Pharmacologically Relevant Receptor Binding Characteristics and 5α-Reductase Inhibitory Activity of Free Fatty Acids Contained in Saw Palmetto Extract

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Saw palmetto extract (SPE), used widely for the treatment of benign prostatic hyperplasia (BPH) has been shown to bind α1-adrenergic, muscarinic and 1,4-dihydropyridine (1,4-DHP) calcium channel antagonist receptors. Major constituents of SPE are lauric acid, oleic acid, myristic acid, palmitic acid and linoleic acid. The aim of this study was to investigate binding affinities of these fatty acids for pharmacologically relevant (α1-adrenergic, muscarinic and 1,4-DHP) receptors. The fatty acids inhibited specific [3H]prazosin binding in rat brain in a concentration-dependent manner with IC50 values of 23.8 to 136 μM/mL, and specific (±)-[3H]PN 200-110 binding with IC50 values of 24.5 to 79.5 μM/mL. Also, lauric acid, oleic acid, myristic acid and linoleic acid inhibited specific [3H]N-methylscopolamine ([3H]NMS) binding in rat brain with IC50 values of 56.4 to 169 μM/mL. Palmitic acid had no effect on specific [3H]NMS binding. The affinity of oleic acid, myristic acid and linoleic acid for each receptor was greater than the affinity of SPE. Scatchard analysis revealed that oleic acid and lauric acid caused a significant decrease in the maximal number of binding sites (Bmax) for [3H]prazosin, [3H]NMS and (±)-[3H]PN 200-110. The results suggest that lauric acid and oleic acid bind noncompetitively to α1-adrenergic, muscarinic and 1,4-DHP calcium channel antagonist receptors. We developed a novel and convenient method of determining 5α-reductase activity using LC/MS. With this method, SPE was shown to inhibit 5α-reductase activity in rat liver with an IC50 of 101 μM/mL. Similarly, all the fatty acids except palmitic acid inhibited 5α-reductase activity, with IC50 values of 42.1 to 67.6 μM/mL. In conclusion, lauric acid, oleic acid, myristic acid, and linoleic acid, major constituents of SPE, exerted binding activities of α1-adrenergic, muscarinic and 1,4-DHP receptors and inhibited 5α-reductase activity.

Key words saw palmetto extract; free fatty acid; receptor; benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) and related lower urinary tract symptoms (LUTS) are common disorders in aging men. Plant extracts are widely used in the treatment of BPH and related LUTS. In fact, phytotherapeutic agents, including saw palmetto extract (SPE), are very popular in many European countries as herbal remedies, representing up to 80% of all drugs prescribed for these disorders.1,2) An analysis of many clinical trials showed mild to moderate improvements in symptom scores and urinary flow in patients with BPH.3) The most widely known effect of SPE is the inhibition of type 1 and 2 isozymes of 5α-reductase.4,5) Other pharmacodynamic mechanisms of SPE have also been proposed, including anti-androgenic effects, anti-proliferative effects and anti-inflammatory effects.5–8) Our recent study has shown that the intraduodenal administration of SPE in the rat cys-tometry resulted in a significant and dose-related increase in micturition interval as well as a decrease in micturition volume and bladder capacity caused by the infusion of 0.1% acetic acid.9) Furthermore, we have reported that SPE inhibited the specific binding of radioligands to α1-adrenergic, muscarinic and 1,4-dihydropyridine (1,4-DHP) calcium channel antagonist receptors in the lower urinary tract of rats.9–11) However, the exact active constituents of SPE remain to be identified. SPE contains mainly saturated and unsaturated fatty acids (90%). Among these fatty acids, the major constituents are oleic acid and lauric acid, which together compose more than 50% of SPE.12) The aim of this study is to investigate binding activities for pharmacologically relevant (α1-adrenergic, muscarinic, 1,4-DHP calcium channel antagonist) receptors of major fatty acids contained in SPE. In addition, with a newly developed method using LC/MS, the effects of SPE and its fatty acids on 5α-reductase activity in the rat liver were also examined.

MATERIALS AND METHODS

Materials SPE (Serenoa repens purified extract) was kindly provided by Indena Japan (Tokyo, Japan). SPE was obtained with hypercritical CO2 (SABAL SELECT, Indena S.p.A., Milan, Italy). The constituents contained in SPE are presented elsewhere.12) Oleic acid, lauric acid, myristic acid, palmitic acid and linoleic acid were purchased from Tokyo Chemical Industry (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), Sigma-Aldrich (St. Louis, MO, U.S.A.) and Wako Pure Chemicals (Osaka, Japan), respectively. [3H]N-Methylscopolamine ([3H]NMS, 3.03 TBq/mmol), [7-methoxy-3H]prazosin ([3H]prazosin, 3.21 TBq/mmol), and (±)[5-methyl-3H]PN 200-110 ([±]-[3H]PN 200-110, 2.71—3.03 TBq/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, U.S.A.). All other chemicals were purchased from commercial sources.

Animals Male and female Sprague-Dawley rats at 8—10 weeks of age (Japan SLC Inc., Shizuoka, Japan) were housed in the laboratory with free access to a normal diet (CE-2, CREA Japan Inc., Tokyo, Japan) and water, and maintained on a 12-h light–dark cycle in a room with controlled temperature and humidity. The study was conducted in accordance with the guidelines of the Experimental Ani-
nal Ethics Committee of the University of Shizuoka.

**Tissue Preparation and Radioreceptor Binding Assay**

Radioligand binding assays for muscarinic receptors, $\alpha_1$-adrenoceptors and 1,4-DHP receptors were performed using $[^3H]$NMS, $[^3H]$prazosin and (+)-$[^3H]$PN 200-110, respectively. Male rats were killed by taking the blood from the descending aorta under temporary anesthesia with diethyl ether and the brain (except for cerebellum) was excised after being perfused with cold saline from the aorta. The brain was homogenized by a Kinematica Polytron homogenizer in 19 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4). The homogenate was then centrifuged at 40000 × $g$ for 20 min at 4 °C, and the suspension of the pellet was centrifuged again under similar conditions. The resulting pellet was finally suspended in 29 volumes of 50 mM Tris–HCl buffer (pH 7.4).

In the presence of various concentrations of SPE and fatty acids, the brain homogenate (5 mg) was incubated with 0.25 mM $[^3H]$NMS in a total volume of 0.5 ml in 30 mM Na$^+$/HEPES buffer (pH 7.5). The incubation was carried out for 60 min at 25 °C. In the presence of various concentrations of SPE and fatty acids, the brain homogenate (10 mg) was incubated with 0.25 mM $[^3H]$prazosin in a total volume of 1 ml in 50 mM Tris–HCl buffer (pH 7.4). The incubation was carried out for 30 min at 25 °C. In the presence of various concentrations of SPE and fatty acids, the brain homogenate (5 mg) was incubated with 0.30 mM (+)-$[^3H]$PN 200-110 in a total volume of 0.5 ml in 50 mM Tris–HCl buffer (pH 7.4). The incubation was carried out in the dark with a sodium lamp for 60 min at 25 °C. Each reaction was terminated by rapid filtration (Cell Harvester, Brandel Co., Gaithersburg, MD, U.S.A.) through Whatman GF/B glass fiber filters, and the filters were then rinsed three times with 3 ml of ice-cold 50 mM Na/K+ phosphate buffer. Tissue-bound radioactivity was extracted from the filters by placing them overnight in scintillation fluid (21 toluene, 11 Triton X-100, 15 g 2,5-diphenyloxazole, 0.3 g 1,4-bis(2-(5-phenyloxazolyl))benzene), and radioactivity was measured with a liquid scintillation counter. The specific binding of $[^3H]$NMS, $[^3H]$prazosin and (+)-$[^3H]$PN 200-110 was determined experimentally from the difference between counts in the absence and presence of 1 mM atropine, 10 μM phentolamine and 1 μM nifedipine, respectively. Protein concentrations were measured by the method of Lowry et al. Microsomal Preparation, 5α-Reductase Activity, and LC/MS Analysis

The liver microsomes of rats were prepared as reported by Liu et al. with some modifications. The liver was removed from female rats and homogenized in 4 volumes of medium A (0.32 M sucrose, 1 mM dithiothreitol, and 20 mM sodium phosphate, pH 6.5). The homogenate was then centrifuged at 10000 × $g$ for 10 min. The supernatant was further centrifuged at 105000 × $g$ for 1 h twice. The microsomal fraction was suspended in 1 volume of medium A, and the dispersion of microsomes was achieved using a syringe with 18 G, 23 G, and 26 G needles in succession. Protein concentrations were measured by the method of Bradford. The microsomal suspension was stored at −80 °C just prior to use. A complete reaction mixture included 1 mM dithiothreitol, 20 mM phosphate buffer (pH 6.5), 50 μM testosterone, 167 μM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and the microsomes (0.2 mg of protein) in a final volume of 0.3 ml. Fatty acids were added to the reaction solution including the microsomal fraction. The incubation was carried out for 10 min at 37 °C, by the addition of microsomes and NADPH to the pre-heated reaction solution. The incubation was terminated by adding 10 μM of 2 M NaOH. To extract metabolites, 10 μl of internal standard (30 μM dexamethasone) and 0.6 ml of ethyl acetate were added, and the tubes were capped and shaken. The organic layer was evaporated with a stream of nitrogen gas. The residue was dissolved in 50 μl of methanol.

**Data Analysis**

Radioligand binding data were subjected to a non-linear regression analysis using Graph Pad PRISM (ver. 4, Graph Pad Software, San Diego, CA, U.S.A.). The apparent dissociation constant ($K_d$) and maximal number of binding sites ($B_{max}$) for $[^3H]$NMS (0.06—1.0 nm), $[^3H]$prazosin (0.03—0.5 nm) and (+)-$[^3H]$PN 200-110 (0.03—1.0 nm) were estimated. The ability of each agent to inhibit the specific binding of a radioligand and the 5α-reductase activity was estimated from the IC$^{50}$ values, namely the concentration of the agent necessary to displace 50% of the specific binding of the radioligand and to inhibit 50% of the 5α-reductase activity (determined by a log probit analysis). The data were expressed as the mean±S.E. The statistical analysis of the data was performed with a one-way analysis of variance (ANOVA), followed by Dunnnett’s test for multiple comparisons. Statistical significance was accepted at $p<0.05$.

**RESULTS**

**Effects on $\alpha_1$-Adrenergic, Muscarinic and 1,4-DHP Calcium Channel Antagonist Receptors**

The effects of SPE and free fatty acids on the specific binding of $[^3H]$prazosin, (+)-$[^3H]$PN 200-110, and $[^3H]$NMS in the rat brain homogenates were examined. Although the lower urinary tract is clinically important target of SPE, in this study, the brain tissue was used because of a similarity between brain and lower urinary tract of pharmacological agents and SPE in binding affinities of $\alpha_1$-adrenergic, muscarinic and 1,4-DHP receptors and also because of less amount of nonspecific binding for each radioligand in the brain compared to the bladder and prostate.

SPE (10—200 μg/ml) inhibited specific $[^3H]$prazosin binding in a concentration-dependent manner (Fig. 1A). The IC$^{50}$ value was 106 μg/ml (Table 1). Similarly, lauric acid, oleic
acid, myristic acid, palmitic acid and linoleic acid (10—300 μg/ml) inhibited specific $[^3H]$prazosin binding in a concentration-dependent manner (Fig. 1A). Their IC$_{50}$ values ranged from 23.8 to 136 μg/ml (Table 1). Linoleic acid was the most potent inhibitor of $[^3H]$prazosin binding sites, followed by oleic acid, myristic acid, lauric acid and palmitic acid. The inhibitory effects of linoleic acid, oleic acid and myristic acid were 1.7—4.5 times greater than the effect of SPE. SPE, lauric acid, oleic acid, myristic acid and linoleic acid (30—300 μg/ml) inhibited specific $[^3H]$NMS binding in the rat brain in a concentration-dependent manner, but palmitic acid had little effect (Fig. 1B). Respective IC$_{50}$ values were 185, 169, 70.6, 105 and 56.4 μg/ml (Table 1). The inhibitory effects of these fatty acids except lauric acid were 2—3 fold greater than the effect by SPE, and the order of inhibitory potency was the same as that described for $[^3H]$prazosin. Moreover, SPE and each fatty acid (10—200 μg/ml) inhibited specific (+)$[^3H]$PN 200-110 binding in the rat brain in a concentration-dependent manner (Fig. 1C), and their IC$_{50}$ values ranged from 24.5 to 79.5 μg/ml (Table 1). The inhibitory effects of linoleic acid and oleic acid were greater than the effect by SPE, lauric acid or palmitic acid.

A Scatchard analysis revealed that oleic acid (52.7 μg/ml) and lauric acid (73.5 μg/ml) around IC$_{50}$ concentrations reduced significantly (39%, 33%, respectively) the $B_{max}$ values for specific $[^3H]$prazosin binding in rat brain, compared with the corresponding control values (Table 2). Similarly, in the presence of oleic acid (72.8 or 33.3 μg/ml) and lauric acid (163 or 82.3 μg/ml), there was a significant decrease of $B_{max}$ values for the specific binding of $[^3H]$NMS (49%, 24%, respectively) and of (+)$[^3H]$PN 200-110 (34%, 41%, respectively) in the brain. There was little significant change in $K_d$ values for the specific binding of each radioligand.

### Table 1. Inhibition of Specific Binding of $[^3H]$Prazosin, $[^3H]$NMS, and (+)$[^3H]$PN 200-110 by SPE and Free Fatty Acids in Rat Brain

<table>
<thead>
<tr>
<th>IC$_{50}$ values (μg/ml)</th>
<th>$[^3H]$Prazosin</th>
<th>$[^3H]$NMS</th>
<th>(+)$[^3H]$PN 200-110</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPE</td>
<td>106±11</td>
<td>185±8</td>
<td>59.7±4.0</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>92.1±10.9</td>
<td>169±8</td>
<td>79.5±4.0</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>46.3±4.3</td>
<td>70.6±4.1</td>
<td>33.3±0.9</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>62.1±5.2</td>
<td>105±4</td>
<td>45.0±2.2</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>136±27</td>
<td>ND</td>
<td>61.3±4.3</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>23.8±1.4</td>
<td>56.4±2.4</td>
<td>24.5±0.9</td>
</tr>
</tbody>
</table>

Specific binding of $[^3H]$prazosin (0.25 μM), $[^3H]$NMS (0.25 μM), and (+)$[^3H]$PN 200-110 (0.30 μM) in rat brain was determined in the absence and presence of various concentrations (10—300 μg/ml) of SPE, lauric acid, oleic acid, myristic acid, palmitic acid, and linoleic acid. Values are the mean±S.E. of three to seven determinations. ND: not determined.
inhibited the 5α-reductase activity in a concentration-dependent manner (Fig. 3), with an IC₅₀ value of 101 μg/ml (Table 3). All the fatty acids (10—300 μg/ml) except palmitic acid, inhibited the activity in a concentration-dependent manner (Fig. 3). Their IC₅₀ values ranged from 42.1 to 67.6 μg/ml (Table 3). The inhibitory effects of these fatty acids were greater than the effect by SPE. Palmitic acid did not inhibit significantly the 5α-reductase activity.

**Discussion**

The major findings of this study are that lauric acid, oleic acid, myristic acid and linoleic acid, the major constituents of SPE, as well as SPE itself, actively bound to pharmacologically relevant (α₁-adrenergic, muscarinic and 1,4-DHP) receptors in rat brain, and significantly inhibited 5α-reductase activity in rat liver. Our previous study has shown that SPE inhibited specific binding of [³H]prazosin in rat prostate and spleen and the binding of [³H]NMS in the bladder and submaxillary gland. The present study, lauric acid, oleic acid, myristic acid, palmitic acid and linoleic acid contained in SPE, as well as SPE itself, were shown to inhibit specific binding of [³H]prazosin, [³H]NMS and (+)[³H]PN 200-110 in rat brain. Based on IC₅₀ values, the activity to bind α₁-adrenergic and muscarinic receptors of linoleic acid, oleic acid, and myristic acid was 1.7—4.5 times greater than that of SPE. Similarly, the 1,4-DHP receptor binding activity of linoleic acid, oleic acid, and myristic acid was approximately 1.3—2.4 times greater than that of SPE. The receptor binding activity of lauric acid and palmitic acid was similar to that of SPE.

The receptor binding activity of unsaturated fatty acids (oleic acid, linoleic acid) tended to be greater than that of saturated fatty acids (lauric acid, myristic acid, palmitic acid). Vijayaraghavan et al.⁹ reported that the activity to bind α₇-containing nicotinic receptor of linolenic acid (C18:3) having three double bonds was greater than that of linoleic acid (C18:2). Thus, the receptor binding activity of fatty acids was suggested to increase with the degree of unsaturation. By contrast, the receptor binding activity of myristic acid (C14:0) was greater than that of palmitic acid (C16:0) or lauric acid (C12:0). There seemed to be little apparent relationship between receptor binding activity and bond lengths in saturated fatty acids.

The Scatchard analysis revealed that lauric acid and oleic acid caused a significant decrease in the Bₘₐₓ for specific [³H]prazosin binding (Table 2). Moreover, both fatty acids also decreased significantly Bₘₐₓ values for specific [³H]NMS binding. We have shown that SPE reduced significantly Bₘₐₓ values for specific [³H]prazosin binding in the rat prostate and for specific [³H]NMS binding in the rat bladder. Goepel et al.²⁴ also have showed that SPE reduced significantly (30—40%) [³H]prazosin binding (Bₘₐₓ) to cloned human α₁-adrenergic receptors. Hence, oleic acid and lauric acid seem to inhibit specific [³H]prazosin binding in a non-competitive manner. Rauch et al.²⁵ reported that oleic acid reduced significantly the Bₘₐₓ for specific [³H]quinuclidinylbenzilate (QNB) binding in purified canine sarcolemmal membranes. Further, Kjome et al.²⁶ showed that arachidonic acid inhibited specific [³H]QNB binding to human brain muscarinic receptors and caused a decrease in the Bₘₐₓ and increase of Kᵦ for [³H]QNB binding. According to Rauch et al.,²⁵ the incubation of membranes with free unsaturated fatty acids induces a conformational transformation of the unoccupied muscarinic receptor form R to R*. As the conformation R* is not able to bind [³H]QNB, its Bₘₐₓ may be decreased. Additionally, each fatty acid including lauric acid and oleic acid was shown to bind 1,4-DHP receptors with a decrease in Bₘₐₓ values for specific (+)[³H]PN 200-110 (Table 2). Pepe et al.²⁷ reported that docosahexaenoic acid inhibited the actions of a Ca²⁺ channel agonist (BAY K 8644) and antagonist (nitrendipine) in rat cardiac myocytes. As the inhibitory mechanism, docosahexaenoic acid was assumed to bind Ca²⁺.

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**Table 3. Inhibition of 5α-Reductase Activity by SPE and Free Fatty Acids in Rat Liver**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>IC₅₀ (μg/ml)</th>
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<tbody>
<tr>
<td>SPE</td>
<td>101 ±2</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>67.6 ± 5.2</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>54.3 ± 3.7</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>56.4 ± 2.5</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>42.1 ± 1.4</td>
</tr>
</tbody>
</table>

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The activity of 5α-reductase in the rat liver was measured in the absence and presence of various concentrations (10—300 μg/ml) of SPE, lauric acid, oleic acid, myristic acid, palmitic acid and linoleic acid. Each point represents the mean±S.E. of five to six determinations.
channels at or near the 1,4-DHP binding sites and to alter the specific protein–lipid and lipid–lipid relationship between L-type Ca\(^{2+}\) channels in cardiac membranes.\(^{27}\)

Previous studies have shown the physiological and pharmacological roles of 1,4-DHP receptors in the bladder. In fact, our previous study has demonstrated that (+)-\(^{3}H\)PN 200-110 bound to the rat bladder homogenates in a rapid, specific, and saturable manner with high affinity.\(^{28}\) The \(K_d\) values for bladder (+)-\(^{3}H\)PN 200-110 binding was in a reasonably agreement with that in cardiovascular tissues.\(^{15}\) Thus, these data suggest the physiological and pharmacological roles (relaxant effects \textit{etc.}) of 1,4-DHP receptors in the rat bladder.

We developed a convenient method of determining 5α-reductase activity using LC/MS and non-labeled testosterone. SPE, lauric acid, oleic acid, myristic acid and linoleic acid inhibited 5α-reductase activity in a concentration-dependent manner (Fig. 3, Table 3). The inhibitory effect of each fatty acid was similar but seemed to be slightly more potent than that of SPE (Table 3). Consistent with our results, Raynaud \textit{et al.}\(^{23}\) reported that palmitic acid was inactive in the inhibition of 5α-reductase. The inhibitory effect on 5α-reductase activity by each fatty acid (Table 3) was roughly similar to their affinity for pharmacologically relevant receptors (Table 1). The possibility cannot be ruled out that each fatty acid inhibited the enzyme activity and the binding of radioligands in a nonspecific manner, by influencing the bio-membrane fluidity of receptors and enzymes.

It is reported that SPE contains 30.2% lauric acid, 28.5% oleic acid, 12.1% myristic acid, 9.5% palmitic acid, and 4.6% linoleic acid.\(^{12}\) SPE inhibited specific binding of \(^{3}H\)prazosin, \(^{3}H\)NMS and (+)-\(^{3}H\)PN 200-110 with IC\(_{50}\) values of 106, 185 and 59.7 µg/ml, respectively. SPE at a concentration of 106 µg/ml was estimated to contain 32.0 µg/ml of lauric acid, 30.2 µg/ml of oleic acid, 12.7 µg/ml of myristic acid, 10.1 µg/ml of palmitic acid and 4.9 µg/ml of linoleic acid. The sum of inhibitory rates for the binding of \(^{3}H\)prazosin at each concentration of these fatty acids (Fig. 1A) was estimated to be 33.7%. That in the case of \(^{3}H\)NMS and (+)-\(^{3}H\)PN 200-110 was 30.2% and 37.8%, respectively. Furthermore, the IC\(_{50}\) value for the inhibition of 5α-reductase activity by SPE was 101 µg/ml, and the sum total of inhibition by each fatty acid contained in 101 µg/ml of SPE was only 11.3%. Thus, the sum of the inhibition by each fatty acid was less than 50%. Namely, the receptor binding activity and inhibitory effect on 5α-reductase activity of SPE was assumed to be due to the synergistic effects of the fatty acids contained in SPE.

SPE contained 28.5% oleic acid, the receptor binding activity of which was relatively greater than that of lauric acid, myristic acid or palmitic acid. Linoleic acid seemed to have the greatest receptor binding activity and inhibitory effect on 5α-reductase activity among the free fatty acids contained in SPE. Based on IC\(_{50}\) values, the pharmacological activities of linoleic acid were slightly (1.3—2.0 times) greater than those of oleic acid. However, the amount of linoleic acid was about one-sixth of that of oleic acid. The contribution of oleic acid to the pharmacological activities of SPE may be 3—4.6 times greater than that of linoleic acid. Thus, it is likely that oleic acid contributes extensively to the pharmacological effects of SPE.

REFERENCES