Online Supplement

**FFA causes leukocyte activation and resultant endothelial dysfunction through enhanced angiotensin II production in mononuclear and polymorphonuclear cells**

Yoko Azekoshi MD1**, Takanori Yasu MD1**, Saiko Watanabe MD1, Tatsuya Tagawa MD1, Satomi Abe BS2, Ken Yamakawa MD1, Yoshinari Uehara MD2, Shinichi Momomura MD3, Hidenori Urata MD2, Shinichiro Ueda MD1

1Department of Clinical Pharmacology & Therapeutics, University of the Ryukyus School of Medicine, Okinawa, Japan, 2Department of Cardiovascular Diseases, Fukuoka University Chikushi Hospital, Fukuoka, Japan, 3Department of First Integrated Medicine, Saitama Medical Center, Jichi Medical University, Saitama, Japan.

*Present address: Department of Nutritional Sciences, Faculty of Health and Welfare, Seinan Jo Gakuin University, Kitakyushu-shi, Japan

**Co-first authors with equal contributions to this work

Running title: Endothelial dysfunction by FFA and angiotensin II

Correspondence: Dr. Shinichiro Ueda
Department of Clinical Pharmacology & Therapeutics
University of the Ryukyus School of Medicine, Okinawa 903-0215, Japan
Tel: +81-98-895-1195; Fax: +81-98-895-1447
E-mail: suedano9@dream.com
Expanded Methods

Measurement of forearm blood flow (FBF) by strain gauge plethysmography during the intra-arterial infusion of drugs

All experiments were performed in a quiet, temperature-controlled room (22°C - 24°C). FBF was measured bilaterally by strain gauge, venous occlusion plethysmography during the intra-arterial infusion of drugs including acetylcholine (Daiichi Pharmaceutical Co., Ltd.), sodium nitroprusside, Ang I and Ang II (Delivert, Toa Eiyo, Fukushima, Japan) through a 27-gauge needle inserted into the brachial artery, as described previously.¹

Measurement of circulating RAS activity and plasma myeloperoxidase (MPO) level

Plasma renin activity² and plasma aldosterone concentrations³ were measured by standardized radioimmunoassay. Serum ACE activity was measured by the standardized method with artificial substrate.⁴ Plasma concentrations of Ang I and II were measured by radioimmunoassay.⁵ Plasma levels of MPO were measured by an enzyme-linked immunosorbent assay kit (Bio Check, Inc., CA).

Cell isolation

Mononuclear and polymorphonuclear cells were separated by gradient centrifugation using Lymphoprep and Polymorphprep (AXS-Shield, Oslo, Norway), respectively, as previously described.⁶ The mononuclear cell layers isolated by Lymphoprep contain 1%-2% polymorphonuclear cells, and the polymorphonuclear cell layers isolated by Polymorphprep contain 1%-4% mononuclear cells, as stated by the manufacturer. We confirmed these purity levels in a pilot study (n=3, data not shown). Each cell fraction was collected and rinsed with an equal volume of physiologic saline and pelleted by centrifugation at 250 × g for 10 min at room temperature and stored at -80°C until assay. The cell fraction was frozen on methanol/dry ice and thawed three times, then centrifuged at 5,000 rpm for 10 min at 4°C. The pellets were resuspended in assay buffer (10 mM Tris buffer, pH 7.4 containing 400 mM KCl and 0.01% Triton X-100) and homogenized with a hand homogenizer on ice. The protein concentration of the homogenate was measured by BCA Protein Assay Reagent by Pierce (Rockford, IL).
Online references


Table S1. Circulating renin-angiotensin system before and after the lipid heparin/infusion

<table>
<thead>
<tr>
<th>Variables</th>
<th>Baseline</th>
<th>60min</th>
<th>180min</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma renin activity (mg/ml/hr)</td>
<td>2.1 ± 0.8</td>
<td>1.5 ± 0.9</td>
<td>1.6 ± 0.7</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma aldosterone (pg/ml)</td>
<td>105.8 ± 88.3</td>
<td>87.1 ± 26.5</td>
<td>89.5 ± 44.5</td>
<td>ns</td>
</tr>
<tr>
<td>Serum ACE activity (IU/L)</td>
<td>12.5 ± 3.1</td>
<td>13.0 ± 3.1</td>
<td>12.9 ± 3.1</td>
<td>ns</td>
</tr>
<tr>
<td>Angiotensin I (pmol/min)</td>
<td>53.6 ± 7.7</td>
<td>51.1 ± 12.8</td>
<td>57.0 ± 17.7</td>
<td>ns</td>
</tr>
<tr>
<td>Angiotensin II (pmol/min)</td>
<td>19.5 ± 4.7</td>
<td>19.8 ± 5.8</td>
<td>17.5 ± 4.0</td>
<td>ns</td>
</tr>
<tr>
<td>Angiotensin II/I ratio</td>
<td>0.37 ± 0.09</td>
<td>0.40 ± 0.13</td>
<td>0.33 ± 0.11</td>
<td>ns</td>
</tr>
</tbody>
</table>

ACE, angiotensin converting enzyme;
Figure S1. Effect of elevated free fatty acid (FFA) on vasoconstriction to angiotensin (ANG) I and II

Percent changes in forearm blood flow (FBF) ratio, which was calculated as the FBF of the infused arm divided by that of the non-infused arm, during intra-arterial infusion of ANG I (A) and ANG II (B) with saline/heparin (open circles) or lipid/heparin (closed circles) infusion for 1 h.