearts elevated total Mito Ca\(^{2+}\) content 38% more than in 6-mo hearts (P < 0.05). However, both the NE-induced rise in Mito Ca\(^{2+}\) and the difference in response between 6- and 24-mo hearts were partially abolished by n-3 PUFA treatment. NE increased the fractional activation of PDH by 44% above control levels in the 6-mo group with 49% in the 24-mo group after n-6 PUFA diet. However, NE stimulation of PDH\(_A\) was attenuated by n-3 PUFA diet, attaining values only 29 and 23% above control levels in 6- and 24-mo mitochondria, respectively (P < 0.05). Global ischemia and reperfusion (I/R) in n-6 PUFA hearts gave rise to higher levels of total Mito Ca\(^{2+}\) concentration (P < 0.0001) and PDH\(_A\) (P < 0.0001) compared with n-3 PUFA. Ruthenium red (3.4 \(\mu\)M) abolished the effects of I/R in all groups. With aging, heart Mito membrane phosphatidylcholine was increased after n-6 PUFA-rich diet (by ~15%, P < 0.05), whereas cardiolipin and n-3 PUFA content were diminished by 31% (P < 0.05) and 73% (P < 0.05), respectively. These effects were prevented by n-3 PUFA-rich diet. The present study, by directly manipulating the cardiac Mito membrane n-3-to-n-6 PUFA ratio, shows that the activation of Ca\(^{2+}\)-dependent PDH can be augmented when the n-3-to-n-6 PUFA ratio is low (n-6 PUFA-rich diet; 24-mo hearts) or attenuated when this ratio is relatively high (n-3 PUFA-rich diet). We propose that one of the consequences of dietary-induced manipulation of membrane phospholipids and PUFAs may be the altered flux of Ca\(^{2+}\) across the Mito membrane and thus altered intramitocondrial Ca\(^{2+}\)-dependent processes.

\(\omega-3\) fatty acids; mitochondria; calcium; pyruvate dehydrogenase; heart; rat

CALCIUM IONS SERVE a dual-messenger role in the heart in that they not only mediate excitation-contraction coupling during cardiac work and ATP hydrolysis but also activate dehydrogenases and the generation of ATP by oxidative phosphorylation, allowing maintenance of a balance of energy supply and demand (14, 16, 18, 22, 31). There are three Ca\(^{2+}\)-activated dehydrogenases, namely, NAD-isocitrate, 2-oxoglutarate, and pyruvate dehydrogenase (PDH), which catalyze nearirreversible reactions in the terminal oxidation of carbohydrate and lipid substrate (16). These enzymes reside in the mitochondrial (Mito) matrix, within the permeability barrier imposed by the inner Mito membrane, and their activation requires the transmission of changes in cytosolic Ca\(^{2+}\) into the mitochondria, a process that involves separate electrophoretic uptake (uniporter) and electroneutral Na\(^{+}/\)Ca\(^{2+}\) release (antiporter) pathways (10, 51). Ruthenium red blocks Mito net uptake of Ca\(^{2+}\) by impeding uniporter function (11) and thus prevents the activation of PDH (17, 30, 51). Ca\(^{2+}\) activates a phosphatase that dephosphorylates three serine residues of the E\(_{1A}\) subunit of PDH to form catalytically active PDH\(_A\) (53). This action confers stable interconversion and thus permits preservation of the in situ fractional activation of PDH\(_A\)/PDH\(_{Total}\) during tissue extraction under appropriate conditions (21).

Although Ca\(^{2+}\) play a positive role in control of cardiac Mito metabolism under physiological conditions, massive Mito accumulation of Ca\(^{2+}\) may occur following ischemia and can lead to Mito damage and cell death. The final step in this series of events has been proposed to be the Ca\(^{2+}\)-dependent opening of a nonselective, high-conductance channel in the Mito inner membrane (3) known as the “mitochondrial permeability transition” (MPT) pore, which causes the loss of proton motive force and failure of ATP generation (10).

With increased age, the n-6 PUFA content of rat cardiac cell and inner Mito membrane has been shown to be elevated in contrast to a decline in n-3 PUFA, and these changes are associated with abnormal cell Ca\(^{2+}\) balance leading to enhanced Ca\(^{2+}\)-dependent arrhythmogenesis during reperfusion following ischemia (32) and disparities in the specific activity and thermotropic kinetics of Mito membrane-bound enzymes (39). Consumption of a diet rich in “\(\omega-3\)” or n-3 polyunsaturated fatty acids (PUFA) has been shown to increase the ratio of n-3 to n-6 PUFA content in cardiac cell membranes (23, 33, 45, 46). Compared with rats fed n-6 PUFA-rich diet, normoxic isolated working hearts from n-3 PUFA-treated rats exhibited a higher efficiency in terms of...

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work performed per unit O₂ consumed (45). These differences were augmented following ischemia and reperfusion (I/R) such that O₂ utilization efficiency was decreased further in n-6 PUFA-treated hearts compared with n-3; however, this difference was abolished following routeisenium red-induced inhibition of Mito Ca\(^{2+}\) uptake (45).

The goal of the present study was to test whether direct modulation of the n-3-to-n-6 PUFA ratio in cardiac Mito membranes by dietary intervention could abolish age-associated changes in Mito membranes and rescue function in aged hearts by increasing their efficiency in Ca\(^{2+}\) handling and energy utilization. We postulated that a higher Mito Ca\(^{2+}\) content in postischemic n-6 PUFA hearts may be associated with higher Ca\(^{2+}\) cycling rates across the Mito membrane compared with n-3 hearts. Although this alone would cause a loss of efficiency, this might be compounded by excessive dehydrogenase activation, high values of proton motive force, and high rates of proton leakage across the Mito membrane. This would constitute a degree of uncoupling and a direct loss of efficiency according to the chemiosmotic mechanism of oxidative phosphorylation. We measured the fractional activation of Ca\(^{2+}\)-dependent PDH (PDH\(_{A}/PDH\)\(_{Total}\)) under conditions of increased work or ischemic stress in isolated perfused hearts from 6-mo and 24-mo rats that were fed a n-3 PUFA- or n-6 PUFA-rich diet for 6 wk. Total Mito Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) was measured in Mito preparations obtained using a rapid isolation technique that minimizes loss or gain of Ca\(^{2+}\) (29). Proton leak in mitochondria was inferred by measuring state 4 respiration (absence of ADP) with a variety of oxidizable substrates. The effects of age and dietary lipid on membrane PUFA, cardiolipin, and other phospholipids were measured in mitochondria isolated from ventricular myocardium.

METHODS

Animals and dietary lipid supplementation. Male Wistar rats from the Gerontology Research Center Aging Colony were utilized in strict accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Isocaloric diets were formulated by an 11.7% (wt/wt) addition of either animal fat (rich in n-6 PUFA) or marine fish oil (rich in n-3 PUFA) to a standard low-fat reference diet (Ref) that fulfilled essential fatty acid requirements for normal growth (46). The fat-supplemented diets contained 15.6% total fat, whereas the low-fat Ref diet contained 3.9% normal growth (46). The fat-supplemented diets contained 15.6% total fat, whereas the low-fat Ref diet contained 3.9% normal growth (46). The fat-supplemented diets contained 15.6% total fat, whereas the low-fat Ref diet contained 3.9% normal growth (46). Animals were fed for a minimum of 6 wk and were 6 and 24 mo old at the time of isolated heart perfusions.

Isolated heart preparation. After rapid cannulation of the aorta, coronary perfusion of the isolated isovolumic hearts was commenced at a constant pressure of 75 mmHg with a filtered (0.45 µm) bicarbonate buffer that consisted of (in mmol/l): 3.48 KCl, 116.4 NaCl, 26.2 NaHCO₃, 1.67 NaH₂PO₄, 0.69 MgSO₄, 1.5 CaCl₂, 11.1 glucose, and 0.2 octanoate. The buffer was gassed with 95% O₂-5% CO₂ and equilibrated at pH 7.38–7.42, 35°C. The coronary flow rate, which was monitored during all experimental protocols, was maintained at 19 ± 0.5 ml/min, but this was decreased to 1 ml/min to produce low-flow global ischemia. Four perfusion protocols were implemented: 3-Hz electrical pacing, with hearts stabilized for 15 min (Control); 5 Hz + 10⁻⁷ M norepinephrine (NE) (stimulated increased work); 15-min low-flow ischemia + 5-min reperfusion, with 3-Hz electrical pacing throughout (I/R); and perfusion with 3.4 µM ruthenium red during control period, 15-min low-flow ischemia, and 5-min reperfusion, with 3 Hz throughout (I/R + RR).

Rapid Mito isolation. Mitochondria were prepared from these hearts by a rapid isolation method (29) that was designed to minimize Ca\(^{2+}\) redistribution. At the end of each protocol, the ventricles were cut free, a portion was freeze-clamped in liquid N₂, and the rest was dropped directly into ice-cold medium containing (in mmol/l) 250 sucrose, 0.03 diltiazem, 0.0032 ruthenium red, 2 K-EGTA, and 10 K-HEPES, pH 7.4, and was homogenized for 20 s with a Brinkman Polytron (12,000–15,000 rpm). The homogenate was subjected to rapid centrifugation to isolate the mitochondria, as described previously (21).

Atomic absorption spectroscopy. A portion of mitochondria isolated from each heart subjected to one of the four perfusion protocols was stored at -80°C until Ca\(^{2+}\) content could be determined. Mito suspensions were digested in high-purity nitric acid. Mito Ca\(^{2+}\) was determined in samples containing 0.1% lanthanum oxide (to prevent phosphate interference) by atomic absorption spectrophotometric analysis (Perkin-Elmer Zeeman 5000) with CaCO₃ as the standard. Absorbance readings were corrected for lanthanum, and final values were expressed as nanomoles per milligram protein.

The supernatant was removed and stored on ice. PDH\(_{A}\) was assayed in a reaction mixture composed of (in mmol/l) 50 K Philip (pH 7.2), 3 EDTA, 25 NaF, 1 K-dichloroacetate, 1 1,4-dithiothreitol, 1 ADP, and 0.05 leupeptin. The ultrafine tissue powder was eventually permitted to reach 0°C on ice and thus thawed into a suspension that was centrifuged at 15,000 g for 5 min at 0°C. The supernatant was removed and stored on ice. PDH\(_{A}\) was assayed spectrophotometrically at 340 nm and 30°C by adding 100 µl of extract to 900 µl of the following medium (in mmol/l): 50 K-HEPES (pH 7.2), 1 MgCl₂, 4 1,4-dithiothreitol, 0.004 rotenone, 1.67 NAD, 0.2 thiamine pyrophosphate, 0.15 coenzyme A (CoA), 2 ADP, 0.08 EDTA, 16.7 L-lactate (pH 7.2), and 2 µM lactate dehydrogenase. Citrate synthase activity was assayed in a reaction mixture composed of 0.1 M Tris-Cl (pH 8), 0.1 mM DTNB, 100 µM acetyl CoA, and 0.05% Triton X-100. Extract (20 µl) was added, followed 1 min later by 0.5 µmol of oxaloacetate. The total volume was 2 ml, and the reaction was spectrophotometrically followed by an increase in absorbance at 412 nm. Substrate oxidation by isolated mitochondria. Mitochondria were isolated from ventricular tissue using the Nagarse
digestion method (29). Oxygen uptake of Mito suspensions was measured using an O2 electrode (Yellow Springs Instruments) and a thermostat-regulated chamber of 2 ml. Temperature was maintained at 25 or 37°C as specified. The basal composition of the medium was (in mmol/l) 120 KCl, 20 K-HEPES (pH 7.2), 10 NaCl, 2 KPO4, and 1 mg/ml of fatty acid-poor bovine serum albumin. The respiratory substrates are specified below.

Mito membrane lipid analysis. Lipids were extracted by the method of Bligh and Dyer (5). The antioxidant butylated hydroxytoluene was included in the extract (0.1% of the lipid dry weight). Phospholipids were separated from the total lipid extract by thin-layer chromatography on silica gel H plates, which were developed in petroleum ether-diethyl ether-acetic acid (90:15:1). The phospholipids remaining at the origin were eluted from the silica gel and methylated in 1%(vol/vol) H2SO4 in methanol by heating at 70°C for 3 h. The fatty acid profiles of the diets and isolated Mito membrane phospholipids were determined by gas-liquid chromatography (5710A Hewlett-Packard) using columns packed with equal parts of SP 2310 and SP 2330 (Supelco).

Chemicals. Reagents were purchased from Sigma, St. Louis, MO, except acetyl CoA and oxaloacetate, which were from Calbiochem (LaJolla, CA). Lactate dehydrogenase and NAD were from Boehringer-Mannheim (Indianapolis, IN).

Statistical analyses. Data are presented as means ± SE. Group contrasts were tested where indicated by three-way ANOVA or by two-way ANOVA (SPSS for Windows). Individual comparisons were determined by Student-Newman-Keuls post hoc test. Significant differences were accepted at P < 0.05.

RESULTS

PDHA and Mito Ca2+ during ß-adrenergic receptor stimulation. Table 1 shows that under control perfusion conditions peak systolic pressure did not differ among any of the dietary or age groups. PDHA levels tended to be higher in 24-mo compared with 6-mo hearts and this was significant after n-6 PUFA-rich diet (P < 0.05 vs. 6 mo). Although NE raised peak systolic pressure from control levels by more than twofold, an equivalent level of PDHA was observed among all of the control perfusion groups. NE markedly raised PDHA in all groups compared with respective control perfused hearts. In 6-mo rat hearts, the highest level of PDHA after control perfusion or NE was measured in the n–6 PUFA group and the lowest in the n–3 PUFA group. In Ref hearts, the effect of NE stimulation was significantly greater when they were aged 24 mo compared with 6 mo (P < 0.05). However, in 6-mo n–6 PUFA hearts, NE treatment elevated PDHA to a level equal to that measured in 24-mo n–6 PUFA hearts. Values of PDHA for NE-stimulated hearts were lower in the n–3 PUFA group than in the n–6 PUFA group, and no significant difference in PDHA occurred among 6- and 24-mo n–3 PUFA hearts. A three-way ANOVA indicated significant effects of age, dietary lipid, and treatment with 10−7 M NE (F = 30.8, P < 0.001) on the activation of PDH (PDHA/Total). There were no significant three-way or two-way interactions.

<table>
<thead>
<tr>
<th>Table 1. Effect of 10−7 M NE in perfused hearts on PSP, PDHA, and Mito [Ca2+]</th>
<th>Dietary Group</th>
<th>Ref</th>
<th>n–6 PUFA</th>
<th>n–3 PUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 mo</td>
<td>24 mo</td>
<td>6 mo</td>
<td>24 mo</td>
</tr>
<tr>
<td>Mito [Ca2+] (mM/mg protein)</td>
<td>0.33 ± 0.02</td>
<td>0.38 ± 0.03</td>
<td>0.43 ± 0.03</td>
<td>0.46 ± 0.04</td>
</tr>
<tr>
<td>PDHA, %Total PDH</td>
<td>11.7 ± 0.66</td>
<td>13.4 ± 1.56</td>
<td>13.6 ± 0.52</td>
<td>20.5 ± 2.34</td>
</tr>
<tr>
<td>Mito [Ca2+]</td>
<td>0.88 ± 0.03*</td>
<td>1.34 ± 0.05†</td>
<td>1.17 ± 0.05*</td>
<td>1.47 ± 0.07†</td>
</tr>
<tr>
<td>PDHA, %Total PDH</td>
<td>40.6 ± 2.24*</td>
<td>65.2 ± 17.5**</td>
<td>57.7 ± 8.8**</td>
<td>69.4 ± 11.1**</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 in reference (Ref) and n–6 polyunsaturated fatty acid (PUFA) groups and 5 in n–3 PUFA group. Activation of pyruvate dehydrogenase (PDHap) is expressed as %total PDH, which was determined by additional perfusion with 5 mM dichloracetate of subgroups of hearts for each age and diet. All PDH measures are normalized to citrate synthase content in each heart. Citrate synthase activity did not differ significantly among any of the groups, regardless of age or dietary lipid treatment. NE, norepinephrine; PSP, peak systolic pressure; Mito [Ca2+]; mitochondrial Ca2+ concentration. *P < 0.05 vs. control. †P < 0.05 vs. 6 mo. ‡P < 0.05 vs. Ref. §P < 0.05 vs. n–6 PUFA.
effects of age, diet, and I/R treatment on PDHA were observed (3-way ANOVA, $F = 40.5, P < 0.0001$; no significant 3-way interactions). PDHA was significantly greater in the postischemic n–6 PUFA group than in the n–3 PUFA group, with the Ref group displaying intermediate values (diet $\times$ I/R, $F = 17, P < 0.0001$). PDHA values tended to be higher in 24-mo Ref and n–6 PUFA hearts compared with 6-mo hearts (NS). I/R made little impact on PDHA levels in n–3 PUFA hearts compared with 24-mo Ref and n–6 PUFA hearts.

Figure 2 shows that the pattern of total Mito $[Ca^{2+}]$ levels during I/R resembled that seen for PDHA. Significant effects of age, diet, and I/R treatment on total Mito $[Ca^{2+}]$ were observed (3-way ANOVA, $F = 218.6, P < 0.0001$; no significant 3-way interactions). In particular, total Mito $[Ca^{2+}]$ values for I/R were significantly greater in the Ref and n–6 PUFA groups than in the n–3 PUFA groups (F = 24.8, P < 0.0001). As with PDHA, in 24-mo Ref and n–6 PUFA hearts there was a trend toward higher total Mito $[Ca^{2+}]$ values compared with their respective 6-mo counterparts. Compared with these two diet groups, total Mito $[Ca^{2+}]$ values in 6- and 24-mo n–3 PUFA groups were both lower.

Respiratory properties of mitochondria from the dietary PUFA groups. For measurement of respiration rates, mitochondria were isolated from additional hearts by a standard technique. Comparison of the coupled respiratory state 3 (0.5 mM ADP) with the resting state 4 (no ADP present) $O_2$ uptake rates, with pyruvate as the oxidative substrate, shows that the preparations were well-coupled (Table 2). Calculated respiratory control ratios (state 3/state 4) exceed a value of 8. In general, state 3 rates of substrate oxidation were unchanged by age or diet. Exceptions to this are a decline with age in the rate of glutamate oxidation in Ref (2-way ANOVA, $F = 3.7, P = 0.012$; significant age $\times$ diet interaction, $F = 8.1, P = 0.002$) and a lower rate of succinate oxidation in the 6-mo n–3 PUFA group relative to the other dietary groups ($F = 4.3, P = 0.014$; no significant interaction for age $\times$ diet). By contrast, state 4 rates of $O_2$ uptake were elevated in n–6 PUFA hearts compared with n–3 PUFA (Table 2), particularly with pyruvate (2-way ANOVA, $F = 4.9, P = 0.009$) and glutamate (2-way ANOVA, $F = 3.8, P = 0.024$) as substrates. Note that the values in Table 2 were obtained at 25°C, a temperature often used for Mito studies. Repetition at 37°C using different preparations of isolated mitochondria also showed that state 3 rates were largely unchanged by age or by diet (Table 3). This is in contrast to the results of an earlier study, which showed a decrease with age in the rate of palmitoylcarnitine oxidation (15). The reason for the disparity is not clear but could relate to a different fat composition of the (undefined) lab chow used in that study. However, state 4 rates of pyruvate (2-way ANOVA, $F = 4.99, P = 0.008$) and palmitoylcarnitine oxidation (2-way ANOVA, $F = 17.1, P < 0.001$) were significantly higher in the n–6 PUFA group than in the n–3 PUFA group. In particular, state 4 rates were highest in 24-mo n–6 PUFA hearts (Table 3). There were no diet $\times$ age interactions for state 4 rates of oxidation of either pyruvate or palmitoylcarnitine at 37°C.

Cardiac Mito membrane phospholipid and fatty acid profiles. There was a significant decrease in Mito cardiolipin (diphasophatidylglycerol) content in senescent Ref and n–6 PUFA groups (2-way ANOVA, $F = 58.1, P < 0.001$; age $\times$ diet interaction $F = 6.94, P = 0.01$). In contrast, no decrease in cardiolipin occurred in the n–3 PUFA group. Significant main effects of age and diet on phosphatidylcholine content were observed (2-way ANOVA, $F = 44.3, P < 0.001$; age $\times$ diet interaction $F = 9.99, P = 0.001$). Phosphatidylcholine increased with aging in the Ref and n–6 PUFA groups but was unchanged in the n–3 PUFA group (Table 4).

Table 5 shows that, compared with n–3 PUFA rich-diet, n–6 PUFA-rich diet produced significantly greater n–6 PUFA incorporation into Mito membranes (linoleic acid (C18:2) 2-way ANOVA main effects: $F = 585.9, P < 0.001$, age $\times$ diet interaction $F = 18.3, P < 0.001$; arachidonic acid (C20:4) 2-way ANOVA main effects: $F = 85.9, P < 0.001$, age $\times$ diet interaction $F = 5.5, P = 0.009$). In contrast, n–3 PUFA diet resulted in increased Mito levels of n–3 PUFAs such as eicosapentaenoic acid (C20:5; 2-way ANOVA, main effects: $F = 3.8, P = 0.009$).
Table 2. Substrate oxidation rates at 25°C

<table>
<thead>
<tr>
<th>State</th>
<th>Substrate</th>
<th>Ref (6 mo)</th>
<th>Ref (24 mo)</th>
<th>n-6 PUFA (6 mo)</th>
<th>n-6 PUFA (24 mo)</th>
<th>n-3 PUFA (6 mo)</th>
<th>n-3 PUFA (24 mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 mo</td>
<td>Pyruvate</td>
<td>218.3 ± 4.4</td>
<td>224.2 ± 13.4</td>
<td>209.2 ± 6.7</td>
<td>202.5 ± 9.8</td>
<td>188.0 ± 7.8</td>
<td>202.4 ± 7.3</td>
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<tr>
<td></td>
<td>Glutamate-malate</td>
<td>100.2 ± 9.3</td>
<td>125.5 ± 10.7</td>
<td>149.9 ± 7.4</td>
<td>158.9 ± 12.4</td>
<td>145.5 ± 2.9</td>
<td>131.7 ± 2.2</td>
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<tr>
<td></td>
<td>Succinate</td>
<td>143.0 ± 11.7</td>
<td>149.6 ± 13.9</td>
<td>133.5 ± 3.4</td>
<td>159.0 ± 16.5</td>
<td>105.8 ± 1.2*</td>
<td>131.7 ± 2.2*</td>
</tr>
<tr>
<td>4 mo</td>
<td>Pyruvate</td>
<td>20.2 ± 11</td>
<td>22.0 ± 1.4</td>
<td>25.6 ± 2.9</td>
<td>23.9 ± 1.0</td>
<td>17.5 ± 1.0†‡</td>
<td>20.0 ± 0.5‡</td>
</tr>
<tr>
<td></td>
<td>Glutamate-malate</td>
<td>24.8 ± 1.8</td>
<td>22.6 ± 1.2</td>
<td>24.3 ± 1.1</td>
<td>32.5 ± 4.9*</td>
<td>19.0 ± 0.3†‡</td>
<td>20.9 ± 0.8‡</td>
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<tr>
<td></td>
<td>Succinate</td>
<td>53.0 ± 3.3</td>
<td>52.4 ± 2.3</td>
<td>51.4 ± 2.3</td>
<td>57.5 ± 8.9</td>
<td>43.6 ± 0.5</td>
<td>47.7 ± 3.2</td>
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Values are means ± SE of substrate oxidation rates at 25°C elicited by addition of ADP to give 0.5 mM (state 3) or in absence of ADP following its consumption (state 4) in ventricular mitochondria isolated after stabilized control perfusion from rats subjected to modification of cardiac membrane lipid profile by dietary treatments; n = 6 in Ref and n-6 PUFA groups and 5 in n-3 PUFA group. Respiration substrates were utilized at the following final concentrations: pyruvate (2.5 mM) + L-malate (5 mM); glutamate (5 mM) + L-malate (5 mM); succinate (5 mM) + rotenone (1 µM). EGTA was present at 0.1 mM. O2 uptake rate is measured in ng atom·min⁻¹·mg protein⁻¹ and was normalized to citrate synthase units/mg protein. *P < 0.05 vs. Ref. †P < 0.05 vs. n-6 PUFA.

Table 3. Substrate oxidation rates at 37°C

<table>
<thead>
<tr>
<th>State</th>
<th>Substrate</th>
<th>Ref (6 mo)</th>
<th>Ref (24 mo)</th>
<th>n-6 PUFA (6 mo)</th>
<th>n-6 PUFA (24 mo)</th>
<th>n-3 PUFA (6 mo)</th>
<th>n-3 PUFA (24 mo)</th>
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<tbody>
<tr>
<td>6 mo</td>
<td>Pyruvate</td>
<td>479.0 ± 15.4</td>
<td>471.7 ± 25.5</td>
<td>428.0 ± 18.3</td>
<td>470.5 ± 42.3</td>
<td>417.6 ± 22.4</td>
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<td>Palmitoyl carnitine</td>
<td>369.3 ± 8.4</td>
<td>404.3 ± 23.8</td>
<td>323.8 ± 15.1</td>
<td>385.0 ± 36.6</td>
<td>337.8 ± 13.1</td>
<td>375.6 ± 7.64</td>
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<tr>
<td>4 mo</td>
<td>Pyruvate</td>
<td>49.9 ± 2.9</td>
<td>55.1 ± 2.3</td>
<td>54.5 ± 5.7</td>
<td>62.7 ± 4.8</td>
<td>43.4 ± 1.8‡</td>
<td>50.5 ± 2.9‡</td>
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<td>Palmitoyl carnitine</td>
<td>45.6 ± 0.4</td>
<td>63.7 ± 1.8*</td>
<td>48.5 ± 0.3</td>
<td>73.1 ± 6.1†‡</td>
<td>46.5 ± 1.7†‡</td>
<td>59.5 ± 3.3*</td>
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</table>

Values are means ± SE of substrate oxidation rates at 37°C elicited by addition of ADP to give 0.5 mM (state 3) or in absence of ADP following its consumption (state 4) in ventricular mitochondria isolated after stabilized control perfusion from rats subjected to modification of cardiac membrane lipid profile by dietary treatments; n = 4 in Ref and n-3 PUFA groups and 3 in n-6 PUFA group. Respiration substrates were utilized at the following final concentrations: pyruvate (2.5 mM) + L-malate (0.5 mM); palmitoyl carnitine (20 µM) + L-malate (0.5 mM). EGTA was present at 0.1 mM. The O2 uptake rate is expressed as ng atom·min⁻¹·mg protein⁻¹ and was normalized to citrate synthase units/mg protein. *P < 0.05 vs. 6 mo. †P < 0.05 vs. Ref. ‡P < 0.05 vs. n-6 PUFA.
Although it is still controversial whether Mito matrix free [Ca\(^{2+}\)] follows changes in cytosol free Ca\(^{2+}\) on a beat-to-beat basis (27), it is clear that the larger systolic transients that occur in the cytosol when cardiac myocytes are stimulated with NE result in a significant increase in Mito matrix free [Ca\(^{2+}\)] over the course of a few seconds (35). Furthermore, the blockade of excessive Mito Ca\(^{2+}\) uptake by ruthenium red before I/R in this study completely prevented the I/R-augmented activation of PDH. The effect of ruthenium red to abrogate a stimulated increase in PDHA, which has been shown to result in decreased tissue ATP/(ADP + P\(_i\)) (51), confirms earlier work (17, 30). A caution in making this sort of relation between Mito total Ca\(^{2+}\) and either PDHA content or respiratory activity is that the former is measured in a preparation that is derived largely from subsarcolemmal mitochondria, whereas the latter parameters derive from both subsarcolemmal and intermyofibrillar mitochondria. These two populations of cardiac myocyte mitochondria have been shown to differ significantly in both Ca\(^{2+}\)-transport and respiratory properties. There is no reason to believe that the relative size of these two populations is changed by our dietary manipulations, although it could be so.

Although PDH is emphasized in this report, findings of altered activation by Ca\(^{2+}\) are thought to relate equally to the Ca\(^{2+}\)-sensitive dehydrogenases of the tricarboxylate cycle (31). This is important, because the majority of the reducing equivalents available for oxidation by the respiratory chain are generated in the tricarboxylate cycle, whether the substrate is carbohydrate or fat. Thus there is no suggestion that increased values of PDHA change the partition of flux between glucose and fatty acid oxidation, but rather that substrate oxidation overall is activated. In this context, we used both glucose and octanoate as substrate for the perfusions to mimic the availability of both glucose and fatty acids in vivo, while avoiding problems with solubility of long-chain fatty acids. In addition, the presence of fatty acid depresses PDHA levels due to the activation by acetyl CoA and NADH (20, 53) and allows more scope for increase in PDHA due to Ca\(^{2+}\). Inclusion of fatty acid in our perfusions does not permit direct comparison with the work of Kobayashi and Neely (28), in which they utilized glucose as the sole oxidizable substrate and did not observe elevated PDHA after reperfusion.

### Table 4. Cardiac ventricular mitochondrial membrane phospholipid profile

<table>
<thead>
<tr>
<th>Dietary Groups</th>
<th>6 mo</th>
<th>24 mo</th>
<th>6 mo</th>
<th>24 mo</th>
<th>6 mo</th>
<th>24 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPG</td>
<td>14 ± 0.4</td>
<td>10 ± 0.5*</td>
<td>12 ± 0.4</td>
<td>8.5 ± 0.4*</td>
<td>15 ± 0.4</td>
<td>14 ± 0.4†</td>
</tr>
<tr>
<td>PC</td>
<td>45 ± 0.8</td>
<td>49 ± 0.8*</td>
<td>46 ± 0.7</td>
<td>53 ± 0.7†</td>
<td>42 ± 0.8</td>
<td>42 ± 0.7†</td>
</tr>
<tr>
<td>PE</td>
<td>36 ± 0.7</td>
<td>34 ± 0.8</td>
<td>35 ± 0.8</td>
<td>33.3 ± 0.6</td>
<td>38 ± 0.8</td>
<td>40 ± 0.8</td>
</tr>
<tr>
<td>PS</td>
<td>2.3 ± 0.2</td>
<td>2.5 ± 0.2</td>
<td>2.4 ± 0.3</td>
<td>2.5 ± 0.2</td>
<td>2.2 ± 0.1</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>PI</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.25 ± 0.1</td>
<td>1.40 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.3</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE and expressed as % of total phospholipids extracted from 5 rat hearts per group. DPG, diphostatidyglycerol (cardiolipin); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol. *P < 0.05 vs. 6 mo. †P < 0.05 vs. Ref. ‡P < 0.05 vs. n–6 PUFA.

### Table 5. Summary of major long chain fatty acids incorporated in mitochondrial membrane phospholipids following dietary lipid treatment and aging

<table>
<thead>
<tr>
<th>Dietary Groups</th>
<th>6 mo</th>
<th>24 mo</th>
<th>6 mo</th>
<th>24 mo</th>
<th>6 mo</th>
<th>24 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref</td>
<td>16:0</td>
<td>9.0 ± 0.29</td>
<td>12.0 ± 0.35*</td>
<td>10.0 ± 0.29</td>
<td>14.0 ± 0.69*</td>
<td>9.5 ± 0.35</td>
</tr>
<tr>
<td>n–6 PUFA</td>
<td>18:0</td>
<td>21.3 ± 0.12</td>
<td>24.0 ± 0.87</td>
<td>23.0 ± 1.15</td>
<td>26.0 ± 1.73</td>
<td>10.1 ± 0.72†</td>
</tr>
<tr>
<td>n–3 PUFA</td>
<td>18:1</td>
<td>0.3 ± 0.07</td>
<td>0.9 ± 0.11</td>
<td>0.22 ± 0.05</td>
<td>0.3 ± 0.12</td>
<td>0.7 ± 0.10</td>
</tr>
<tr>
<td>n–6 PUFA</td>
<td>18:2</td>
<td>9.4 ± 0.09</td>
<td>10.0 ± 0.39</td>
<td>9.0 ± 0.81</td>
<td>9.0 ± 0.43</td>
<td>9.5 ± 0.40</td>
</tr>
<tr>
<td>n–3 PUFA</td>
<td>18:3</td>
<td>25.3 ± 0.37</td>
<td>12.0 ± 0.29*</td>
<td>17.0 ± 1.21</td>
<td>11.0 ± 1.44*</td>
<td>12.0 ± 0.58†</td>
</tr>
<tr>
<td>n–6 PUFA</td>
<td>20:4</td>
<td>18.0 ± 1.15</td>
<td>29.0 ± 1.44*</td>
<td>24.0 ± 1.44</td>
<td>32.0 ± 1.79*</td>
<td>9.2 ± 0.64†</td>
</tr>
<tr>
<td>n–3 PUFA</td>
<td>22:4</td>
<td>0.7 ± 0.12</td>
<td>1.1 ± 0.16</td>
<td>1.1 ± 0.18</td>
<td>1.6 ± 0.15</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>n–6 PUFA</td>
<td>18:3</td>
<td>0.2 ± 0.01</td>
<td>0.1 ± 0.04</td>
<td>0.2 ± 0.03</td>
<td>0.2 ± 0.03</td>
<td>1.8 ± 0.06</td>
</tr>
<tr>
<td>n–3 PUFA</td>
<td>20:5</td>
<td>0.3 ± 0.03</td>
<td>0.3 ± 0.01</td>
<td>0.1 ± 0.03</td>
<td>0.1 ± 0.05</td>
<td>6.0 ± 0.17†</td>
</tr>
<tr>
<td>n–6 PUFA</td>
<td>22:5</td>
<td>2.0 ± 0.16</td>
<td>0.5 ± 0.09*</td>
<td>2.0 ± 0.12</td>
<td>0.8 ± 0.12*</td>
<td>2.2 ± 0.05</td>
</tr>
<tr>
<td>n–3 PUFA</td>
<td>22:6</td>
<td>10.0 ± 0.23</td>
<td>4.0 ± 0.29*</td>
<td>9.0 ± 0.64</td>
<td>2.0 ± 0.43*</td>
<td>2.70 ± 1.01†</td>
</tr>
<tr>
<td>n–6 PUFA</td>
<td>Σ n–6 PUFA</td>
<td>44.0 ± 0.84</td>
<td>42.1 ± 1.23</td>
<td>42.1 ± 0.85</td>
<td>44.6 ± 1.58</td>
<td>21.3 ± 0.50†</td>
</tr>
<tr>
<td>n–3 PUFA</td>
<td>Σ n–3 PUFA</td>
<td>12.5 ± 0.17</td>
<td>4.9 ± 0.34*</td>
<td>11.3 ± 0.69</td>
<td>3.1 ± 0.44*</td>
<td>37.1 ± 1.11†</td>
</tr>
<tr>
<td>n–3 n–6 PUFA</td>
<td>0.29 ± 0.01</td>
<td>0.12 ± 0.01*</td>
<td>0.27 ± 0.02</td>
<td>0.07 ± 0.01*</td>
<td>1.74 ± 0.04†</td>
<td>2.01 ± 0.09‡</td>
</tr>
</tbody>
</table>

Values (mol/100 mol) are presented as means ± SE; n = 6. Key PUFAs: 18:2 (n–6), linoleic acid; 20:4 (n–6), arachidonic acid; 18:2 (n–3), α-linolenic acid; 20:5 (n–3), eicosapentaenoic acid; 22:6 (n–3), docosahexaenoic acid. *P < 0.05 vs. 6 mo. †P < 0.05 vs. Ref. ‡P < 0.05 vs. n–6 PUFA.
Total Mito Ca\(^{2+}\) remained within the physiological range, regardless of the diet or age group, most likely because of the brevity of the ischemic period. However, the mitochondria from n–6 PUFA hearts clearly showed a greater net uptake of Ca\(^{2+}\) than those in the n–3 PUFA group. On this basis, we may predict that n–6 PUFA-rich hearts are likely to reach Ca\(^{2+}\) overload before n–3 PUFA hearts, following longer periods of ischemia, with the consequent opening of the MPT channel, decrease in proton-motive force, and cellular de-energization (10). Thus the high proportion of n–3 PUFA in cardiac Mito membranes after n–3 PUFA-rich diet may confer protection against I/R injury by minimizing the impact of Mito Ca\(^{2+}\) overload. We may also predict an impact of altered n–3 PUFA in Mito membranes on both necrotic and apoptotic cell death. By affecting the tendency of the mitochondria to overload with Ca\(^{2+}\) after I/R, the n–3-to-n–6 PUFA ratio may determine whether the MPT channel opens or not. This has been implicated in the process of apoptosis, which can be opposed by Ca\(^{2+}\) chelators, as well as by oxygen radical scavengers and the protein BCL-2 (48, 49). However, it remains controversial whether Mito membrane depolarization precedes MPT pore opening, or vice versa, in apoptosis.

In response to stimulation by NE, the hearts from animals fed n–6 PUFA-rich diet appear to have an advantage, in that they showed a more powerful activation of PDH and presumably also of the tricarboxylate cycle. However, the workload sustained in n–6 PUFA hearts with NE was not significantly different compared with n–3 PUFA hearts because left ventricular systolic pressure (Table 1), coronary flow rates, coronary perfusion pressure, and electrical stimulation rates were similar in both dietary groups regardless of age. This indicates that activation of PDH, and by inference activation of the Krebs cycle, was inefficiently high in n–6 PUFA hearts for an equivalent amount of mechanical work, compared with n–3 PUFA.

In the context of the lowered thermodynamic efficiency of the n–6 PUFA hearts, three possibilities can be envisaged from the present results. 1) The elevated Mito matrix free [Ca\(^{2+}\)], which can be directly inferred from the elevated total Mito [Ca\(^{2+}\)], will result in increased rates of Ca\(^{2+}\) cycling across the Mito membrane. Because the uniporter is driven by ΔpH and the antiporter by Δψ (10), this will lead to dissipation of proton-motive force (the energy currency of the mitochondrion). Under physiological conditions, the energy usage of this futile cycle is usually regarded as minimal (2), but it may become substantial after I/R. 2) The greater degree of activation of PDH, and presumably of the tricarboxylate cycle, after n–6 PUFA-rich diet or with increased age would be expected to generate high proton-motive forces and high rates of proton leak across the inner Mito membrane. Such an effect is seen in studies where high, nonphysiological concentrations of substrate are used in experimental work with isolated mitochondria. Under such conditions, the conductance of the membrane to protons becomes “nonohmic” and proton leak increases rapidly with proton-motive force (38). It is indeed possible to generate very high Mito NADH-to-NAD\(^{+}\) ratios, and presumably proton-motive forces, by exposing heart mitochondria to buffered 1 µM free Ca\(^{2+}\), pyruvate, and 2-oxoglutarate as substrates (20, 37). Although we suspect that such a mechanism is a plausible source of inefficiency, it remains somewhat speculative in the absence of a direct measurement of Mito protonmotive force in situ in the perfused isolated beating heart. 3) We have presently shown that state 4 rates of respiration are raised in mitochondria from the n–6 PUFA hearts, even when Ca\(^{2+}\) ions are excluded from the incubation. This indicates an intrinsic increase in proton permeability of the membrane, which is likely a direct consequence of the changed lipid composition of these organelles following dietary treatment.

The degree to which the feeding of n–3 PUFA-rich diet prevents the age-associated decrease in the n–3-to-n–6 PUFA ratio that occurs with n–6 PUFA and Ref diets is striking. Our present finding of dietary lipid modulation of cardiac membrane ratio of n–3 to n–6 PUFA is supported by other studies that have used a similar dietary model with younger adult rats, monkeys, or dogs and have shown that following n–3 PUFA-rich diet, postischemic recovery of Mito energy metabolism and myocardial pump function is improved and that, in addition, there is a reduced incidence of reperfusion arrhythmias compared with n–6 PUFA (9, 23, 33, 34, 45, 46, 57, 58). The Ref diet is a standard rat chow (except that all long-carbon chain n–3 PUFAs are excluded), and thus is relevant to previous studies of heart mitochondria in aging. Nohl (39) reported that the ratio of unsaturated to saturated fatty acids of lipids from the inner membrane of rat heart mitochondria was significantly decreased and n–6 PUFA content rose in contrast to a significant decline in n–3 PUFA in senescent rats. A higher n–3-to-n–6 PUFA ratio (longer carbon chain vs. shorter carbon chain PUFA) implies a greater degree of membrane fluidity; thus the n–3 PUFA-rich diet may be expected to prevent a decrease in membrane fluidity otherwise seen in aging. Increased cardiac Mito membrane local lipid viscosity may alter lipid-protein relationships and thus the activity of intrinsic membrane proteins (25, 26). For example, it has been shown that following increased dietary incorporation of n–3 PUFA into cardiac sarcoplasmic reticulum membranes, there is a lower relative activity of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase and a 30% decrease in ATP-dependent Ca\(^{2+}\)-uptake (50). Na\(^+\)/Ca\(^{2+}\) exchange in cardiac sarcolemmal vesicles has been shown to be stimulated by acute exposure to PUFA (47). Single cardiac myocyte studies have demonstrated that the activities of dihydropyridine-sensitive L-type Ca\(^{2+}\) channels (44), transient outward K\(^+\) channels (8, 24, 52), and Na\(^+\)/Ca\(^{2+}\) channels (54) are specifically attenuated following acute exposure to nanomolar concentrations of free n–3 PUFA. Although the mechanism of action underlying acute exposure may not be the same as that of dietary-induced incorporation of PUFA into membrane phospholipids, the ability to modulate the activity of...
such protein complexes, according to the type of PUFA content in related phospholipids, indicates that n–3 PUFA-rich membranes may minimize the extent of I/R-induced perturbation of intracellular ion homeostasis. The impact of dietary lipid modulation of the n–3-to-n–6 PUFA ratio is far reaching and multifarious because of effects on Ca\textsuperscript{2+}-homeostasis, altered Ca\textsuperscript{2+}-dependent phospholipase A\textsubscript{2} activity, the release of free PUFAs from membranes, and their subsequent interaction with cyclooxygenases, lipooxygenases, and other enzymes in the formation of prostanooids, leukotrienes, cytokines, fatty acid hydroperoxides, and 4-hydroxyalkenals (1, 42, 49). A detailed review of these important “cascade” effects and their feedback is, however, beyond the scope of this discussion.

We also measured, in hearts treated to n–3 PUFA-rich diet, a significant increase in the presence of cardiolipin (diphosphatidylglycerol), a phospholipid present only in the inner Mito membrane that is required for the activity of cytochrome-c oxidase (the terminal member of the respiratory electron-transfer chain), carnitine translocase, and adenine nucleotide translocase (4, 41). Our present finding of an n–3 PUFA-induced increase in cardiolipin content is in agreement with a previous study of fish oil-fed dogs (34). In contrast, Yamaoka et al. (55, 56), using an essential fat-deficient diet supplemented with long-chain n–3 PUFAs, found no alteration in cardiolipin but instead a significant decrease in cytochrome-c oxidase activity and oxidative phosphorylation. However, in our study, we avoided essential fatty acid deficiency. Previous work has shown that the rates of transport of adenine nucleotides, pyruvate, and carnitine are decreased with increased age (15, 40, 43). Substrate transport and electron transport chain function are dependent on membrane viscosity (12). Thus the decreased cardiolipin content found with increased age (43) may alter the activity of inner Mito membrane proteins. Generally, the lipids of metabolically active intracellular membranes such as mitochondria are considerably unsaturated (7); thus specific proportions of long-chain highly unsaturated n–3 PUFAs may be essential in metabolic and ionic homeostasis. It is important to note that we observed no major difference in Mito membrane n–6 PUFA or n–3 PUFA between the n–6 PUFA and the Ref dietary groups. These two diets were devoid of all n–3 PUFA diets with carbon chains longer than α-linolenic acid (18:3); i.e., n–3 PUFAs of marine origin were excluded. However, Ref, a comparatively low-fat version of the n–6 PUFA-rich diet, had the same n–3-to-n–6 PUFA ratio of 0.2 as the n–6 PUFA diet. Thus these Mito differences following n–3 PUFA-rich diet that we presently report may be ascribed to increased incorporation of long-carbon chain n–3 PUFAs into Mito membrane phospholipids.

In conclusion, this study has shown the following. 1) Direct manipulation of the cardiac Mito membrane content ratio of n–3 to n–6 PUFA results in augmented activation of Ca\textsuperscript{2+}-dependent PDH when the n–3-to-n–6 PUFA ratio is very low (n–6 PUFA-rich diet; 24-mo hearts) or attenuated activation when this ratio is relatively high (n–3 PUFA-rich diet). 2) Aged Ref or n–6 PUFA-rich dietary groups had significantly elevated Mito [Ca\textsuperscript{2+}] and PDH\textsubscript{A} (particularly after I/R or positive inotropic stimulation by NE). 3) Notably, this could be prevented in 24-mo rats by raising the n–3-to-n–6 PUFA ratio with n–3 PUFA-rich diet. 4) Increased state 4 rates of respiration (coupled, minus ADP), in the absence of Ca\textsuperscript{2+}, contributed to a loss of efficiency intrinsic to the Mito membranes from n–6 relative to n–3 PUFA hearts. Age-linked increases in state 4 respiration observed in n–6 PUFA hearts were not apparent in n–3 PUFA-rich hearts. 5) Mito membrane phosphatidylcholine and n–6 PUFAs were increased in Ref hearts from aged animals, whereas cardiolipin and n–3 PUFA content were decreased. These age-associated effects were augmented by n–6 PUFA and prevented by n–3 PUFA-rich diet. Thus enhanced PDH activation with aging or increased n–6 PUFA content is considered to be mediated through increased net Mito Ca\textsuperscript{2+} uptake. The relative contributions to this effect of changes in cytosol Ca\textsuperscript{2+} and the intrinsic alterations in Mito membrane phospholipid composition documented here remain to be elucidated. The key role of membrane lipids and constituent fatty acids in defining and modulating membrane permeability, membrane protein function, and intercompartment signaling, especially in aging, where specific PUFA accumulation and deficits occur, requires extensive study.

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