Lipids Stimulate the Production of 6-keto-prostaglandin F$_{1\alpha}$ in Human Dorsal Hand Veins

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Abstract—Obese hypertensives have increased nonesterified fatty acids (NEFAs) and α-adrenergic vascular reactivity. Raising NEFAs locally with intralipid and heparin augments dorsal hand venoconstrictor responses to phenylephrine, an α$_1$-adrenoceptor agonist. The enhanced venoconstrictor responses were reversed by indomethacin. The findings suggest that raising NEFAs leads to the generation of cyclooxygenase (COX) product(s) that enhance vascular reactivity. To test this notion, 6-keto-PGF$_{1\alpha}$ and TxB$_2$, the stable metabolites of prostaglandin H$_2$ (PGH$_2$); prostacyclin (PGI$_2$); and thromboxane (TxA$_2$), were measured ≈1.5 to 2 cm downstream of a dorsal hand vein infusion of intralipid and heparin (n=10) or saline and heparin (n=5) for 2 hours each. During the third hour, intralipid and heparin (experimental) and saline and heparin (control) were continued, and either saline (control) or indomethacin (intervention) were infused. Intralipid and heparin raised local 6-keto-PGF$_{1\alpha}$ concentrations by 350% to 500% (P<0.005), but saline and heparin did not (P=NS). TxB$_2$ levels did not change significantly with any infusion. Infusion of indomethacin during the third hour of intralipid and heparin lowered plasma 6-keto-PGF$_{1\alpha}$ (P<0.05), whereas infusion of saline with intralipid and heparin did not (P=NS). Oleic and linoleic acids at 100 μmol/L, increased 6-keto-PGF$_{1\alpha}$ in vascular smooth muscle cells (VSMCs) through a protein kinase C and extracellular, signal-regulated kinase independent pathway. However, oleic and linoleic acids increased intracellular Ca$^{2+}$ in VSMCs. The data indicate that NEFAs induce the production of COX products, perhaps via Ca$^{2+}$-dependent activation of phospholipase A$_2$. The COX product(s) may contribute to increased vascular α-adrenergic reactivity among insulin-resistant individuals when NEFAs are elevated. (Hypertension. 2001;38:858-863.)

Key Words: fatty acids ■ prostaglandins ■ muscle, vascular, smooth ■ cyclooxygenase

Obese hypertensives have increased vascular α-adrenergic reactivity and tone as well as elevated non-esterified fatty acids (NEFAs) which are resistant to suppression by insulin.$^{1-3}$ In lean normotensives, raising NEFAs with intralipid and heparin enhances local and systemic sensitivity to phenylephrine, an α$_1$-adrenoceptor agonist.$^{4-6}$ In the hand vein studies, raising NEFAs locally enhanced α$_1$-adrenergic vasoconstriction and endothelium-dependent dilation via an indomethacin-sensitive mechanism, which implicates cyclooxygenase (COX) product(s) in the altered vascular reactivity.$^{6}$

cis-Unsaturated NEFAs, eg, oleic acid, activate a sequential signaling pathway that includes protein kinase C (PKC), reactive oxygen species (ROS), and extracellular signal-regulated kinases (ERKs).$^{7,8}$ ERKs can induce a Ca$^{2+}$-independent activation of phospholipase A$_2$ with hydrolysis of arachidonic acid from complex lipids, leading to the subsequent generation of eicosanoid products. Alternatively, linoleic but not oleic acid can be elongated and desaturated to arachidonic acid.$^{10}$ Arachidonic acid is converted by the COX pathway to the highly labile prostaglandin PGH$_2$. PGH$_2$ can induce vasodilation by direct action at endothelial thromboxane (TxA$_2$) receptors with liberation of NO or via conversion by prostacyclin synthetase to prostacyclin (PGI$_2$), a vasodilator eicosanoid. In vascular smooth muscle cells (VSMCs), PGH$_2$ can promote vasoconstriction by binding to the TxA$_2$ receptors or through conversion to TxB$_2$ by thromboxane synthetase. Based on these observations, the present study was designed to determine if raising NEFAs in dorsal hand veins with an infusion of intralipid and heparin leads to an indomethacin-sensitive, ie, COX-dependent, increase in the stable metabolites of prostacyclin (6-keto-PGF$_{1\alpha}$) and/or thromboxane (TxB$_2$).$^{11}$ Oleic acid induces a mitogenic signaling pathway in VSMCs that includes PKC, reactive oxygen species, and ERK.$^{7}$ As noted, activation of ERK can, in turn, activate phospholipase A$_2$.$^{9}$ Thus, experiments in cultured VSMCs were performed to determine if NEFAs increase 6-keto PGF$_{1\alpha}$ via a PKC and ERK-dependent pathway.

Methods

Subjects
Ten (7 men, 3 women) healthy subjects, 23 to 45 years of age, were recruited through advertisement and were paid. Volunteers under-
went a medical history and physical examination. Participants signed an approved written informed consent.

Hand Vein Protocol
Volunteers were studied on 2 days, beginning at 8:00 AM. A 23-gauge catheter was inserted antegrade into a dorsal vein of each hand, and an infusion of 0.9% saline was begun at 0.3 mL/min. A 21-gauge catheter was placed retrograde with the tip ~2 cm distal to the antegrade catheter in both hands. In all volunteers, 10% intralipid at 0.1 mL/min and heparin 10 U/mL at 0.1 mL/min (IL/H) was infused in the right hand, and the saline was reduced to 0.1 mL/min. In the left hand, half of the subjects received 0.9% saline at 0.1 mL/min and 10 U/mL heparin at 0.1 mL/min, while the other half received 10% intralipid with saline at 0.2 mL/min. After 120 minutes, indomethacin 20 μg/mL at 0.1 mL/min was substituted for saline during the last hour. The saline and heparin and intralipid with/without heparin were continued. On the other study day, everything was the same except the saline infusion was continued during the last hour. The sequence of the 2 study days was randomized. On both days, blood was obtained at baseline and at 60, 90, 120, 150, and 180 minutes for 6-keto-PGF₁α and TₓB₂.

Biochemical Measurements
Plasma NEFAs were measured as described.⁴,⁵

6-Keto-PGF₁α and TₓB₂
Blood was obtained and plasma was prepared as described.¹³ The extraction was performed by Powell’s method.¹⁴ The eicosanoids were assayed using an enzyme-linked immunosorbent assay (ELISA) kit (Oxford Biomedical Research). The recoveries of 6-keto-PGF₁α and TₓB₂ were 76% and 75%, respectively.

The assay for 6-keto-PGF₁α in rat aortic VSMCs was performed as described.¹⁵ Cells in 6-well plates were serum starved overnight to induce quiescence. The VSMCs were exposed to 10⁻² M bradykinin or 100 μmol/L concentrations of sodium salts of the 4 different 18-carbon NEFAs (oleic, stearic, elaidic, and linoleic acids)³ for 5, 10, 15, 30, and 60 minutes.⁶

Intracellular Ca²⁺ Response of VSMCs to NEFAs
Intracellular Ca²⁺ response of VSMCs to NEFAs were measured using FLIPR (Fluorometric Imaging Plate Reader System, Molecular Devices Corp.)¹⁷ as described.¹⁸ Following 1-hour incubation, cells were washed 4 times with buffer and exposed to buffer, oleic, stearic, elaidic and linoleic acids at 25 to 200 μmol/L. Buffer was the negative control, and bradykinin and angiotensin II at 10⁻⁸ M were the positive controls.

Statistical Analysis
Data are presented as mean±SEM. Analyses were performed with SPSS 6.0. The effects of intralipid and heparin, saline and heparin, and intralipid alone on plasma TₓB₂ and 6-keto-PGF₁α were analyzed with 2-factor ANOVA (time, infusion), as the effects of saline or indomethacin during the third hour. Student’s paired t test was used to compare levels of 6-keto-PGF₁α before and after indomethacin infusions at 120 and 180 minutes. The 6-keto-PGF₁α and intracellular calcium measurements in VSMCs were performed in triplicate and analyzed by 1-way ANOVA. Values of P<0.05 were considered statistically significant.

Results
6-keto-PGF₁α in Human Hand Veins
Plasma 6-keto-PGF₁α concentrations in the hand veins were 365±91 pg/mL. The infusion of intralipid and heparin alone for the first 2 hours increased 6-keto-PGF₁α (n=10) from the basal levels, as shown in Figure 1A. Plasma 6-keto-PGF₁α concentrations did not change during the third hour when saline (control) was infused. In contrast, infusion of indomethacin during the third hour of intralipid and heparin reduced plasma 6-keto-PGF₁α, as shown in Figure 1B.

The infusion of saline and heparin (n=5) did not change plasma 6-keto-PGF₁α from the basal levels as shown in Figure 1A and 1B. Neither the infusion of saline (control) nor indomethacin during the third hour of saline and heparin had any effect on plasma 6-keto-PGF₁α (Figure 1A and 1B).

Intralipid alone (n=5) for 2 hours did not significantly increase plasma 6-keto-PGF₁α (not shown). Furthermore, infusions of indomethacin or saline during the third hour of intralipid alone did not alter 6-keto-PGF₁α.

Local plasma levels of TₓB₂ at baseline were 122±41 pg/mL. These values were not significantly changed by the infusions of intralipid and heparin together or intralipid alone. Likewise, TₓB₂ levels were not reduced by the co-infusion of indomethacin during the third hour of the intralipid with or without heparin infusions (data not shown).

6-Keto-PGF₁α in VSMCs
As shown in Figure 2A, the addition of either oleic or linoleic acid at 100 μmol/L to primary cultures of VSMCs led to a 2.5-fold increase in 6-keto-PGF₁α levels from baseline. The increase of 6-keto-PGF₁α, in response to oleic and linoleic acids was significantly greater (P<0.05) than that observed with equimolar concentrations of stearic acid and elaidic acids, which did not change 6-keto-PGF₁α.

To examine the signaling mechanisms responsible for the increase in 6-keto-PGF₁α levels, the VSMCs were preincubated with the direct PKC inhibitor bisindolylmaleimide and the MAP kinase/ERK kinase (MEK) inhibitor PD 098,059 before the addition of oleic acid as described.¹⁹ Blocking PKC and MEK did not affect the production of 6-keto-PGF₁α in VSMCs stimulated with oleic and linoleic acids (Figure 2B).
Calcium Flux in VSMCs Stimulated With NEFAs

The results noted above indicate that the PKC and ERK signaling pathways were probably not mediating the rise in 6-keto-PGF1α. Therefore, additional experiments were performed to determine if oleic and linoleic acids raise intracellular Ca2+ in VSMCs, which could activate phospholipase A2 and lead to the generation of COX products.20,21 As illustrated in Figure 3, the addition of oleic or linoleic acids to VSMCs at 100 μmol/L concentrations was associated with significant increases in intracellular Ca2+ detected by the FLIPPR. In contrast, equimolar concentrations of stearic and elaidic acids had no effect on calcium flux. The peak Ca2+ response was greater and more prolonged with the addition of linoleic acid than with oleic acid.

**Discussion**

There are 3 principle findings in the present study. First, raising plasma NEFAs with a local infusion of intralipid and heparin leads to the generation of 6-keto-PGF1α in human dorsal hand veins. Second, cis-unsaturated NEFAs increase 6-keto-PGF1α production in cultured VSMCs. Third, although cis-unsaturated NEFAs activate a PKC and ERK signaling pathway in VSMCs, this does not explain the increase of 6-keto-PGF1α. Rather, an increase of intracellular Ca2+ in VSMCs stimulated with cis-unsaturated NEFAs is the more likely stimulus to the rise in of 6-keto-PGF1α.

In this study, the local infusion of intralipid and heparin increased levels of 6-keto-PGF1α in dorsal hand veins. We showed that infusion of intralipid and heparin in dorsal hand veins at the same rates used in this protocol doubled oleic acid and tripled linoleic acid concentrations 1.5 to 2 cm downstream.22 The local infusions of intralipid and heparin significantly reduced the amount of phenylephrine, a relatively selective α1-adrenoceptor agonist, required to induce a given level of vеноconstriction.4,6,12,22 Moreover, indomethacin reversed the enhancement of vеноconstrictor responses to phenylephrine of dorsal hand veins in which NEFAs were raised with a local infusion of intralipid and heparin.6 Collectively, these observations suggested that raising NEFAs locally induced the production of a COX product, which enhanced α1-adrenoceptor reactivity. This study confirms the impression that the infusion of intralipid and heparin produces an indomethacin-sensitive increase of 6-keto-PGF1α, the stable metabolite of prostacyclin. The link between the increase of 6-keto-PGF1α when NEFAs are raised locally and enhanced α1-adrenoceptor-mediated constrictor responses of dorsal hand veins is not
immediately evident. In our previous study, raising NEFAs locally enhanced the venoconstrictor response to phenylephrine and the venodilator response to acetylcholine, an endothelium-dependent event. Both the enhanced venodilator response to acetylcholine and the augmented venoconstrictor responses to phenylephrine were reversed by indomethacin, a COX inhibitor. The findings suggested that COX product(s) generated in response to the intralipid and heparin infusion contributed both to enhanced α₁-adrenoceptor–mediated venoconstriction and endothelium-dependent venodilatation.

Based on the findings noted, we hypothesized that a rise of local NEFAs during the infusion of intralipid and heparin led to a COX-dependent increase of the endoperoxides PGH₂ and PGH₃. PGH₂ could enhance venoconstrictor responses via VSMC TxA₂ receptors and venodilatation via endothelial TxA₂ receptors with liberation of NO. The conversion of PGH₂ to PGI₂ via the action of prostacyclin synthetase could also contribute to enhanced venodilator responses to acetylcholine. Unfortunately, PGH₃ is a highly labile product that is very rapidly metabolized to TxB₂ and PGI₂ and subsequently to their respective stable metabolites, TxB₃ and 6-keto-PGF₁α. Therefore, this study was designed to measure local venous changes in TxB₂ and 6-keto-PGF₁α as indicators of the precursor eicosanoids PGH₂, TxA₂, and PGI₂ during the infusion of intralipid and heparin.

Intravenous administration of intralipid and heparin to raise plasma NEFAs locally in dorsal hand veins increased plasma 6-keto-PGF₁α (Figure 1A). The effect of intralipid and heparin to raise 6-keto-PGF₁α was attenuated by co-administration of indomethacin, a COX inhibitor. In these experiments, TxB₂ concentrations were not significantly altered, which is consistent with other data that indicate a paucity of thromboxane synthetase in some vascular beds. Therefore, this study was designed to measure local venous changes in TxB₂ and 6-keto-PGF₁α as indicators of the precursor eicosanoids PGH₂, TxA₂, and PGI₂ during the infusion of intralipid and heparin.

The second new finding of this study is that oleic and linoleic acids, but not elaidic or stearic acids, increase 6-keto-PGF₁α in cultured VSMCs in vitro by a PKC- and ERK-independent mechanism. Although raising NEFAs locally with intralipid and heparin produces an indomethacin-sensitive increase of 6-keto-PGF₁α in dorsal hand veins, the source of that eicosanoid is not established by our study. Platelets contain the enzymes required to synthesize both prostacyclin and thromboxane. However, the stable metabolite of TxA₂, i.e., TxB₂, was not consistently altered, which suggests that platelets were probably not the primary source for the increase of 6-keto-PGF₁α. This observation suggested that the vascular wall may have contributed to the rise of 6-keto-PGF₁α. To begin exploring that possibility, the effects of 4 different 18-carbon NEFAs on 6-keto-PGF₁α production were measured in cultured VSMCs. One hundred μmol/L concentrations of oleic and linoleic acids, 2 cis-unsaturated fatty acids, increased 6-keto-PGF₁α in cultured VSMCs within 5 minutes, and these increases were maintained for at least 1 hour (Figure 3). In contrast, equimolar concentrations of stearic acid, an 18-carbon saturated NEFA, and elaidic acid, an 18-carbon trans-unsaturated NEFA (isomer of oleic acid) did not alter 6-keto-PGF₁α concentrations. These findings suggest that cis-unsaturated fatty acids, eg, oleic and linoleic acids, can increase PGH₂ and PGI₂ production by VSMCs, which could alter vascular reactivity.

There are various ways in which oleic and linoleic acids could enhance eicosanoid production. Among the possibilities, arachidonic acid is essential in the genesis of COX products. Linoleic acid, an essential fatty acid in humans, is the principal source of arachidonic acid, which is formed by the combined actions of microsomal enzymes, including fatty acid elongase and 85- and 86-desaturase. However, oleic and linoleic acids produced approximately equivalent increases of 6-keto-PGF₁α in cultured VSMCs. The response to oleic acid is unlikely to be explained by conversion to arachidonic acid, because oleic acid is not a substrate for enzymes producing arachidonic acid. However, both oleic and linoleic acids can activate a PKC signaling pathway. Stimulation of ERKs can lead to a Ca²⁺-independent activation of phospholipase A₂. This possibility was examined using specific inhibitors of PKC and ERK in VSMCs stimulated with oleic and linoleic acid. The findings suggest that cis-unsaturated NEFAs raise 6-keto-PGF₁α in VSMCs by a PKC and ERK-independent mechanism (Figure 2B).

The third finding of this study is that the increase of 6-keto-PGF₁α in VSMCs stimulated with oleic and linoleic acids may be explained by an increase of intracellular calcium. Because the activation of PKC and ERK did not appear to mediate the rise in 6-keto-PGF₁α, other explanations were sought. The most probable alternative mechanism is Ca²⁺-dependent activation of phospholipase A₂, which is well described in VSMCs. cis- Unsaturated NEFAs have multiple effects on membrane transport, including effects on Ca²⁺ influx. Consequently, the effects of NEFAs on intracellular Ca²⁺ levels in VSMCs were examined. One hundred μmol/L concentrations of oleic and linoleic acids, which increased 6-keto-PGF₁α concentrations, raised intracellular Ca²⁺ (Figure 3). In contrast, equimolar concentrations of stearic and elaidic acids, which did not increase 6-keto-PGF₁α in VSMCs, also did not significantly alter intracellular Ca²⁺. Despite a larger relative increase of intracellular Ca²⁺ in response to linoleic than oleic acid, the rise in 6-keto-PGF₁α was comparable. These results may be explained by the relationship between intracellular Ca²⁺ and phospholipase A₂ activation. This relationship is steep and saturable within the physiological range of intracellular Ca²⁺. Thus, the Ca²⁺ response to oleic acid may be sufficient to elicit full phospholipase A₂ activation. Although additional studies are required to further delineate the signaling mechanism, these data suggest that oleic and linoleic acids can evoke a Ca²⁺-dependent activation of phospholipase A₂ in VSMCs with hydrolysis of arachidonic acid from complex lipids which leads to the generation of COX products.
PGF$_1_{\alpha}$ response to NEFAs. Indomethacin has been associated with inhibition and not activation of phospholipase A$_2$. The indomethacin infusion rate of 2 $\mu$g/min, given a hand vein flow rate of 1 to 2 mL/min, would result in local concentrations of 1 to 2 $\mu$g/mL, ie, $\equiv$ 3 to 5 $\mu$mol/L. These levels are above the IC$_{50}$ of $<1 \mu$mol/L required for inhibition of COX, yet are substantially below the concentration required to inhibit phospholipase.$^{31,32}$ In a previous study, we reported that nitroglycerin-induced venodilator responses were not affected by intralipid and heparin or indomethacin, which suggests that this COX inhibitor did not have a major effect on phosphodiesterase, ie, cGMP degradation.$^6$

Other limitations of the study include the fact that endothelial cells can also generate 6-keto-PGF$_1_{\alpha}$, but this was not addressed. However, we have previously observed that oleic and linoleic acids at 100 $\mu$mol/L concentrations do not raise intracellular calcium concentrations in cultured endothelial cells and inhibit the increase of calcium induced by ATP (G. Mille, G. Lu, unpublished data, 1997). The effects of heparin on 6-keto-PGF$_1_{\alpha}$ levels in cultured vascular smooth muscle cells were also not addressed. Although systemic infusion of heparin can increase 6-keto-PGF$_1_{\alpha}$, this effect of heparin may be mediated through activation of phospholipase A$_2$ in leukocytes. In contrast, heparin suppresses 6-keto-PGF$_1_{\alpha}$ in cultured vascular smooth muscle and endothelial cells.$^{34-36}$ The control infusion of heparin and saline did not increase 6-keto-PGF$_1_{\alpha}$ concentrations in dorsal hand veins (Figure 1), which is consistent with the in vitro data.$^{34-36}$

In summary, raising NEFAs in the dorsal hand veins of normal volunteers led to an indomethacin-sensitive increase in local plasma 6-keto-PGF$_1_{\alpha}$ concentrations. However, Tx$_B_2$ concentrations did not increase suggesting that the vascular wall, and not the platelet, was a probable contributor to the increased 6-keto-PGF$_1_{\alpha}$. Experiments in VSMCs showed that oleic and linoleic acids, but not elaidic and stearic acids, increased 6-keto-PGF$_1_{\alpha}$. Inhibitors of PKC and ERK did not inhibit the NEFA-induced rise of 6-keto-PGF$_1_{\alpha}$, which suggested that ERK-mediated activation of phospholipase A$_2$ was not involved. However, oleic and linoleic acids, but not stearic or elaidic acids, raised intracellular calcium in VSMCs. Thus, Ca$^{2+}$-dependent activation of phospholipase A$_2$ may explain the rise of 6-keto-PGF$_1_{\alpha}$ in VSMCs stimulated with oleic and linoleic acids. These findings, combined with other reports in the literature, raise the possibility that NEFAs, eg, oleic acid, which are elevated and highly resistant to suppression in insulin in obese hypertensive individuals, may increase vascular $\omega$-adrenergic reactivity and tone by altering the production of vasoactive eicosanoids.

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