Acylcarnitine Chain Length Influences Carnitine-enhanced Drug Flux through the Spinal Meninges In Vitro

Wolfgang C. Ummenhofer, M.D.,* Christopher M. Bernards, M.D.†

Background: Palmitoyl carnitine has been shown to improve the penetration of hydrophilic drugs through the spinal meninges. Naturally occurring acylcarnitines, however, exist as a homologous series of different acyl chain lengths. The purpose of this study was to determine the most effective acylcarnitine chain length to increase meningeal permeability.

Methods: The transmeningeal flux of mannitol, morphine, and sufentanil through monkey spinal meninges was determined before and after adding acylcarnitines with chain lengths of 6 to 18 carbon atoms. Flux was measured using a previously established in vitro diffusion cell model.

Results: For mannitol, acylcarnitines generally showed a greater penetration-enhancing effect with increasing chain length, with palmitoyl carnitine (16 carbons) being the most effective compound with an increase of 24 ± 29% (mean ± SE). Morphine flux was increased most significantly by lauroyl (12 carbons) and myristoyl-carnitine (14 carbons) with 165 ± 25% and 188 ± 44% flux increases, respectively. In contrast, none of the studied acylcarnitines significantly altered the meningeal penetration of the more hydrophobic drug sufentanil.

Conclusions: The results suggest that, to promote hydrophilic drug penetration, acylcarnitines must surpass a critical chain length (10 carbon units) but should not exceed 16 carbon units. The activity of the acylcarnitines at the spinal meninges is reduced on either side of this range. The ability of acylcarnitines to increase the transmeningeal flux of morphine in vitro suggests that lauroyl or myristoyl carnitine may increase the spinal bioavailability of morphine after epidural administration. (Key words: Analgesics, opioids; morphine; sufentanil. Carnitine. Meninges, spinal. Animals, monkeys: Macaca nemestrina.)

SPINALY active drugs administered epidurally must cross the spinal meninges to reach their sites of action in the spinal cord dorsal horn. Diffusion has been shown to be the principal mechanism by which drugs cross the meninges,1–3 with the arachnoid mater serving as the principal meningeal permeability barrier.4 Thus increasing drug penetration through the spinal meninges offers the theoretical possibility of increasing the spinal bioavailability of epidurally administered drugs.

Previously we showed that palmitoyl carnitine selectively increases the transmeningeal flux of the hydrophilic drugs morphine and mannitol.5,6 However, palmitoyl carnitine is but one of a homologous series of naturally occurring acylcarnitines with different acyl chain lengths. The intestinal mucosa, fatty acyl chain length is critical for determining the magnitude of the absorption-enhancing effects of acylcarnitines.7 The purpose of the present study was to determine how acyl chain length affects the ability of acylcarnitines to enhance drug permeability through the spinal meninges.

To address this question, we used a previously described diffusion cell model1,3,4,5,8 to measure the transmeningeal flux of mannitol, morphine, and sufentanil across monkey spinal meninges before and after adding an acylcarnitine. Acylcarnitines with fatty acid chain lengths from 6 (hexanoyl-carnitine) to 18 (stearoyl-carnitine) were compared.

Materials and Methods

Studies were approved by the University of Washington Animal Care and Use Committee, and guidelines of the American Association for Accreditation of Laboratory Animal Care were followed throughout.

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**Tissue Preparation**

Monkey (*Macaca nemestrina*) tissues were obtained from animals scheduled to be killed as part of the tissue distribution program of the University of Washington Regional Primate Research Center. All animals (n = 42) were anesthetized with thiopental and ketamine before the meningeal specimens were removed.

The spinal cords were exposed from T5 to L5 by laminectomy. The spinal cord was removed en bloc and all three meningeal layers were carefully resected from the spinal cord, preserving their normal anatomic relations. From this sheet of intact meningeal tissues, specimens measuring approximately 4 cm² were cut for mounting in the diffusion cell.

**Flux Measurements**

The intact spinal meninges were placed between two halves of a temperature-controlled (37°C) diffusion cell. Ten milliliters of mock cerebrospinal fluid (140 mEq NaCl, 25 mEq NaHCO₃, 0.4 mEq MgCl₂, 3.5 mEq urea, 4 mEq glucose, 2 mEq CaCl₂; pH = 7.38–7.42; 292–298 mOsm) were placed in the fluid reservoirs on either side of the meningeal tissue. Air and carbon dioxide (5%) were bubbled through each fluid reservoir to maintain normal pH and to provide oxygen to the meningeal cells.

After allowing at least 20 min for the chambers to equilibrate to 37°C, one or two study drugs and the corresponding H or ¹³C-labeled radiotracer were added to the donor reservoir on the dura mater side of the diffusion cell. In most experiments, the flux of two different drugs were measured simultaneously. The drugs studied were mannitol (2.6 μM), morphine (2.6 μM), and sufentanil (2.6 μM). The radiotracers used were ¹³C-mannitol (specific activity, 56.7 μCi/mmol; radiochemical purity, 98.4%; New England Nuclear, Boston, MA), ¹³C-morphine (specific activity, 62 Ci/mmol; radiochemical purity, 97.9%; New England Nuclear), and ¹³C-sufentanil (specific activity, 9 Ci/mmol; radiochemical purity, 99%; Janssen Pharmaceutica, Olen, Belgium). After the study drugs and radiotracers were added, 200-μl samples were removed from the donor and recipient reservoirs at 10-min intervals for 100 min. The samples were placed in borosilicate scintillation vials for later scintillation counting to determine drug concentration.

At t = 100 min, 100 μl of hexanoyl-, octanoyl-, decanoyl-, lauroyl-, myristoyl-, palmitoyl-, or stearoyl-carnitine (Sigma Chemical Co., St. Louis, MO) was added to the donor reservoir to produce a final concentration of 5 mM. Thereafter, 200-μl samples were collected from both reservoirs at 10-min intervals for an additional 100 min. These samples were also placed in borosilicate scintillation vials for later scintillation counting to determine drug concentration. Sufentanil flux was measured with all but palmitoyl-carnitine, which was studied previously.⁷

Drug flux was determined from drug concentration data by plotting the amount of drug in the recipient reservoir at each time point. The slope of the line relating concentration versus time data was determined by least-squares linear regression and is equal to the test drug’s flux through the meninges. Because of the unavoidable delay in reaching the new flux rate after adding an acylcarnitine, flux was determined from the samples collected between 50 and 100 min (before adding carnitine) and between 130 and 200 min (after adding carnitine). Thus the reported flux values represent steady-state and not initial flux conditions.

To determine the role of acylcarnitine concentration in increasing drug flux, we performed additional studies exactly as described above with a short (octanoylcarnitine), intermediate (lauroylcarnitine), and long (stereoylcarnitine) chain acylcarnitine at concentrations of 0.5 and 2.5 mM. These studies were conducted with morphine and mannitol but not with sufentanil.

**Drug Analysis**

Hydrofluor scintillation fluid (National Diagnostics, Atlanta, GA) (5–10 ml) was added to each sample and the samples were counted in a Packard liquid scintillation counter (Tri Carb 2000, Packard, Downers Grove, IL) for 10 min or until the standard deviation of depurations per minute was 2% or less. Background radioactivity was determined by counting mock cerebrospinal fluid without added radiotracer and was subtracted from the depurations per minute of each sample. After converting depurations per minute to millimoles, linear regression was used to determine drug flux (mM⁻¹·min⁻¹·cm⁻²) before and after adding the different acylcarnitines.

**Statistical Analysis**

Differences in drug flux before and after acylcarnitine were added were assessed using the Student’s paired t test. The effect of different chain lengths on drug flux was assessed by one-way analysis of variance, and Fisher’s protected least-squares difference was used as a post-hoc test. Differences were considered statistically significant if p < 0.05.
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Table 1. Sufentanil Flux before and after Addition of 5 mM Acylcarnitines

<table>
<thead>
<tr>
<th>Acylcarnitine</th>
<th>Flux before Acylcarnitine (nmol/min·cm²)</th>
<th>Flux after Acylcarnitine (nmol/min·cm²)</th>
<th>Flux (%) change</th>
<th>Significance* (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexanoyl (n = 7)</td>
<td>4.2 ± 0.5</td>
<td>4.7 ± 1.1</td>
<td>10 ± 18</td>
<td>0.611</td>
</tr>
<tr>
<td>Octanoyl (n = 7)</td>
<td>4.6 ± 0.6</td>
<td>4.6 ± 1.0</td>
<td>-2.6 ± 11</td>
<td>0.911</td>
</tr>
<tr>
<td>Decanoyl (n = 6)</td>
<td>5.0 ± 1.1</td>
<td>4.7 ± 0.7</td>
<td>7.7 ± 16</td>
<td>0.724</td>
</tr>
<tr>
<td>Lauroyl (n = 6)</td>
<td>5.1 ± 0.6</td>
<td>4.2 ± 0.3</td>
<td>-11 ± 12</td>
<td>0.259</td>
</tr>
<tr>
<td>Myristoyl (n = 7)</td>
<td>3.1 ± 0.9</td>
<td>2.8 ± 0.4</td>
<td>21 ± 21</td>
<td>0.749</td>
</tr>
<tr>
<td>Stearoyl (n = 7)</td>
<td>5.0 ± 0.5</td>
<td>5.5 ± 0.6</td>
<td>14 ± 8.5</td>
<td>0.308</td>
</tr>
</tbody>
</table>

Values are mean ± SE; fluxes before addition of acylcarnitine did not differ significantly between groups (P = 0.48).

* P value for the percent change in sufentanil flux after the addition of acylcarnitine.

Results

Tables 1–3 show the influence of the different acylcarnitines on the transmeningeal flux of sufentanil, mannitol, and morphine. None of the acylcarnitines significantly altered the meningeal permeability of sufentanil. In contrast, all acylcarnitines significantly (P < 0.05) increased the flux of mannitol and morphine.

With mannitol, there was a general trend for the penetration-enhancing effects of acylcarnitines to increase as chain length increased (fig. 1), but myristoyl-carnitine (C14) was an exception to this trend. For morphine, carnitines with intermediate-length acyl chains (12 and 14 carbon) produced the greatest penetration-enhancing effects.

Figures 2 and 3 show the effect of acylcarnitine concentration on morphine (fig. 2) and mannitol (fig. 3) flux. The most effective concentration for increasing morphine flux with octanoyl-, lauroyl-, and stearoylcarnitine was 2.5 mM. Above and below that concentration the increase in morphine flux was less. With mannitol, octanoyl- and lauroylcarnitine also produced maximal effects at a concentration of 2.5 mM. However, with stearoylcarnitine there was a linear increase in mannitol flux as stearoylcarnitine concentration increased between 0.5 and 5.0 mM.

Discussion

In this study we confirmed our earlier report that acylcarnitines can enhance the meningeal permeability to hydrophilic but not hydrophobic molecules. This permeation enhancement is believed to be related to the demonstrated ability of acyl carnitines to partition into lipid bilayers and thereby disrupt the tight packing order of the lipid bilayer. In addition, we found that there is a nonmonotonic relation between acyl chain length

Table 2. Mannitol Flux before and after Addition of 5 mM Acylcarnitines

<table>
<thead>
<tr>
<th>Acylcarnitine</th>
<th>Flux before Acylcarnitine (pmol/min·cm²)</th>
<th>Flux after Acylcarnitine (pmol/min·cm²)</th>
<th>Flux (%) change</th>
<th>Significance* (P)</th>
<th>Significant Differences between Acylcarnitines†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Hexanoyl</td>
<td>3.2 ± 0.4</td>
<td>4.8 ± 0.5</td>
<td>55 ± 12</td>
<td>0.002</td>
<td>4, 6, 7</td>
</tr>
<tr>
<td>2 Octanoyl</td>
<td>4.6 ± 0.6</td>
<td>5.9 ± 0.9</td>
<td>30 ± 11</td>
<td>0.034</td>
<td>4, 6, 7</td>
</tr>
<tr>
<td>3 Decanoyl</td>
<td>2.3 ± 0.6</td>
<td>4.2 ± 1.0</td>
<td>81 ± 20</td>
<td>0.009</td>
<td>4, 6, 7</td>
</tr>
<tr>
<td>4 Lauroyl (n = 8)</td>
<td>4.6 ± 0.9</td>
<td>10.6 ± 0.7</td>
<td>196 ± 66</td>
<td>0.001</td>
<td>1, 2, 3</td>
</tr>
<tr>
<td>5 Myristoyl</td>
<td>3.3 ± 1.2</td>
<td>5.9 ± 1.2</td>
<td>119 ± 24</td>
<td>0.002</td>
<td>1, 2, 3, 5</td>
</tr>
<tr>
<td>6 Palmitoyl (n = 11)</td>
<td>2.3 ± 0.4</td>
<td>7.2 ± 0.7</td>
<td>244 ± 29</td>
<td>0.001</td>
<td>1, 2, 3, 5</td>
</tr>
<tr>
<td>7 Stearoyl</td>
<td>2.6 ± 0.6</td>
<td>7.2 ± 1.2</td>
<td>224 ± 66</td>
<td>0.004</td>
<td>1, 2, 3, 5</td>
</tr>
</tbody>
</table>

Values are mean ± SE; fluxes before addition of acylcarnitines did not differ between groups (P = 0.16).

* P value for the percent change in mannitol flux after the addition of acylcarnitine.

† Numbers indicate the acylcarnitines for which the increase in morphine flux was significantly different.

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and the ability of acyl carnitines to increase meningeal permeability. Finally, we found that the optimal acyl carnitine for increasing meningeal permeability differs for different drug molecules.

The arachnoid mater has been shown to be the primary barrier to movement of drug molecules from the epidural to the subarachnoid space. The arachnoid mater consists of overlapping layers of flattened epithelial-like cells connected to one another by frequent tight junctions and occluding junctions. There is little evidence to support the concept of specific carriers, or porter, for morphine (or, for that matter, mannitol) across this meningeal barrier. Thus we think it rational to assume a major role for the arachnoid lipid bilayer and the tight junctions in restricting movement of solutes across the barrier. Our finding that the optimal acyl carnitine for increasing meningeal permeability differs between morphine and mannitol probably relates to differences in the way these two molecules negotiate the arachnoid cell membrane and/or the intercellular tight junctions.

The lipid bilayer moiety of biological membranes is considered the primary barrier to free diffusion of water and solutes. This conclusion arises from observations of lipid bilayer model membrane systems, which are generally less permeable than biological membranes.

Table 3. Morphine Flux before and after addition of 5 mM Acylcarnitines

<table>
<thead>
<tr>
<th>Acylcarnitine</th>
<th>Flux before Acylcarnitine (pmol/min·cm²)</th>
<th>Flux after Acylcarnitine (pmol/min·cm²)</th>
<th>Flux (% change)</th>
<th>Significance* (P)</th>
<th>Significant Differences between Acylcarnitines†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexanoyl (n = 7)</td>
<td>4.1 ± 0.5</td>
<td>7.7 ± 0.7</td>
<td>93 ± 16</td>
<td>0.0003</td>
</tr>
<tr>
<td>2</td>
<td>Octanoyl (n = 7)</td>
<td>3.2 ± 0.4</td>
<td>5.3 ± 0.7</td>
<td>73 ± 21</td>
<td>0.003</td>
</tr>
<tr>
<td>3</td>
<td>Decanoyl (n = 7)</td>
<td>4.2 ± 1.3</td>
<td>7.6 ± 2.0</td>
<td>87 ± 9.4</td>
<td>0.005</td>
</tr>
<tr>
<td>4</td>
<td>Lauroyl (n = 8)</td>
<td>3.7 ± 0.3</td>
<td>9.5 ± 0.7</td>
<td>165 ± 25</td>
<td>0.0001</td>
</tr>
<tr>
<td>5</td>
<td>Myristoyl (n = 7)</td>
<td>3.6 ± 0.6</td>
<td>8.8 ± 0.3</td>
<td>188 ± 44</td>
<td>0.0001</td>
</tr>
<tr>
<td>6</td>
<td>Palmitoyl (n = 11)</td>
<td>3.6 ± 0.5</td>
<td>7.4 ± 0.5</td>
<td>109 ± 16</td>
<td>0.0001</td>
</tr>
<tr>
<td>7</td>
<td>Stearoyl (n = 7)</td>
<td>4.3 ± 0.8</td>
<td>6.5 ± 1.2</td>
<td>50 ± 16</td>
<td>0.031</td>
</tr>
</tbody>
</table>

Values are mean ± SE; fluxes before addition of acylcarnitines did not differ between groups (P = 0.92).

* P value for the percent change in morphine flux after the addition of acylcarnitine.

† Numbers indicate the acylcarnitines for which the increase in morphine flux was significantly different.

Fig. 1. Acylcarnitine chain length and its influence on drug flux enhancement across the spinal meninges. Values are the mean of 6–11 animals per group.

Fig. 2. Effect of octanoyl-, lauroyl-, and stearoylcarnitine concentration on enhancement of morphine flux across the spinal meninges.
However, the nature of the permeability barrier remains unclear, particularly with respect to ionic solutes, and it has been suggested that there are fundamental differences between the permeation mechanisms of electrically charged (e.g., morphine) and electrically neutral (e.g., mannitol) solutes. It is generally accepted that ligands, with the singular exception of protons, permeating across phospholipid bilayers must become, at some point, immersed in a low dielectric medium in the interior of the bilayer. Indeed, the close relation between permeation rates and oil-water partition coefficients was instrumental in the formulation of the currently accepted models of membrane structure. The dependence of polar solute permeation rates on membrane phospholipid acyl chain lengths suggests that the hydrophobic domain of the acyl chains themselves are the primary barrier to solute permeation. Permeation mechanisms that involve transient defects in the lipid bilayer arising from thermal fluctuations have been described, and there are thought to be at least two varieties of transient defects required to explain differential permeation of water-soluble neutral and ionic solutes. Thus our observation of the manner in which different acyl carnitines promote different permeability changes toward ionized solute (morphine) when contrasted with unionized solute (mannitol) is consistent with what is known about the mechanisms by which these two classes of solute traverse lipid bilayers.

The biphasic relation between carnitine acyl chain length and morphine permeation enhancement (fig. 1) deserves comment. The critical micelle concentration and the ability to solubilize and form vesicles from amphipathic lipids depends, in large part, on the length of the amphipathic acyl chain. For example, it was shown previously that solubilization of membrane components by acyl glycosides shows the same biphasic relation to acyl chain length demonstrated here for morphine permeation enhancement. Thus one explanation for the smaller effect of longer acyl chains is that these longer chain carnitines spontaneously form micelles in solution, thereby decreasing the number of free acyl carnitine monomers available for partition into the arachnoid lipid bilayer. In fact, the smaller penetration-enhancing effect of palmitoyl carnitine in this study, compared with our earlier study, is probably explained by this same phenomenon. In our earlier study, palmitoyl carnitine was added to the diffusion cell as a dry powder at 100 min, whereas in the present study palmitoyl carnitine was added as a predissolved solution. When added as a solution, some fraction of the palmitoyl carnitine undoubtedly existed as a stable micelle, thereby reducing the amount or palmitoyl carnitine available to partition into the arachnoid cells. This marked difference in the effect of identical amounts of palmitoyl carnitine when added as a dry powder rather than as a solution has been confirmed in multiple control experiments (C.M. Bernards, M.D., and W.C. Ummenhofer, M.D., unpublished observations). Thus the biphasic relation between carnitine chain length and morphine permeability enhancement may be affected somewhat by the differential bioavailability of the acyl carnitines. However, the absence of the same biphasic effect of acyl chain length on mannitol permeability is striking and presumably reflects differences in the mode of passage of the two solutes through the arachnoid mater, as noted previously. Of interest with mannitol is the marked decrease in the effect of myristoyl carnitine compared with lauroyl (12 carbon) and palmitoyl (16 carbon) (fig. 1). The mechanism for this finding is not clear, but it is consistent with work by LeCluyse et al., who found the same relative effect of myristoyl carnitine, lauroyl carnitine, and palmitoyl carnitine on increasing solute permeability through the intestinal mucosa.

Micelle formation may also explain the nonlinear effect of acylcarnitine concentration on enhancing drug flux. Micelle formation depends on several parameters,
including the concentration of the amphipile. At concentrations exceeding the critical micelle concentration, amphiphile monomers begin to aggregate into micelles, resulting in a decreased number of available monomers. Thus the marked decrease in the effect of acylcarnitines between concentrations of 2.5 mm and 5 mm could be explained readily if the critical micelle concentration of the tested acylcarnitines falls between these two concentrations. However, it is difficult to explain why the concentration-dependent effect of sterylcarntine is qualitatively different for morphine and mannitol. The fact that there is a clear difference supports the idea that these two drug molecules interact with the arachnoidal membrane in different ways.

As noted before, acyl carnitines have been shown to partition into lipid bilayers and to disturb the tight packing order of the membrane phospholipids. This effect of acyl carnitines has been presumed to explain the ability of these compounds to enhance solute permeability. However, it is also possible that acyl carnitines effectively “shield” polar solutes, thereby decreasing the activation energy required for them to traverse the hydrophobic core of the lipid bilayer. In essence, acyl carnitines may form clathrates around solutes, thereby increasing their permeability much as cyclodextrins shield hydrophobic drugs and increase their permeability through biologic membranes. This hypothesis is also consistent with the observation that permeability enhancement varies with acyl chain length. For example, the critical micelle concentration and its temperature dependence have been determined for a homologous series of alkylmethylglucamides and shown to vary inversely with acyl chain length in various amphiphilic compounds. Future studies with the system described here will seek to illuminate the relative importance of acyl carnitine critical micelle concentration variability and solute solubilization effects on enhancing meningeal permeability to anesthetic agents.

In conclusion, the results of this study suggest that long-chain acylcarnitines may significantly improve the transmeningeal flux of hydrophilic drugs after epidural administration. For morphine, still one of the most commonly used hydrophilic drugs for epidural administration, addition of intermediate-chain-length acylcarnitines might increase drug delivery to the spinal cord, thus reducing drug redistribution from the epidural space to the systemic circulation. As a result, the therapeutic index for epidurally administered morphine would be expected to be increased. However, it must be kept in mind that the epidural space is a complex site composed of multiple “compartments” including fat, ligaments, venous plexus, periosteum, and so forth. Thus it is possible that acylcarnitines may increase drug movement into one or more of these compartments as well and thereby obviate the benefits of increased meningeal permeability. Thus appropriate in vivo experiments must be conducted to address this issue. In addition, formal toxicity studies must be performed before any acylcarnitines are considered for therapeutic uses.

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