Free Fatty Acid Causes Leukocyte Activation and Resultant Endothelial Dysfunction Through Enhanced Angiotensin II Production in Mononuclear and Polymorphonuclear Cells

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

Abstract—Release of free fatty acid (FFA) from adipose tissue is implicated in insulin resistance and endothelial dysfunction in patients with visceral fat obesity. We demonstrated previously that increased FFA levels cause endothelial dysfunction that is prevented by inhibition of the renin-angiotensin system (RAS) in humans. However, the mechanisms for FFA-mediated activation of RAS and the resultant endothelial dysfunction were not elucidated. We investigated effects of elevated FFA on activity of circulating and vascular RAS, angiotensin II–forming activity of leukocytes, and leukocyte activation of normotensive subjects. We showed that increased FFA levels significantly enhanced both of RAS components in human mononuclear and polymorphonuclear cells that were cultured without FFA (mean fold increase: 3.5 at 180 minutes; \( P = 0.0016 \)) and polymorphonuclear (2.0; \( P = 0.0012 \)) cells, whereas parameters of the circulating and vascular RAS were not affected. We also showed that FFA caused angiotensin II– dependent leukocyte activation, which impaired endothelial function partly via increased myeloperoxidase release and presumably enhanced adhesion of leukocytes. We propose that the enhanced production of angiotensin II by FFA in mononuclear and polymorphonuclear cells causes activation of leukocytes that consequently impairs endothelial function. RAS in leukocytes may regulate the leukocyte-vasculature interaction as the mobile RAS in humans. (Hypertension. 2010;56:136-142.)

Key Words: FFA | angiotensin | leukocyte | endothelial function

The levels of circulating free fatty acid (FFA), mainly originating from lipolysis in adipose tissue, are increased in patients with metabolic syndrome and type 2 diabetes mellitus,\(^1\)\(^-\)\(^3\) reflecting resistance to the antilipolytic action of insulin. Increased plasma FFA concentrations cause endothelial dysfunction,\(^4\) insulin resistance,\(^5\) and endothelial apoptosis.\(^6\) These observations, together with results from epidemiological studies,\(^7,8\) suggest that FFA is involved in atherosclerosis in subjects with insulin resistance. Recently, we have found that FFA-induced endothelial dysfunction is prevented by the inhibition of the renin-angiotensin (Ang) system (RAS) in humans,\(^9\) suggesting that RAS activation by FFA may predominantly contribute to FFA-induced endothelial dysfunction. This hypothesis appears plausible because of the close relationship between obesity and RAS activity in humans.\(^10,11\) In addition, RAS activation is also associated with enhanced oxidative stress,\(^12\) which is an intermediary mechanism by which FFA adversely alters vascular function.\(^13\) However, although the proatherogenic action of excessive Ang II has been well documented, there is little information regarding the mechanism of RAS activation in individuals with obesity. Indeed, only a few studies have investigated the effects of elevated FFA on RAS activity.\(^14\) The aim of the present study was to investigate effects of elevated FFA on RAS and to elucidate mechanisms for FFA-induced endothelial dysfunction in humans. We also investigated the interaction between FFA and leukocytes, because FFA is involved in leukocyte activation through protein kinase C redistribution\(^15\) and because leukocytes are involved in the development of atherosclerosis. We demonstrate here that elevated FFA enhances Ang II production in mononuclear and polymorphonuclear cells without any effect on the circulating RAS and causes Ang-dependent leukocyte activation, which leads to endothelial dysfunction.

Methods

Subjects

The study subjects were 49 healthy men (20 to 36 years old). None of the subjects were taking medications on a regular basis. Subjects had normal results from a routine physical examination and standard
laboratory tests. All of the participants gave written informed consent. The ethics committee of the University of the Ryukyus approved the study protocol.

**Lipid/Heparin Infusion**
Participants fasted overnight and abstained from alcohol or caffeine for ≥12 hour before the study. The participants came to our laboratory at 9:00 AM and received a continuous infusion of an intravenous fat emulsion (Intralipid 20%, Fresenius Kabi AB) at 90 mL/h to increase their serum FFA concentrations. Heparin (Shimizu Pharmaceutical Co., Ltd.) at 0.3 U/kg per minute was coinfused to activate lipoprotein lipase and thereby catalyze the hydrolysis of triglyceride.

**Preparation of Palmitate and Oleate for Ex Vivo Experiments**
Palmitate (Chem Service S-33) and oleate (Wako Pure Chemical Industries 153-01241) solutions were prepared as described elsewhere with minor modifications. In brief, sodium palmitate and oleic acid were dissolved to 50 mmol/L in 0.1 N NaOH 70% ethanol at 70°C. FFA solutions were stored in small aliquots at ~80°C. FFA preparations were thawed and complexed with FFA-free, low-endotoxin BSA (Sigma A8806–5G) before use.

**Measurement of Plasma FFA Concentration**
Plasma FFA concentration was measured before and after 1- and 3-hour lipid/heparin infusions in 8 healthy men. Blood samples for FFAs were placed in tubes containing 50 μL of paraaxon (diethyl p-nitrophenyl phosphate; Sigma Chemical Company) diluted to 0.04% in diethyl ether to prevent ex vivo lipolysis. Samples were centrifuged immediately at 4°C, and the plasma obtained from these tubes was stored at ~80°C until assay.

**Fluorogenic Enzymatic Assay for Ang II–Forming Activity**
Ang II–forming activity in mononuclear and polymorphonuclear cells was measured according to the previously described method with minor alterations. A modified Ang I substrate was developed by the addition of a fluorescent tag, N-methylantranilic acid, at the N terminal and 2,4-dinitrophenyl (Dnp) at the C terminal of Ang I (Peptide Institute, Inc). Ang II–forming enzymes cleave between the Phe and His residues, permitting the release of His-Leu-Dnp, the quenching residue. Then, fluorescence was detected with excitation at 355 nm and emission at 460 nm. The reaction yielded N-methylantranilic acid-Ang II that was measured by using the synthesized N-methylantranilic acid-Ang II as a standard. The final Ang II–forming activity data were represented as the picomole yield of N-methylantranilic acid-Ang II per minute per milligram of protein. The intra-assay and interassay coefficients (n=12 each) were 3.6% and 5.8% for total Ang II–forming activity.

**Assessment of Leukocyte Adhesiveness and Deformability by Ex Vivo Microchannel Capillary Model**
We used a microchannel flow analyzer (HR200, Kowa Co, Ltd) as an ex vivo capillary model to assess adhesiveness and deformability of leukocytes in whole blood. Within 10 minutes after blood collection into a heparinized tube, 0.1 mL of blood was drawn through the narrow microchannels (7854–parallel, 7-μm equivalent diameter, 20-μm long channels) under a constant suction of 20 cm H2O. The microscopic motion images of blood passing through the microditches were monitored and stored on a computer system. In offline analysis, an investigator blinded to treatments randomly selected 5 different still images when 0.08 to 0.10 mL of blood flew out and then counted the number of adherent or plugging leukocytes on the microchannel terrace. Adherent leukocytes were defined as static leukocytes with clear surface borders on still images.

**Study Protocols**

**Effect of a Single Dose of Losartan on FFA-Induced Endothelial Dysfunction**
This protocol was performed on 2 days in 8 normotensive men with an interval of ≥7 days between the study days. We measured changes in forearm blood flow (FBF) during intra-arterial infusion of acetylcholine at 50, 100, 200, and 400 nmol/min and sodium nitroprusside at 3, 10, and 30 nmol/min before and after a 3-hour lipid/heparin infusion. This experiment was repeated 4 hours after a single 50-mg oral dose of losartan. Our preliminary experiment showed that there was no order effect of vasodilatation to acetylcholine and that losartan had no effect on endothelial function in healthy men in the absence of lipid infusion.

**Effects of Increased FFA on Circulating RAS**
Lipid with heparin was infused for 3 hours, and blood samples were collected before the infusion and 1 and 3 hours after the commencement of the infusion in 15 healthy men. Plasma renin activity, serum Ang-converting enzyme activity, plasma concentration of Ang I and II, and aldosterone were measured.

**Effects of Increased FFA on Forearm Vascular Responses to Ang I and II**
To assess the effects of increased FFA levels on vascular RAS (ie, Ang-converting enzyme activity in vascular endothelial cells and the sensitivity of vascular smooth muscle cells to Ang II), we measured changes in FBF by strain gauge plethysmography during intra-arterial infusions of Ang I and II at 0.5, 1.0, 5.0, and 10.0 pmol/min with or without systemic lipid/heparin infusion in 10 normotensive men.

**Effects of Increased FFA on Ang II–Forming Activity**
This protocol was performed on 2 days with an interval of ≥7 days between them. Fifteen normotensive subjects received a 3-hour infusion of lipid/heparin or saline/heparin as a control at the same rate in an open-label, randomized, crossover design. Blood samples for the measurement of Ang II–forming activity in mononuclear and polymorphonuclear cells were collected in tubes containing EDTA-2Na before and 60 and 180 minutes after the start of the infusion. Ang II–forming activity in leukocytes was also measured after incubation of fresh whole blood drawn from 5 healthy men with 0.2 or 0.4 mmol/L of oleate/palmitate mixture (1:1 ratio) or vehicle (2% BSA) at 37°C for 30 minutes.

**Effects of Increased Serum FFA on Leukocyte Activity**
This protocol was performed on 3 days in 8 normotensive men with ≥7 days in a washout period between study days. First, blood samples were collected to assess leukocyte activity using the microchannel flow analyzer and determining the plasma levels of myeloperoxidase (MPO) before and 60 and 120 minutes after starting a systemic infusion of saline/heparin (study day 1) and lipid/heparin (study day 2 or 3). Immediately after blood sampling at 120 minutes, bolus heparin (70 U/kg of body weight) was injected to release vessel wall–immobilized MPO into the circulating blood. Each subject received 160-mg oral doses of valsartan (Novartis Pharmaceutical Co, Ltd) or placebo at 28 and 4 hours before the experiment in a double-blind crossover design on study day 2 or 3. The order of the pretreatment was randomized. As a parameter of newly released MPO from activated leukocytes by FFA provocation, we calculate δMPO (serum MPO after bolus injection of heparin during FFA provocation)–(serum MPO after bolus injection of heparin during normal saline infusion). Leukocyte adhesiveness was also assessed after incubation of fresh whole blood from 5 healthy men with 0.2 or 0.4 mmol/L of oleate/palmitate mixture (1:1 ratio) or vehicle (2% BSA) at 37°C for 30 minutes. This experiment was repeated after the oral administration of valsartan in the same subjects.

**Effects of Increased Serum FFA and a Bolus Injection of Heparin on FBF in Response to Acetylcholine**
Eight healthy men participated in this protocol, which was performed on 3 days with an interval of ≥7 days among them. Endothelial
function was evaluated by the vasodilation to intra-arterial infusion of acetylcholine after a 1-hour systemic infusion of saline/heparin as a control (day 1) and of lipid/heparin followed by bolus heparin or vehicle (day 2 or 3). We initiated the FBF measurements 15 minutes after bolus heparin or vehicle. The investigators who assessed endothelial function were blinded to treatments.

**Statistical Analysis**

Data are presented as the mean±SD unless otherwise indicated. Probability values <0.05 were considered to be statistically significant. Comparisons of the dose-response curves of acetylcholine, Ang I, and Ang II were made by repeated measure of ANOVA. Comparison of each parameter of the circulating RAS before and after lipid/heparin infusion and comparison of Ang II–forming activity after the lipid infusion with that after the control infusion were also made by repeated measure of ANOVA. The number of adhesive leukocytes and plasma concentration of MPO were analyzed by ANOVA with a post hoc multiple comparison test.

**Results**

Plasma FFA concentration before and after 1-hour and 3-hour lipid/heparin infusions were 0.54±0.23, 1.56±0.30, and 1.97±0.55 milliequivalents/L (n=8), respectively.

**Role of Ang II in FFA-Induced Endothelial Dysfunction**

As we reported previously,10 increased FFA levels significantly attenuated vasodilatation to acetylcholine (P<0.0001, ANOVA; Figure 1). This FFA-induced endothelial dysfunction was prevented by a single 50-mg dose of losartan (Figure 1). Lipid/heparin infusion did not significantly affect blood pressure and baseline FBF. Elevated FFA did not affect the FBF response to sodium nitroprusside either with or without losartan (data are not shown).

![Figure 1. Dose-response curves of intra-arterial infusion of acetylcholine after saline/heparin infusion (□) and lipid/heparin infusion (■) with (left) and without (right) a single dose of losartan (P<0.0001).](image1)

![Figure 2. Ang II–forming activity in mononuclear (left) and polymorphonuclear (right) cells after a 180-minute lipid/heparin infusion (■) and the activity after the saline/heparin infusion (□); P=0.0016 and P=0.0012; A). Ang II–forming activity in mononuclear (left) and polymorphonuclear (right) cells after the incubation with 0.2 or 0.4 mmol/L of oleate/palmitate mixture (1:1 ratio) or vehicle (2% BSA) at 37°C for 30 minutes (B; n=5; *P<0.05 vs vehicle).](image2)
Increased FFA Levels Do Not Affect the Activity of Circulating RAS

There was no significant effect of increased FFA levels on any component of the circulating RAS (please see Table S1, available in the online Data Supplement at http://hyper.ahajournals.org).

Effect of FFA on Vascular Response to Ang I and II

Increased FFA levels did not significantly affect the dose-response curves of Ang I or II (Figure S1).

Effect of FFA on Ang II–Forming Activity in Mononuclear and Polymorphonuclear Cells

Increased FFA levels significantly enhanced the total Ang II–forming activity at each time point in mononuclear (P=0.0016) and polymorphonuclear (P=0.0012) cells, whereas the infusion of saline/heparin had no effect (Figure 2A). The incubation of whole blood with FFA ex vivo significantly enhanced the Ang II–forming activity in both cells (Figure 2B).

Effect of FFA Levels on Leukocyte Activation and the Role of RAS

The lipid/heparin infusion significantly increased the number of adherent or plugging leukocytes (Figure 3A and B). Pretreatment with valsartan significantly inhibited (P<0.05) the FFA-induced leukocyte activation at 120 minutes (Figure 3B). The number of adherent or plugging leukocytes increased in a dose-dependent manner after a 30-minute incubation with the oleate/palmitate mixture (Figure 3C). Pretreatment with valsartan significantly inhibited (P<0.05) leukocyte activation by the oleate/palmitate mixture (Figure 3C). MPO concentration after the FFA provocation and the bolus heparin was significantly higher than that after the control infusion and the bolus heparin (P<0.05 by ANOVA), and the δ serum MPO was significantly decreased by pretreatment with valsartan (Figure 3D).

Effects of Liberation of MPO From Endothelial Cells by the Bolus Heparin on Endothelial Function

The FFA-induced endothelial dysfunction was restored by the bolus heparin injection, which was deemed to liberate MPO from endothelial cells (Figure 4).
Forearm Blood Flow (ml/min/100ml)

![Dose-response curves of acetylcholine after a 1-hour intravenous infusion of NS/heparin (△), of lipid/heparin followed by bolus heparin (●), and after bolus saline (○; *P<0.05).](http://hyper.ahajournals.org/)

**Discussion**

FFA released from expanded adipose tissue is a key adipokine in the facilitation of endothelial dysfunction and insulin resistance in individuals with visceral fat obesity. However, the mechanism by which FFA contributes to endothelial dysfunction and subsequent atherosclerosis in humans is unclear. Here, we show that FFA-induced enhancement of Ang II production in mononuclear and polymorphonuclear cells is a key mechanism for leukocyte activation associated with high FFA concentration, which leads to endothelial dysfunction, in part through the immobilized MPO on endothelium and oxidative stress, although the possibility of a direct effect of FFA on endothelial cell NO synthase cannot be excluded. These results suggest that FFA activates RAS in leukocytes, and the activated RAS modulates the dysfunctional interaction between endothelium and leukocytes, which is believed to be a crucial early step in the development of atherosclerosis.

We demonstrated that increased FFA levels significantly shift the dose-response curve of acetylcholine toward the right without any changes in that of the sodium nitroprusside and that an Ang II type 1 blocker prevented such vascular dysfunction. Our results strongly suggest that activated RAS predominantly contributes to FFA-induced endothelial dysfunction; however, the specific RAS systems that are involved must be identified. The experiments in the present study have partly identified these RAS systems.

First, we investigated the effects of FFA on circulating RAS. Previous evidence has suggested an association between obesity and activated circulating RAS in humans. Given the evidence for abnormal FFA metabolism in visceral fat obesity, it can be assumed that long-term exposure to high FFA levels might affect the circulating RAS activity. Short-term elevation of FFA for a few hours, however, did not affect the activity of circulating RAS in the present study. Even a longer duration of lipid infusion may not have affected the results, because Umpierrez et al. have found no significant increment in plasma renin activity and aldosterone concentration after a 48-hour lipid/heparin infusion. These results suggest that the FFA-induced endothelial dysfunction might not be mediated by the activation of circulating RAS.

We then hypothesized that vascular RAS activity would be activated and modulate vascular function. Indeed, Nielsen et al. showed a significant correlation between the vascular response to Ang II and the plasma palmitate concentration. In terms of Ang II–forming activity from Ang I, Barton et al. showed the activation of renal Ang-converting enzyme activity in obese animals. Increased FFA levels, however, did not alter the FBF response to either Ang I or II in the present study. This result suggests that acutely increased FFA levels do not enhance Ang II production from Ang I by Ang-converting enzyme in vascular endothelial cells and in the circulation. The lack of an enhanced response to Ang II may also indicate that acute short-term increases in FFA levels do not enhance the sensitivity of the Ang II type 1 receptor in vascular smooth muscle cells. However, the assessment of the effect of FFA on the sensitivity of the vasculature to Ang II seems complex because it has been suggested that some FFAs inhibit the binding of Ang II to its receptor.

Another local RAS that is relevant to the pathophysiology of endothelial dysfunction is the RAS in leukocytes. We showed that enzymes responsible for Ang II production were functionally present in human mononuclear and polymorphonuclear cells and significantly enhanced by increased FFA levels. Because human leukocytes contain all components of RAS, enhanced activity of Ang II–forming enzyme is assumed to result in the enhanced production and release of Ang II in the presence of sufficient amounts of substrates. Therefore, one simple and plausible explanation for FFA-induced, RAS-mediated endothelial dysfunction is the enhancement of NADPH oxidase activity and reactive oxygen species production in endothelial cells by Ang II from leukocytes. In fact, we demonstrated previously that vitamin C prevented FFA-induced endothelial dysfunction in humans and that palmitate enhanced reactive oxygen species production and NADPH oxidase expression. However, the enhanced release of Ang II from leukocytes, if any, was not detectable as changes in the plasma concentration of Ang II, the systemic blood pressure, or the forearm vascular tone in the present study, which raises doubts that the increased Ang II production in leukocytes could be functionally relevant to vascular function.

We then assumed that FFA could affect leukocyte functions other than Ang II–forming activity through increased Ang II production. We clearly demonstrated that increased FFA levels significantly promoted the adhesion of leukocytes and enhanced MPO release presumably from neutrophils and monocytes, which both were partially prevented by previous administration of Ang II type 1 blocker. Furthermore, we showed that incubation of an FFA mixture with blood directly caused leukocyte activation and that the previous administration of valsartan significantly attenuated this reaction. These results...
strongly suggest that increased Ang II production in white blood cells is relevant to leukocyte activation and not a paraphenomenon and that valsartan inhibits FFA-induced leukocyte activation via the leukocyte Ang II type 1 receptor. It can also be proposed, therefore, that Ang II has an autocrine/paracrine effect in white blood cells.

In terms of the integration of leukocytes, RAS, and endothelial function, MPO may be one of the key players. We showed that the liberation of MPO from endothelial cells by bolus injection of heparin restored the FFA-induced endothelial dysfunction. These results, together with clear experimental evidence that MPO impairs NO availability in an H2O2-dependent manner,23 suggest that RAS-mediated leukocyte activation by FFA may facilitate endothelial dysfunction partly through the release of MPO and possibly enhanced leukocyte adhesion. It is reasonably assumed that the release of cytokines and reactive oxygen species from activated T lymphocytes, which has been shown to be Ang dependent,30 may also adversely modulate vascular function.31

The extrapolation of our results to patients with visceral fat obesity is not necessarily straightforward. Individuals with visceral fat obesity have chronically elevated FFA levels. However, we studied healthy subjects after an acute (not chronic) elevation of FFA. Further investigations in patients with chronically elevated concentrations of FFA are apparently warranted.

In conclusion, increased FFA levels caused by lipid/heparin infusion in humans, which mimic the lipid profile of individuals with visceral fat obesity and insulin resistance, significantly enhanced Ang II–forming activity in mononuclear and polymorphonuclear cells. This enhancement was implicated in FFA-induced endothelial dysfunction as a mobile RAS presumably through leukocyte activation.

Perspectives

Our present results may provide a better understanding of the underlying mechanisms for the development of atherosclerosis in subjects with visceral fat obesity and in patients with established diabetes mellitus whose FFA levels are assumed to be high. In particular, we propose that leukocyte RAS activation plays a pivotal role in the development of endothelial dysfunction in subjects with high FFA levels as the mobile RAS presumably through leukocyte activation.

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Disclosures

None.

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

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Running title: Endothelial dysfunction by FFA and angiotensin II

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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>
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<th>Variables</th>
<th>Baseline</th>
<th>60min</th>
<th>180min</th>
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<td>Plasma renin activity (mg/ml/hr)</td>
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<td>Plasma aldosterone (pg/ml)</td>
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<td>87.1 ± 26.5</td>
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<td>Serum ACE activity (IU/L)</td>
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<td>Angiotensin I (pmol/min)</td>
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<td>51.1 ± 12.8</td>
<td>57.0 ± 17.7</td>
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<tr>
<td>Angiotensin II (pmol/min)</td>
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<td>Angiotensin II/I ratio</td>
<td>0.37 ± 0.09</td>
<td>0.40 ± 0.13</td>
<td>0.33 ± 0.11</td>
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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.