Effect of Fatty Acid Composition on Insulin and IGF-I Binding in Retinoblastoma Cells

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Receptors for insulin and other hormones are often influenced by their environment, including fatty acid content and membrane fluidity. Y79 retinoblastoma cells enriched with arachidonic or docosahexaenoic acid show an increase in membrane fluidity determined by fluorescence polarization using the plasma membrane-specific probe, trimethylammonium-diphenylhexatriene. Unlike cells enriched with polyunsaturated fatty acids, cells cultured in media containing palmitic or oleic acid showed no changes in membrane fluidity or fatty acid composition. Cells enriched with docosahexaenoic acid show an increase in insulin binding at 15°C that is due to an increase in the number of available insulin receptor sites on the plasma membrane. In contrast, insulin binding is not altered in cells enriched with arachidonic, palmitic or oleic acid. Furthermore, the binding of insulin-like growth factor-I (IGF-I) in Y79 cells is not changed by the fatty acid unsaturation of the cell membrane. Because docosahexaenoic acid is naturally present in high concentrations in the retina these studies suggest it may have a role in modulating insulin binding and function.

Insulin and insulin-like growth factor-I (IGF-I) exert their actions on tissues by binding to specific cell surface receptors. The insulin and IGF-I receptors have similar structures. They are both tetramers containing two alpha and two beta subunits. The alpha subunit contains the binding site for the hormone and is thought to be localized on the external portion of the plasma membrane and not to penetrate the plasma membrane. The beta subunit is linked to the alpha subunits by disulfide bonds and contains the transmembrane portion of the receptor. The lipid environment of the plasma membrane influences many properties and functions of the membrane. Alteration of the fatty acid composition of membrane phospholipids causes selective changes in enzyme activity and receptor and carrier-mediated transport properties. Insulin binding to a variety of cell types is influenced by the unsaturated fatty acid content of the cell membrane. Generally, with only a few exceptions, it has been found that increasing the polyunsaturated fatty acid content of the cell membrane causes an increase in membrane fluidity and an increase in insulin receptor concentration but a decrease in receptor affinity. Studies using liposomes containing partially purified insulin receptors have also indicated that increasing the polyunsaturated fatty acid content is associated with an increase in insulin binding. Insulin bioactivity is also influenced by the fluidity of the cell membrane. In contrast, Bar et al have shown that increasing the membrane fluidity of large vessel endothelial cells has no effect on insulin binding. Furthermore, IGF-I binding to its receptor is apparently not influenced by the fluidity of the plasma membrane even though the structures of the insulin and IGF-I receptors are similar.

In the present studies we ascertained the effect of increasing membrane fluidity on insulin and IGF-I binding in a cell of retinal origin. Specifically we determined the effect of increasing the n-3 or n-6 polyunsaturated fatty acid content on plasma membrane fluidity and insulin and IGF-I binding in human Y79 retinoblastoma cells. The retina and most neural cells are highly enriched with polyunsaturated fatty acids and cells of the neural retina contain high amounts of docosahexaenoic acid (22:6, n-3). The purpose of the high content of polyunsaturated fatty acids in neural cells and the influence they may have on insulin binding and bioactivity is unknown. The Y79 retinoblastoma is a human cell line derived from a tumor of the retina. Previous studies have shown that this cell has retained many neural characteristics and likely originated from a primitive multipotential reti-
nal cell.15-20 The undifferentiated cell simultaneously expresses both neuronal and glial markers as well as the ability to specifically bind insulin and IGF-I.17-23 The present studies show that the polyunsaturated fatty acid content of membrane phospholipids modulates insulin binding without influencing IGF-I binding in a cultured cell line of retinal origin and that docosahexaenoic acid may specifically affect insulin binding and function in these cells.

Materials and Methods

Cell Culture

Human Y79 retinoblastoma cells,24 which grow in suspension as small clusters of six to ten undifferentiated cells, were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES), 100 U/ml penicillin, 100 µg/ml streptomycin, and 294 µg/ml glutamine. The cells were grown in 150 cm² tissue culture flasks in an incubator maintained at 37°C, with 5% CO₂ in humidified air as the gas phase. For experimental purposes cells were transferred into serum-free media containing 100 µg/ml transferrin, 20 nM progesterone, 100 µM putrescine, 30 nM disodium selenite, 10 mM HEPES and 5 µM “fatty acid-free” bovine serum albumin (Miles Laboratories, Naperville, IL). The bovine serum albumin contains <0.1% fatty acid. Cells were conditioned into the serum-free media for 1 week prior to experimentation. Aliquots of the cell suspension were also taken to determine protein content and cell number.29 As previously reported, exposing Y79 cells to media supplemented with polyunsaturated fatty acids in particular docosahexaenoic acid had no effect on cell number or growth.17,22,23 The incubations were conducted at 15°C for 90 min, in a total volume of 1.0 ml. Afterwards, triplicate 200 µl aliquots of the incubation medium were layer over 800 µl of buffer at 0°C and sedimented for 2 min in an Eppendorf microfuge (Hamburg, W. Ger.). The supernatant was removed by aspiration and the tips containing the cell pellet counted in a Micromedic Mark IV gamma radiation counter (Horsham, PA) at 75% efficiency.

Lipid Analysis

Lipids were extracted from intact cells with 20 ml of a 2:1 (v/v) mixture of redistilled chloroform and methanol.30 Phase separation was produced by the addition of 4 ml of acidic saline (154 mM NaCl, 4 mM HCl). After isolation of the CHCl₃ phase, the polar lipids were separated from the neutral lipids by thin layer chromatography using Silica Gel Absorbisil Plus plates (Alltech Assoc., Deerfield, IL) and a solvent system containing hexane:diethyl ether: methanol:acetic acid (85:20:2:2 v/v/v/v). The lipids were extracted from the silica gel by transferring the samples to separatory funnels containing 4 ml acidic saline. After mixing, phase separation was produced by adding 10 ml chloroform/methanol (1:1 v/v). The lipid phase was collected and the CHCl₃ removed by evaporation under N₂. The lipids were then transmsfered by the addition of 1 ml acetonitrile, 1 ml methanol and 1 ml 14% BF₃ in methanol, and heating at 95°C for 45 min.31 The fatty acid methyl esters were extracted with n-heptane and separated by gas-liquid chromatography.17 The separation was performed using a temperature programmed Hewlett-Packard 5890 gas-liquid chromatograph (Palo Alto, CA) equipped with a 1.8 m x 2 mm id glass column packed with 10% SP-2330 on 100/120 mesh Chromosorb W-AW (Supelco, Bellefonte, PA). Peaks were identified by comparison with fatty acid methyl ester.
standards obtained from Supelco. Relative peak areas were computed with a Hewlett-Packard 3390A integrator.

Membrane Fluidity

Membrane fluidity was measured by fluorescence polarization using whole cells and the plasma membrane specific probe, trimethylammonium-diphenylhexatrine (TMA-DPH). Cells were resuspended at approximately $5 \times 10^5$ cells/ml in Hanks buffer and incubated for 2 min with 1 ml of $2 \times 10^{-6}$ M TMA-DPH. The final volume was equal to 2 ml. Fluorescence polarization measurements were taken using a SLM 4800 spectrofluorometer. The excitation wavelength was set at 340 nM and a 389 nM filter was used. Arrhenius plots were constructed using a thermocouple to continuously monitor the temperature with readings taken at every 1°C change. The sample temperature was controlled by a water-jacketed cuvette holder and the temperature increased at a rate of 2°C/min.

Data Analysis

Scatchard analysis of the binding data was performed with the computer program, SCATFIT.33

Results

Membrane Fatty Acid Composition and Fluidity

The fatty acid composition of the membrane phospholipids of the cells used in these studies are given in Table 1. Supplementing the growth medium with palmitic acid caused little change in the fatty acid composition and unsaturation index of the cell phospholipids. By contrast, supplementing the cell culture medium with arachidonic acid produced a 30% increase in the unsaturation index and a large increase in the percentage of 20:4 and docosatetraenoic acid (22:4) in the cell phospholipids. Likewise, docosahexaenoic acid supplementation produced an increase of 90% in the unsaturation index and a large increase in the content of 22:6 in the cell membrane phospholipids.

The increase in the content of polyunsaturated fatty acids in cell membrane phospholipids from either arachidonic or docosahexaenoic acid supplementation was associated with a large increase in membrane fluidity at all temperatures analyzed (Fig. 1). The increase in fluorescence anisotropy, as measured by fluorescence polarization, in the polyunsaturated fatty acid enriched cells was approximately equivalent to the membrane fluidity of unsupplemented cells or cells cultured for 24 hr in the presence of palmitic acid measured at a temperature 8°C higher. Cells were also cultured in the presence of oleic acid, a monounsaturate, and the membrane fluidity and fatty acid composition was similar to unsupplemented cells (data not shown).

Insulin and IGF-I Binding

Figure 2 shows the Scatchard plots for [$^{125}$I] iodoinsulin binding by Y79 retinoblastoma cells grown in unsupplemented serum-free medium or in medium supplemented with palmitic, arachidonic or docosahexaenoic acid. The characteristic curvilinear Scatchard plot for insulin binding is indicative of a two-site or two-affinity binding system. In contrast, IGF-I binding has a linear plot indicating a one-site, one-affinity binding system (Fig. 3). Cells cultured for 24 hr in medium containing docosahexaenoic acid subsequently bind more insulin than control cells or cells exposed to palmitic or arachidonic acid. The binding capacity for insulin reported as fmol/100 μg of protein, increased significantly from 19.3 ± 1.8 for control cells to 61.0 ± 8.3 (n = 13) for cells exposed to docosahexaenoic acid. There was no increase in the binding capacity for insulin in cells exposed to palmitic acid (22.5 ± 2.9) or arachidonic acid (24.8 ± 3.8). Similarly, the number of insulin binding sites/cell was increased after the cells were exposed to docosahexaenoic acid. The number of binding sites/cell for unsupplemented cells was calculated to be 1.4

Table 1. Fatty acid composition of membrane phospholipids from human Y79 retinoblastoma cells

<table>
<thead>
<tr>
<th>Fatty acid supplement</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>20:4</th>
<th>22:4</th>
<th>22:6</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (0.69)*</td>
<td>19.1</td>
<td>19.1</td>
<td>9.9</td>
<td>27.2</td>
<td>2.9</td>
<td>2.1</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Palmitic (0.69)</td>
<td>19.8</td>
<td>18.4</td>
<td>10.4</td>
<td>27.4</td>
<td>3.0</td>
<td>2.2</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Arachidonic (0.92)</td>
<td>21.0</td>
<td>13.5</td>
<td>10.6</td>
<td>23.9</td>
<td>2.1</td>
<td>8.6</td>
<td>2.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Docosahexaenoic (1.33)</td>
<td>21.8</td>
<td>12.9</td>
<td>10.8</td>
<td>23.5</td>
<td>2.1</td>
<td>2.0</td>
<td>0.7</td>
<td>13.6</td>
</tr>
</tbody>
</table>

Cells were grown for 24 hr in serum-free medium containing 10% fetal bovine serum and either no supplemental fatty acid (control) or 10 nmol/ml of the indicated fatty acid. The values do not add up to 100% because only the major fatty acids are presented. Each value is the average of six determinations. The SEM were within 10% or less of the mean value.

* The numbers in parentheses are unsaturation indices: mean number of double bonds per fatty acid.
Fig. 1. Arrhenius plot of membrane fluidity in fatty acid supplemented and unsupplemented Y79 retinoblastoma cells. Cells were cultured in normal media or media enriched in fatty acids. Afterwards membrane fluidity was measured by fluorescence polarization using the plasma membrane specific probe, trimethylammonium diphenylhexatriene. Measurements were taken for every 1°C change. The units are \( r = \text{anisotropy} \) and \( T = \text{absolute temperature (Kelvin)} \).

\[ r = \frac{1}{T} \times 10^{-3} \]

Fig. 2. Scatchard analysis of insulin binding in fatty acid supplemented and unsupplemented Y79 retinoblastoma cells. Cells were cultured in normal media or media enriched in fatty acids. Afterwards insulin binding analysis was conducted as described in Materials and Methods.

\[ B/F \]

Fig. 3. Scatchard analysis of IGF-I binding in fatty acid supplemented and unsupplemented Y79 retinoblastoma cells. Cells were cultured in normal media or media enriched in fatty acids. Afterwards IGF-I binding analysis was conducted as described in Materials and Methods.

\[ B/F \]

Discussion

Enrichment of Y79 cell membrane phospholipids with docosahexaenoic acid causes an increase in membrane fluidity and insulin binding. An increase in the polyunsaturated fatty acid content of membrane phospholipids and presumably membrane fluidity has been shown to increase insulin binding (ie, number of insulin receptors) in a variety of cells. Some of the cell types in which membrane fatty acid unsaturation has been reported to influence insulin binding include Friend erythroleukemia, 3T3-L1 and Ehrlich ascites as well as fatty acid-modified liposomes containing reconstituted insulin receptors from turkey erythrocyte membranes. In contrast,
enrichment of Y79 retinoblastoma cells with arachidonic acid, which also causes an increase in membrane fluidity similar to docosahexaenoic acid enrichment, does not result in an increase in insulin binding or number of insulin receptors. This suggests that enrichment of Y79 cell membrane phospholipids with docosahexaenoic acid selectively causes an increase in the availability of insulin receptors.

Docosahexaenoic acid is present in high concentration in retina and neural cells; however, its function is unknown. We have previously studied the effect of docosahexaenoic acid enrichment on retinoblastoma cell membrane properties. We have shown that increasing the docosahexaenoic acid content of membrane phospholipids alters the transport of selective neurologically important compounds. However, these transport processes were also similarly affected by enrichment with other polyunsaturated fatty acids, including arachidonic acid. This suggested that an increase in plasma membrane fluidity caused by the enrichment of the cells with polyunsaturated fatty acids was probably responsible for the changes in the membrane transport processes. However, the increase in insulin binding is only observed following enrichment of membrane phospholipids with docosahexaenoic acid. This suggests that membrane fluidity in some cell types is not the only factor influencing insulin binding. It is possible that docosahexaenoic acid is increasing insulin binding by being incorporated into phospholipids that are near or surround the insulin receptor, whereas arachidonic acid may not be incorporated into this microenvironment region and thus has no influence on insulin binding. Docosahexaenoic acid and other n-3 fatty acids are preferentially incorporated into the ethanolamine phospholipids and to some extent phosphatidylserine in Y79 cells. This may suggest a role for ethanolamine or serine phospholipids and their orientation in the membrane as determined by their fatty acid composition in the regulation of insulin binding. Several studies have shown that phosphatidylserine and to some extent phosphatidylethanolamine are crosslinked to membrane proteins. Therefore, the fatty acid composition of these phospholipid/protein complexes may be important for regulation of protein properties.

The binding of IGF-I to its receptor in Y79 cells was not influenced by the polyunsaturated fatty acid content of the cell membrane, even though the insulin and IGF-I receptors are highly homologous. The homology, however, between these two receptors in the transmembrane region, the part of the receptor which is in physical contact with the membrane, is only 20%. Therefore, the IGF-I receptor, because of its different amino acid sequence compared to the insulin receptor in the region that spans the plasma membrane, may not be as dependent on the phospholipid and fatty acid content or membrane fluidity for its binding properties. It is also possible that the IGF-I receptor in Y79 cells is localized in a different microenvironment of the membrane that is not affected by changes in the fatty acid content.

Insulin as well as the insulin-like growth factor receptors are present in the retina and central nervous system, including brain, although the function of insulin and the insulin-like growth factors in these tissues is unknown. We have previously reported that insulin and IGF-I binding to Y79 cells specifically increases glycine transport, a putative retinal neurotransmitter. Therefore, it is possible that enriching Y79 cells with docosahexaenoic acid could modulate insulin action and influence neurological responses such as glycine transport in these cells. Currently, studies are being conducted to determine if enrichment of human Y79 retinoblastoma cells with docosahexaenoic acid influences insulin's biological activity.

Key words: insulin binding, IGF-I binding, docosahexaenoic acid, arachidonic acid, membrane fluidity

References