Effects of ω-3 polyunsaturated fatty acids on cardiac sarcolemmal Na⁺/H⁺ exchange

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Goel, Danny P., Thane G. Maddaford, and Grant N. Pierce. Effects of ω-3 polyunsaturated fatty acids on cardiac sarcolemmal Na⁺/H⁺ exchange. Am J Physiol Heart Circ Physiol 283: H1688–H1694, 2002; 10.1152/ajpheart.00664.2001.—Myocardial ischemia-reperfusion activates the Na⁺/H⁺ exchanger, which induces arrhythmias, cell damage, and eventually cell death. Inhibition of the exchanger reduces cell damage and lowers the incidence of arrhythmias after ischemia-reperfusion. The ω-3 polyunsaturated fatty acids (PUFAs) are also known to be cardioprotective and antiarrhythmic during ischemia-reperfusion challenge. Some of the action of PUFAs may occur via inhibition of the Na⁺/H⁺ exchanger. The purpose of our study was to determine the capacity for selected PUFAs to alter cardiac sarcolemmal (SL) Na⁺/H⁺ exchange. Cardiac membranes highly enriched in SL vesicles were exposed to 10–100 μM eicosapentanoic acid (EPA) or docosahexanoic acid (DHA). H⁺-dependent 22Na⁺ uptake was inhibited by 30–50% after treatment with ≥50 μM EPA or ≥25 μM DHA. This was a specific effect of these PUFAs, because 50 μM linoleic acid or linolenic acid had no significant effect on Na⁺/H⁺ exchange. The SL vesicles did not exhibit an increase in passive Na⁺ efflux after PUFAs treatment. In conclusion, EPA and DHA can potentely inhibit cardiac SL Na⁺/H⁺ exchange at physiologically relevant concentrations. This may explain, in part, their known cardioprotective effects and antiarrhythmic actions during ischemia-reperfusion.

ischemia-reperfusion; myocardial cell death; antiarrhythmic; eicosapentanoic acid; docosahexanoic acid

POLYUNSATURATED FATTY ACIDS (PUFAs) have special clinical importance in heart disease. Mortality by coronary heart disease is reduced as a consequence of dietary long-chain PUFAs administration (1, 8, 24, 46). This may occur through an antiarrhythmic action of PUFAs. Long-chain PUFAs prevent or retard arrhythmias induced by ischemia. Intravenous infusion of docosahexanoic acid (DHA, 22 carbons and 6 double bonds (DB)] and eicosapentanoic acid (EPA, 20 carbons and 5 DB) into canine myocardium prevented ventricular fibrillation after ischemia (2, 20, 21, 26). This has been shown by many laboratories in a variety of animal species (2, 15, 27, 28) and is thought to function in a similar manner in humans (5, 6, 8, 46). PUFAs also prevent ventricular fibrillation that has been observed in rats and monkeys after coronary ligation (27, 28). However, the mechanism for the antiarrhythmic effects of PUFAs and the potential for PUFAs to inhibit ischemia-induced cardiac damage and necrosis require further investigation.

Several potential sites for action have been identified. Fatty acids have the capacity to affect the activity of certain ion exchangers. However, it is uncertain whether PUFAs induce a stimulation or an inhibition of specific transporters. For example, the PUFAs linoleic acid (LA) and linolenic acid (LNA) stimulated the Na⁺/Ca²⁺ exchanger (37). Other fatty acids such as palmitoleic and oleic acid also caused a large stimulation of the Na⁺/Ca²⁺ exchanger (36, 38). These fatty acids also activated Ca²⁺-ATPase in erythrocyte membranes (35). However, others have disputed these stimulatory effects. Rats restricted to an unsaturated ω-3 fatty acid diet that contained DHA and EPA exhibited a decrease in cardiac sarcoplasmic reticulum Ca²⁺-ATPase activity (48). Hallaq observed results that indirectly support an inhibition of Na⁺/Ca²⁺ exchange by DHA and EPA (13). The Na⁺/Li⁺ antiporter is inhibited in diabetic patients fed a DHA-supplemented diet (47). EPA inhibited the voltage-dependent Na⁺ channels (I⁰Na) in human embryonic kidney (HEK-293T) cells (52). The antiarrhythmic effects of PUFAs have also been suggested to occur via Ca²⁺ channel inhibition (51) or K⁺ channel blocking (4).

The effects of PUFAs on the cardiac Na⁺/H⁺ exchanger remain undetermined. This is unfortunate in view of the critical role that this transporter plays in ischemia-reperfusion-induced injury in the heart (9, 16, 31–33, 40). H⁺ accumulation during the ischemic period is thought to stimulate the Na⁺/H⁺ exchanger to remove H⁺ from the cell in exchange for extracellular Na⁺. The resulting increase in intracellular Na⁺ stimulates Ca²⁺ entry into the cardiomyocyte through the Na⁺/Ca²⁺ exchanger. Ca²⁺ overload is a well-known factor in the generation of cardiac arrrhythmia, damage, and necrosis (14, 34, 45). Inhibition of the

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Materials and Methods

Materials. Materials used for the Na\(^+\)/H\(^+\) exchange assay including Millipore filters were supplied by Fisher Scientific (Napen, ON). The \(^{22}\)Na\(^+\) tracer was supplied by Mandel Scientific (Guelph, ON). The fatty acids EPA, DHA, AA, LA, and LNA were purchased from Doosan Serdary Laboratories (Toronto, ON). All other materials were purchased from Sigma (St. Louis, MO).

Sarcolemmal membrane preparations. Pigs (body wt 65–85 kg) were used to harvest sarcolemmal membranes. While the animals were under anesthesia (Telazol, 1 ml/23 kg body wt), hearts were removed and placed in an ice-cold water bath. A series of steps were conducted to isolate the cardiac sarcolemma from the left ventricle using a previously described method (18, 24). After membranes were isolated, a number of assays were carried out to determine the purity of the vesicles. The K\(^+\)-p-nitrophenylphosphatase and Na\(^+\)-K\(^+\)-ATPase assays were used to determine sarcolemmal purity as previously described in detail (18, 42). Compared with homogenate, the activities of both of these sarcolemmal marker enzymes were increased ~100 fold. This is consistent with previous reports from our laboratory for sarcolemmal membrane purification using this procedure (18). The final sarcolemmal fraction was suspended in a medium that contained (in mM) 200 sucrose, 25 MES, 8 KOH, pH 5.5 and was centrifuged at 175,000 g for 2 h. The pelleted membranes were collected and resuspended in the same medium at a final protein concentration of 1–6 mg/ml. Protein concentration was quantified using the Lowry method described elsewhere (43). Subsequent to quantification, vesicles were immersed in liquid N\(_2\) and stored at −80°C for subsequent analysis.

Measurement of Na\(^+\)/H\(^+\) exchange. H\(^+\)-dependent Na\(^+\) uptake was examined in both the control and the fatty acid-treated cardiac sarcolemmal vesicles as described by Pierce and colleagues (25, 43, 44). Briefly, 5 \(\mu\)l of \(^{22}\)Na\(^+\) (0.1 \(\mu\)Ci) were added to the bottom of a polystyrene tube that contained 25 \(\mu\)l of uptake medium, 200 mM sucrose, 30 mM 2-(N-hexylamino)ethanesulfonic acid (CHES), 40 mM KOH, 0.1 mM EGTA, and 0.1 mM Na\(^+\) (pH 10.61). A 20-\(\mu\)l aliquot of cardiac sarcolemmal membrane (~10 \(\mu\)g) was placed on the side of the polystyrene tube, and H\(^+\)-dependent Na\(^+\) uptake was initiated by vortexing the mixture. The concentration of the final assay medium was (in mM) 180 sucrose, 10 MES, 17.5 CHES, 17 KOH, 0.05 EGTA, and 0.05 Na\(^+\) with a final extravesicular pH of 9.33. To ensure pH accuracy, all solutions were calibrated using an Orion 82-10 pH electrode. Subsequent to Na\(^+\)/H\(^+\) exchange, the reaction was quenched with stop solution after a preset time of 2–5 s. Approximately 3 ml of stop solution (100 mM KCl and 20 mM HEPES, pH 7.5) was added to the polystyrene tube and subsequently filtered through 0.45-\(\mu\)m Millipore filters. This sequence was repeated two more times with 3 ml of stop solution. Filters were removed, placed in scintillation vials, and dried, and radioactivity was quantified using scintillation spectroscopy. Blanks were treated in a similar fashion except that 3 ml of stop solution were added before the inclusion of 20 \(\mu\)l of protein.

It is important to note that the H\(^+\)-dependent Na\(^+\) uptake measured by this technique is not influenced by other trans-sarcolemmal Na\(^+\)-transport pathways. We cannot detect any Na\(^+\) transport through the Na\(^+\)/Ca\(^2+\) exchanger, \(I_{\text{Na,Ca}}\), or the ATP-dependent Na\(^+\) pump under the assay conditions used here to measure H\(^+\)-dependent Na\(^+\) uptake (25, 43, 44). Thus this methodology is appropriate to selectively isolate and measure the activity of Na\(^+\)/H\(^+\) exchange in cardiac sarcolemmal membranes.

Treatment with PUFAs. Each individual fatty acid (LA, LNA, DHA, EPA, and AA) was prepared in a similar fashion. The PUFAs were suspended in a vehicle of 200 mM KOH, pH 5.5. Each PUFAs was sonicated and vortexed extensively to ensure suspension before use. The PUFAs were prepared fresh immediately before each experiment. Approximately 100 \(\mu\)g of cardiac sarcolemmal vesicles were exposed to 10, 25, 50, and 100 \(\mu\)M of fatty acid. Fatty acids were preincubated with the sarcolemmal vesicles for 90 ± 30 s at 25°C. Control tubes were treated in a similar fashion except the sarcolemmal vesicles were preincubated with the fatty acid vehicle only. After preincubation, H\(^+\)-dependent Na\(^+\) uptake was examined immediately.

Passive efflux of Na\(^+\). Passive efflux of \(^{22}\)Na\(^+\) from the vesicles was carried out as described (23, 25, 30, 40) to assess potential changes in membrane integrity. Briefly, Na\(^+\)/H\(^+\) exchange was carried out for 1 min (see Measurement of Na\(^+\)/H\(^+\) exchange). After uptake, 450 \(\mu\)l of an efflux medium was added that consisted of a 1:10:50 volume ratio of 20 \(\mu\)M dimethylamiloride (DMA), H\(_2\)O, sucrose solution that contained (in mM) 200 sucrose, 25 MES, 8 KOH, pH 5.5, and a Na\(^+\)-free uptake solution, respectively. This efflux solution contained no Na\(^+\), which created an optimum gradient for \(^{22}\)Na\(^+\) to passively exit the vesicles. DMA was added as a precautionary measure to ensure that the Na\(^+\)/H\(^+\) exchanger was inoperable. Passive efflux was measured for 2 s after the addition of efflux medium, stopped with 9 ml of stop solution (see Measurement of Na\(^+\)/H\(^+\) exchange), and subsequently filtered. An uptake time of 1 min followed by the addition of no efflux media served as our zero-time points (maximum uptake before efflux was started). If PUFAs were added (50 \(\mu\)M final), they were present for the 90-s preincubation period, during the 1-min uptake, and during efflux.

Statistics. Data are expressed as means ± SE. Statistical determinations were done using Student’s t-test and were considered significant at P < 0.05. Sample numbers represent the number of measurements obtained from sarcolemma membranes collected from different pigs.
RESULTS

Individual fatty acids were preincubated with cardiac sarcolemmal vesicles, and Na\(^+/\)H\(^+\) exchange was examined. H\(^+/\)Na\(^+\) exchange was examined across a number of reaction times. Na\(^+/\)H\(^+\) exchange was significantly depressed by 100 \(\mu\)M EPA at all reaction times (2–60 s) compared with control vesicles (Fig. 1).

This inhibition of exchange was observed at other EPA concentrations ([EPA]). A significant depression of Na\(^+/\)H\(^+\) exchange occurred after exposure of sarcolemmal vesicles to 50 and 100 \(\mu\)M EPA, but 10 or 25 \(\mu\)M EPA had no effect (Fig. 2).

Na\(^+/\)H\(^+\) exchange was also examined at varying extravesicular pH values to further evaluate the influence of EPA on H\(^+/\)dependent Na\(^+\) uptake. As expected in control vesicles, introduction of a transsarcolemmal H\(^+/\)gradient produced an appropriate increase in H\(^+/\)dependent Na\(^+\) uptake (43) (Fig. 3). EPA treatment inhibited H\(^+/\)dependent Na\(^+\) uptake at all extravesicular pH values examined except pH 6 (Fig. 3). With an intravesicular pH of 5.5 and an extravesicular pH of 6, H\(^+/\)dependent Na\(^+\) uptake would not be expected to be very active. These results, therefore, provide further assurance that the observed inhibitory effects on Na\(^+\) movements are a true reflection of an effect on the Na\(^+/\)H\(^+\) exchange pathway.

It is important to determine whether these effects on the exchanger are limited to one fatty acid or shared with other \(\omega-3\) fatty acid species. DHA is also an \(\omega-3\) fatty acid and has a structure similar to EPA; it would therefore be expected to induce similar effects. H\(^+/\)dependent Na\(^+\) uptake was examined across variable reaction times after DHA treatment. Na\(^+/\)H\(^+\) exchange was significantly depressed by 100 \(\mu\)M DHA at all reaction times (2–60 s) compared with control vesicles (Fig. 4).

Na\(^+/\)H\(^+\) exchange was also significantly inhibited after treatment with varying concentrations of DHA. After exposure of sarcolemmal vesicles to 25–100 \(\mu\)M DHA, a 12–50% inhibition of \(^{22}\)Na\(^+\) uptake was ob-
$\omega$-3 FATTY ACIDS AND Na$^+$/$H^+$ EXCHANGE

H1691

Fig. 4. $H^+$-dependent Na$^+$ uptake as a function of variable reaction time in docosahexanoic acid (DHA)-treated sarcolemmal vesicles. Sarcolemmal vesicles were incubated with 100 $\mu$M of DHA at 25°C (pH 5.5). Na$^+$ uptake was examined in a final solution consisting of 0.05 mM Na$^+$, pH 9.33. Data are means ± SE of 3–5 separate experiments; *$P < 0.05$ vs. control.

Fig. 5. $H^+$-dependent Na$^+$ uptake in sarcolemmal vesicles as a function of variable concentrations of DHA ([DHA]). Sarcolemmal vesicles were incubated with various [DHA] at 25°C (pH 5.5). Na$^+/H^+$ exchange was examined for 5 s with a final [Na$^+$] of 0.05 mM, pH 9.33. Controls were examined in a similar fashion but contained fatty acid vehicle only. Data are means ± SE of 3–5 separate experiments; *$P < 0.05$ vs. control.

Table 1. $H^+$-dependent Na$^+$ uptake as a function of [Na$^+$] in presence or absence of DHA

<table>
<thead>
<tr>
<th>[Na$^+$], mM</th>
<th>Control</th>
<th>DHA Treated</th>
<th>% of Control</th>
</tr>
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<tbody>
<tr>
<td>0.05</td>
<td>0.88 ± 0.17</td>
<td>0.52 ± 0.09</td>
<td>61.5 ± 5.2*</td>
</tr>
<tr>
<td>2.5</td>
<td>9.06 ± 1.92</td>
<td>5.82 ± 1.33</td>
<td>64.5 ± 6.7*</td>
</tr>
<tr>
<td>10.0</td>
<td>14.85 ± 2.15</td>
<td>9.58 ± 0.92</td>
<td>67.5 ± 11.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 3$ or 4 experiments with sarcolemmal membrane preparations obtained from different animals. $H^+$-dependent Na$^+$ uptake was carried out for 30 s with (treated) or without (control) DHA (docosahexanoic acid). *$P < 0.05$. [Na$^+$], Na$^+$ concentration.

though [Na$^+$] in the assay medium varied from 0.05 to 10 mM.

PUFA specificity for altering Na$^+$/$H^+$ exchange was examined further. $\gamma$-LNA and LA were incubated with cardiac sarcolemmal vesicles. Whereas 50 $\mu$M DHA and 50 $\mu$M EPA significantly inhibited Na$^+$/$H^+$ exchange, 50 $\mu$M LNA or LA produced no significant difference in $H^+$-dependent Na$^+$ uptake compared with controls (Table 2). AA (50 $\mu$M) was also tested, because it is structurally similar to DHA and EPA. It also significantly inhibited Na$^+$/$H^+$ exchange.

The destabilizing effect of fatty acids on phospholipid membranes has been well documented (17, 19, 22, 50). It was therefore important to determine whether the inhibition we observed with DHA and EPA was due to a direct effect on the exchanger or merely an increase in the passive efflux of ions. Vesicles were loaded with $^{22}$Na$^+$ via $H^+$-dependent Na$^+$ uptake for 1 min before the initiation of efflux (43). This was necessary to provide a large enough transsarcolemmal Na$^+$ gradient to permit its passive efflux. Efflux was then initiated for $\leq 15$ s in the presence of 20 $\mu$M DMA to inhibit any residual Na$^+$/$H^+$ exchange activity during efflux. As shown in Fig. 6, there were no significant differences in passive Na$^+$ efflux as a function of treatment of the vesicles with 50 $\mu$M DHA or EPA compared with efflux in the absence of the PUFA.

DISCUSSION

This study demonstrates that specific fatty acids can significantly inhibit the activity of the cardiac sarcolemmal Na$^+$/$H^+$ exchanger. Two observations support the contention that the inhibition of the exchanger was not artifactual but instead reflects a real and

Table 2. $H^+$-dependent Na$^+$ uptake in PUFA-treated cardiac sarcolemmal vesicles

<table>
<thead>
<tr>
<th>Linoleic Acid</th>
<th>Linolenic Acid</th>
<th>Arachidonic Acid</th>
<th>Eicosapentanoic Acid</th>
<th>Docosahexanoic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>99 ± 5</td>
<td>96 ± 1</td>
<td>72 ± 4*</td>
<td>76 ± 3*</td>
<td>66 ± 8*</td>
</tr>
</tbody>
</table>

Values are percentages of control ± SE. Sarcolemmal vesicles were incubated with 50 $\mu$M of either linoleic, linolenic, arachidonic, eicosapentanoic, or docosahexanoic acid at 25°C (pH 5.5). $H^+$-dependent Na$^+$ uptake was carried out for 5 s in final solution consisting of 0.05 mM Na$^+$, pH 9.33. PUFA, polyunsaturated fatty acid. *$P < 0.05$. 

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Data are means \( \pm \) SE for 6 separate experiments.

The inhibition of exchange by DHA and EPA was not due to an increase in the passive permeability under the assay conditions employed in the present study (Fig. 6). Second, the effects of the PUFAs were specific to the PUFAs tested. After exposure to LA and LNA, the activity of the Na\(^{+}/\)H\(^{+}\) exchanger remained unaltered. This lack of effect of LA and LNA on cardiac Na\(^{+}/\)H\(^{+}\) exchange agrees with the findings of Gore et al. (12) that enrichment of cells with LA or LNA did not alter Na\(^{+}/\)H\(^{+}\) exchange. In contrast, we found that the addition of DHA and EPA significantly inhibited H\(^{+}\)-dependent Na\(^{+}\) uptake. After DHA and EPA treatment, all of the parameters of Na\(^{+}/\)H\(^{+}\) exchange that were examined were altered (fatty acid concentration, reaction time, and extravesicular pH). This difference in the effects of specific fatty acids on Na\(^{+}/\)H\(^{+}\) exchange implies a structural specificity of the effect. EPA and DHA are longer-chain fatty acids (20 and 22 carbons, respectively) than LA and LNA (both 18 carbons). The number of DBs within these PUFAs also differs; EPA has 5, DHA has 6, LA has 2, and LNA has 3 DBs. The capacity of the cis DBs that are found in PUFAs together with the longer chain length may induce a sufficient disturbance in membrane order near the exchanger to alter its activity. The inhibition of Na\(^{+}/\)H\(^{+}\) exchange observed in the presence of AA would further support this contention. AA, which is 20 carbons in length and has 4 DBs, is structurally similar to DHA and EPA. The conclusion that the exchanger is sensitive only to specific lipid species is also consistent with previous studies of the effects of phospholipases on cardiac sarcolemmal Na\(^{+}/\)H\(^{+}\) exchange. Although treatment of sarcolemmal vesicles with phospholipase D resulted in a significant change in Na\(^{+}/\)H\(^{+}\) exchange, there was no effect after phospholipase C treatment (11). Again, this implies that the Na\(^{+}/\)H\(^{+}\) exchanger is sensitive to specific alterations in its lipid environment.

Our current results may have important clinical significance from two perspectives. First, the concentrations of PUFAs that induced an effect on the exchanger in the present study are within the range expected to be found in plasma from individuals consuming a diet enriched in PUFAs (7) and in pathological conditions such as ischemia-reperfusion (2). Plasma EPA and DHA concentrations have been reported to rise to 0.5–0.7 mM after dietary supplementation (49). Second, pharmacological inhibition of Na\(^{+}/\)H\(^{+}\) exchange significantly reduces ischemia-induced arrhythmias, contractile dysfunction, damage, and necrosis (10, 29, 31, 32, 39, 40). Billman and colleagues (2, 3) have reported the antiarrhythmic effects of DHA and EPA after ischemia. Although inhibition of Ca\(^{2+}\) or K\(^{+}\) channels has been suggested as the mechanism by which PUFAs exert antiarrhythmic effects (4, 51), our results provide a clear and important alternative mechanistic explanation. By inhibiting the Na\(^{+}/\)H\(^{+}\) exchanger, Ca\(^{2+}\) overload will be reduced or prevented (23), and the arrhythmias (23), contractile dysfunction, damage, and necrosis associated with the ischemia-reperfusion insult will be avoided. This conclusion, however, is limited to DHA and EPA and cannot explain the cardioprotective actions of the short-chain PUFAs (27, 28).

A potential limitation inherent in our sarcolemmal work is the use of relatively low [Na\(^{+}\)]. To increase our ability to detect the radioactive signal, we had to use lower total [Na\(^{+}\)] (0.05–10 mM) relative to that found in vivo (140 mM). This may limit the applicability of the data and the effects of DHA and EPA to our in vitro conditions. However, it must be recognized that even though relatively low [Na\(^{+}\)] were employed in our study, varying these concentrations up to 200-fold did not alter the inhibitory action of DHA (see Table 1). Thus it is likely that further increases in [Na\(^{+}\)] up to those present during in vivo conditions would not alter the inhibitory action of these PUFAs.

In summary, our study is the first to demonstrate a potent effect of DHA and EPA on Na\(^{+}/\)H\(^{+}\) exchange. Our results provide insight into the cardioprotective action of PUFAs such as DHA and EPA during ische-
mia-reperfusion and further our knowledge regarding the interactions of lipids with the Na+/H+ exchanger.

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