Objective—An elevated free fatty acid (FFA) level impairs endothelium-dependent vasodilation in humans, which may be pathophysiologically relevant to the development of endothelial dysfunction in patients with insulin resistance. We investigated the effect of inhibition of the renin-angiotensin system (RAS) on FFA-induced endothelial dysfunction.

Methods and Results—Changes in forearm blood flow during intra-arterial infusion of acetylcholine were measured by plethysmography before and after systemic infusion of lipid/heparin in 10 healthy subjects given a single dose of placebo, losartan (50 mg), or perindopril (8 mg). Endothelial function after lipid/heparin infusion was also investigated with the coinfusion of vitamin C or N\textsubscript{G}-monomethyl-L-arginine (L-NMMA). Elevated FFA significantly reduced the response to acetylcholine by 37.7% (P=0.0096) without L-NMMA, but not the response with L-NMMA, whereas FFA did not affect the response to nitroprusside. The single dose of either losartan or perindopril completely prevented FFA-induced endothelial dysfunction. Vitamin C also prevented FFA-induced endothelial dysfunction.

Conclusions—Elevated FFA levels by lipid/heparin infusion, which may partly mimic the abnormal lipid profile in patients with insulin resistance, caused endothelial dysfunction via RAS activation and the presumably resultant oxidative stress in humans. Our results suggest the therapeutic rationale for RAS inhibition in patients with high FFA levels. (Arterioscler Thromb Vasc Biol. 2005;25:2376-2380.)

Key Words: fatty acids ■ endothelium ■ angiotensin II ■ insulin resistance ■ nitric oxide

An increased plasma level of free fatty acid (FFA) may play pivotal roles in the development and progression of endothelial dysfunction and insulin resistance in patients with type 2 diabetes as well as in the prediabetic state, including the metabolic syndrome and visceral adiposity.\textsuperscript{1,2} In addition to a negative correlation between plasma FFA levels and endothelial function,\textsuperscript{3} a direct vascular effect of FFA on endothelial function in humans has already been demonstrated. FFAs elevated by lipid/heparin infusion significantly attenuated the vasodilator response to acetylcholine (ACh) in healthy subjects.\textsuperscript{2,4} Although it has been suggested that reactive oxygen species\textsuperscript{4–6} and activation of a stress-sensitive pathway like the transcriptional factor nuclear factor-\textkappa\textsubscript{B}\textsuperscript{7,8} may be involved in FFA-induced endothelial dysfunction, no specific therapeutic intervention has yet been established.

Several randomized, clinical trials have suggested that inhibition of the renin-angiotensin system (RAS) is particularly effective in patients with diabetic hypertension compared with nondiabetic hypertension.\textsuperscript{9,10} Improvement in endothelial function by angiotensin-converting enzyme (ACE) inhibitors\textsuperscript{11} and angiotensin II type 1 receptor (AT\textsubscript{1}) antagonists (ARB)\textsuperscript{12} in type 2 diabetic patients may partly explain the results of previous randomized, clinical trials. Moreover, there is much evidence in support of enhanced activity of the RAS in obese, hypertensive patients.\textsuperscript{13} In the present study, we investigated the effects of inhibition of RAS by an ACE inhibitor or an ARB on FFA-induced endothelial dysfunction in humans.

Methods

Subjects

Ten young, male nonsmokers participated in our study. Characteristics of the subjects are shown in Table 1. All subjects had normal blood pressure and were not taking any medications. They had normal results of routine physical examination and standard laboratory tests, including a serum lipid profile and glucose concentrations. Written, informed consent was obtained from all participants. The study protocol was approved by the ethics committee of the University of the Ryukyus.

Forearm Blood Flow Measurement

All subjects fasted overnight and abstained from drinking beverages containing alcohol or caffeine for at least 12 hours before the study. All experiments were performed in a quiet, temperature-controlled room. Forearm blood flow (FBF) was measured bilaterally by venous occlusion plethysmography with strain gauges, as described previously.\textsuperscript{14,15}

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2376
Assessment of Endothelial Function Before and After Lipid/Heparin Infusion

All sterile solutions were freshly prepared before each study. ACh (Daiichi Pharmaceutical Co, Ltd) was infused at 50, 100, 200, and 400 nmol/min through a 27-gauge needle inserted into the brachial artery of the nondominant arm before and after systemic infusion of a fat emulsion (Intralipid 20%, Fresenius Kabi AB) at 90 mL/h and heparin (Shimizu Pharmaceutical Co, Ltd) at 0.3 U/mL/h and continued throughout the experiment. The reduction in FBF by L-NMMA was restored by the nitric oxide (NO) clamp technique.14

Protocol 1: Effect of Elevated FFAs on Endothelium-Dependent Vasodilation With or Without RAS Inhibition

This protocol was performed over 3 study days with an interval of at least 7 days between them. Each subject received a single oral dose of losartan (50 mg, Banyu Pharmaceutical Co, Ltd), perindopril (8 mg, Daiichi Pharmaceutical Co, Ltd), or placebo 4 hours before the experiment in a cross-over fashion. The order of treatment was randomized. Then endothelial function was assessed before and after lipid/heparin infusion. Blood samples for plasma FFA concentration, serum ACE activity, and plasma levels of angiotensin (Ang) I and II.16 were obtained which was measured by an enzymatic method,16 were obtained before and after systemic infusion of a fat emulsion (Intralipid 20%, Fresenius Kabi AB) at 90 mL/h and heparin (Shimizu Pharmaceutical Co, Ltd) at 0.3 U·kg⁻¹·min⁻¹ for 1 hour.9

Protocol 2: Effect of Elevated FFAs on Endothelium-Independent Vasodilation With or Without RAS Inhibition

This protocol was performed over 2 study days. Nine of 10 subjects participated in this study. After the measurement of baseline FBF, intra-arterial coinfusion of vitamin C (Daiichi Pharmaceutical Co, Ltd) at 24 mg/min or vehicle (isotonic saline) was started and continued throughout the experiment. Changes in the FBF response to ACh were measured before and after lipid/heparin infusion.

Protocol 3: Effect of Vitamin C on FFA-Induced Endothelial Dysfunction

Six of 10 subjects participated in this study. After the measurement of baseline FBF, intra-arterial coinfusion of vitamin C (Daiichi Pharmaceutical Co, Ltd) at 24 mg/min or vehicle (isotonic saline) was started and continued throughout the experiment. Changes in the FBF response to ACh were measured before and after lipid/heparin infusion.

Protocol 4: Effect of Elevated FFAs on ACh-Induced Vasodilation During Coinfusion With N⁶-Monomethyl-L-Arginine

Five of 10 subjects participated in this study. After the measurement of baseline FBF, intra-arterial coinfusion with N⁶-monomethyl-L-arginine (L-NMMA) (Clinalfa) at 8 μmol/min or vehicle was started and continued throughout the experiment. The reduction in FBF by L-NMMA was restored by the nitric oxide (NO) clamp technique.14 Changes in FBF response to ACh were measured before and after lipid/heparin infusion.

FFB Data Analysis

All FFB data were obtained with a Mac Laboratory 5 chart recorder (AD Instruments). The effect of each drug was expressed as the percentage change from the baseline FBF ratio, which was calculated as the FBF of the infused arm divided by that of the noninfused arm.14

Statistical Analysis

Data are presented as the mean±SD unless otherwise indicated. P<0.05 was considered to indicate statistical significance. The dose-response curves for ACh and SNP were compared before and after lipid/heparin infusion by repeated-measures ANOVA. These analyses were performed with Stat View J-5.0 software (SAS Institute Inc).

Results

Protocol 1: Effect of Elevated FFAs on Endothelium-Dependent Vasodilation With or Without RAS Inhibition

Table 2 shows baseline blood pressure, heart rate, and FBF on each day before and after lipid/heparin infusion. Table 3 shows plasma FFA levels on each study day before and after

<table>
<thead>
<tr>
<th>TABLE 1. Characteristics of Subjects (n=10)</th>
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<tbody>
<tr>
<td>Age, y</td>
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<tr>
<td>Body mass index, kg/m²</td>
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<td>Systolic blood pressure, mm Hg</td>
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<td>Free fat acid, mEQ/L</td>
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<td>Glucose, mg/dL</td>
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Values are mean±SD.

<table>
<thead>
<tr>
<th>TABLE 2. Baseline Blood Pressure, Heart Rate, and Absolute FBF of Before and After Lipid/Heparin Infusion on Each Day</th>
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<tr>
<td>FFA + placebo</td>
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<tr>
<td>FFA + losartan</td>
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<tr>
<td>FFA + perindopril</td>
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Values are mean±SD. BP indicates blood pressure; HR, heart rate.
lipid/heparin infusion. The plasma FFA concentrations either before or after lipid infusion were not statistically different among the 3 study days. Lipid/heparin infusion increased plasma FFA levels by 5- and 7-fold of that of basal levels in the range that has been observed in obese and/or insulin-resistant patients.17,18

A single dose of either losartan or perindopril did not significantly affect baseline blood pressure, heart rate, or FBF. There were no differences between before and after lipid/heparin infusion in these parameters (Table 2). The increase in FFAs after lipid/heparin infusion caused a significant reduction in the FBF response to ACh by 37.7% (P=0.0096; Figure 1a). A single dose of either losartan or perindopril completely prevented the FFA-induced decrease in endothelium-dependent vasodilation (Figure 1b and 1c).

Protocol 2: Effect of Elevated FFAs on Endothelium-Independent Vasodilation With or Without RAS Inhibition
Lipid/heparin infusion did not affect the response to SNP after either placebo or losartan (data not shown).

Protocol 3: Effect of Vitamin C on FFA-Induced Endothelial Dysfunction
The increase in FFAs did not have any influence on ACh-induced vasodilation when vitamin C was intra-arterially coinfused (Figure 2b).

Protocol 4: Effect of Elevated FFAs on ACh-Induced Vasodilation With Coinfusion of L-NMMA
The increase in FFAs did not alter ACh-induced vasodilation when L-NMMA was coinfused (Figure 3b).

Discussion
We have demonstrated that RAS inhibition completely prevented the response to ACh after it was impaired by elevation of FFAs by systemic infusion of lipid/heparin. These results suggest that an elevation of FFAs activates the RAS, which in turn causes endothelial dysfunction. An increase in FFAs did not affect the response to ACh when NO synthase was inhibited or when vitamin C was coinfused, suggesting that

\[
\text{Protocol 1: Effect of Elevated FFAs on Endothelial Dysfunction}
\]

The increase in FFAs did not have any influence on ACh-induced vasodilation when vitamin C was intra-arterially coinfused (Figure 2b).

![Figure 1. Dose-response curves of ACh before (○) and after (●) systemic infusion of FFA with coadministration of placebo (a), losartan (b), or perindopril (c) in 10 subjects. NS indicates not significant.](image)

![Figure 2. Dose-response curves of ACh before (○) and after (●) systemic infusion of FFA with coinfusion of vehicle (a) or vitamin C (b) in 6 subjects. NS indicates not significant.](image)
FFAs specifically reduce the availability of NO, presumably by the enhancement of oxidative stress.

**Validity and Feasibility of the Method**

FFA-induced endothelial dysfunction has been well documented in some,2–4 but not all,19,20 previous studies. Unlike our present results and other reports, Kearney et al19 recently found an enhanced response to ACh after lipid/heparin infusion, which could be explained by changes in systemic hemodynamics that confounded the vascular effect of elevated FFAs on the response to ACh. In fact, there was a significant positive correlation between basal FBF and the response to ACh in human forearm vessels.21 Therefore, it is possible that FFA-induced systemic vasodilation subsequently enhanced the response to ACh, independent of an influence on endothelial function, even in the presence of an elevated plasma FFA level. In other words, the response to ACh in their experiment was more strongly influenced by basal FBF than by the effect of FFAs on vascular endothelial cells. In our experiment, however, the local effect of elevated FFAs on endothelial function was clearly demonstrated, because the changes in basal FBF were modest compared with those in the study performed by Kearney et al. In addition, we measured changes in FBF bilaterally to exclude any systemic effect.

Increased FFA levels by lipid infusion are also associated with impaired peripheral and hepatic insulin sensitivity in humans.22 Given that we have previously shown a positive correlation between endothelial function and insulin sensitivity,23 one would claim that endothelial dysfunction by lipid infusion might result secondarily from impaired insulin sensitivity by elevated FFAs. However, should be noted that lipid should be infused for >3 hours to cause insulin resistance in healthy subjects. On the other hand, we showed that lipid infusion for only 1 hour significantly impaired endothelial function. Therefore, it is likely that the elevated FFAs directly caused endothelial function, independent of insulin sensitivity, in our experiment.

**How Do FFAs Activate the RAS?**

A single dose of either an ARB or ACE inhibitor prevented the FFA-induced endothelial dysfunction. These findings strongly suggest that elevation of plasma FFA levels impairs endothelium-dependent vasodilation through activation of the RAS. On the other hand, however, we found in our preliminary study that elevated FFAs was not associated with any changes in plasma circulating RAS components. This discrepancy might be explained by the lack of a correlation between the activities of the plasma RAS and the vascular RAS. Elevated FFAs might specifically activate the RAS in vascular endothelial cells. Thus, we hypothesize that FFAs might have increased the production of Ang II, enhanced the sensitivity of AT1 to agonists, or, like mechanical stress, activated the AT1 receptor directly without involvement of any agonist in human forearm resistance vessels. The third possibility is unlikely, because not only an ARB but also an ACE inhibitor completely prevented the FFA-induced endothelial dysfunction. Recently, Nielsen et al24 reported that Ang II–stimulated forearm vasoconstriction was enhanced in men with visceral obesity and a family history of hypertension and that there was a significant, positive correlation between the plasma palmitate concentration and the vasoconstrictor response to Ang II, which supports our hypothesis.

**How Does RAS Activation Impair Endothelial Function?**

We found that vitamin C could restore the FFA-induced endothelial dysfunction, similar to previous reports,4 and that FFAs did not affect the response to ACh when NO synthase was inhibited by L-NMMA. Therefore, it is tempting to speculate that activation of the RAS by FFAs led to enhanced oxidative stress, which impaired the response to ACh by reducing NO availability. Indeed, there is recent evidence to suggest that Ang II stimulates superoxide anion production by activating NADH/NAD(P)H-dependent membrane oxidase through the AT1 receptor in human vascular endothelial cells.25 Hirooka et al26 have shown that an increase in Ang II impaired the response to ACh in human forearm resistance vessels, which is consistent with our hypothesis.

**Clinical Implications**

One of the features of insulin resistance is a defective insulin-mediated reduction of fatty acid levels, which results in a high plasma FFA level in patients with type 2 diabetes, the metabolic syndrome, and visceral adiposity. An elevation of FFAs could be 1 of the mechanisms that promote insulin resistance and endothelial dysfunction4 and possibly be a therapeutic target under such circumstances. Although our
present results only showed a preventive effect of a single dose of either losartan or perindopril on FFA-induced acute endothelial dysfunction, it can be reasonably hypothesized that RAS inhibition might be a possible therapeutic strategy to improve vascular endothelial function in patients with impaired FFA metabolism. In this context, improvement of endothelial function by either an ARB or ACE inhibitor thereby in type 2 diabetic patients has been consistently demonstrated,11,12 and RAS inhibition for the management of hypertension seems to be more effective in hypertensive patients with type 2 diabetes than in nondiabetic hypertensive patients.9,10

Conclusion
We showed that an elevation of circulating FFA levels by lipid/heparin infusion, which may partly mimic the abnormal lipid profile in patients with insulin resistance, caused endothelial dysfunction via activation of the RAS and presumably the resultant oxidative stress in humans. These results may provide an explanation for the endothelial dysfunction in patients with insulin resistance and for the activated RAS in obese, hypertensive patients who also have endothelial dysfunction. Further investigation is required to show the applicability of our results in patients with high FFA levels and to elucidate the molecular mechanisms of FFA-induced, RAS-mediated endothelial dysfunction.

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Inhibition of the Renin-Angiotensin System Prevents Free Fatty Acid–Induced Acute Endothelial Dysfunction in Humans

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