Free Fatty Acid Impairment of Nitric Oxide Production in Endothelial Cells Is Mediated by IKKβ

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Objective—Free fatty acids (FFA) are commonly elevated in diabetes and obesity and have been shown to impair nitric oxide (NO) production by endothelial cells. However, the signaling pathways responsible for FFA impairment of NO production in endothelial cells have not been characterized. Insulin receptor substrate-1 (IRS-1) regulation is critical for activation of endothelial nitric oxide synthase (eNOS) in response to stimulation by insulin or fluid shear stress.

Methods and Results—We demonstrate that insulin-mediated tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt, eNOS, and NO production are significantly inhibited by treatment of bovine aortic endothelial cells with 100 μmol/L FFA composed of palmitic acid for 3 hours before stimulation with 100 nM insulin. This FFA preparation also increases, in a dose-dependent manner, IKKβ activity, which regulates activation of NF-κB, a transcriptional factor associated with inflammation. Similarly, elevation of other common FFA such as oleic and linoleic acid also induce IKKβ activation and inhibit insulin-mediated eNOS activation. Overexpression of a kinase inactive form of IKKβ blocks the ability of FFA to inhibit insulin-dependent NO production, whereas overexpression of wild-type IKKβ recapitulates the effect of FFA on insulin-dependent NO production.

Conclusions—Elevated levels of common FFA found in human serum activate IKKβ in endothelial cells leading to reduced NO production, and thus may serve to link pathways involved in inflammation and endothelial dysfunction. (Arterioscler Thromb Vasc Biol. 2005;25:989-994.)

Key Words: diabetes • endothelial dysfunction • endothelial nitric oxide synthase • free fatty acids • IKKβ • nitric oxide

Endothelial dysfunction is a hallmark of diabetic vascular disease and can be described as impairment in the generation and function of nitric oxide (NO) as a vasodilator and vascular homeostatic agent. Insulin’s physiological action in the vasculature promotes vasodilation through increased NO production and resultant enhanced blood flow may couple metabolic and hemodynamic homeostasis. Insulin increases NO production in endothelial cells through an IRS-1 and phosphatidylinositol 3-kinase (PI3-kinase)–dependent pathway that results in phosphorylation of endothelial nitric oxide synthase (eNOS) by Akt in a calcium-independent manner.1,2 Similarities have been demonstrated between insulin signaling in endothelial cells and in classic insulin-responsive cells such as skeletal muscle cells, hepatocytes, and adipocytes. Mechanisms of impaired insulin signaling in these better-characterized insulin responsive cells are likely to be relevant to endothelial dysfunction in diabetes that is not well understood.

Metabolic abnormalities found in diabetes and obesity include increases in the circulating levels of cytokines such as tumor necrosis factor-α (TNF-α) and metabolites such as free fatty acids (FFAs), diacylglycerol, and fatty acyl-coenzyme A. Resistance of target tissues to the effects of insulin has been attributed to alteration(s) in cellular responsiveness to insulin, mediated by reduction of insulin stimulated signaling. FFA increases insulin receptor substrate (IRS-1) serine phosphorylation in cultured skeletal myocytes, which subsequently reduces insulin-dependent IRS-1 tyrosine phosphorylation, decreasing the activation of PI3-kinase.3 Similarly, we observed that endothelial cell exposure to TNF-α impairs insulin and fluid shear-dependent NO production, with reduced IRS-1 tyrosine phosphorylation and increased IRS-1 serine phosphorylation.4 These observations led us to test the hypothesis that FFA would impair insulin-dependent tyrosine phosphorylation of IRS-1 with a subsequent decrease in NO production.

Serum glucose and FFA levels have been reduced in type 2 diabetic patients by treatment with high-dose aspirin (7 g/d) for 2 weeks.5 The inhibitory effects of aspirin have been attributed to its direct interaction with IKKβ and possibly JNK,6,7 but not to inhibition of cyclooxygenases. FFA and
TNF-α has also been shown to activate IKKβ in adipocytes and hepatocytes, which can then increase the serine phosphorylation of IRS-1, with subsequent reduction in insulin-dependent tyrosine phosphorylation of IRS-1, PI3-kinase activation, and ultimately glucose transport. Conversely, reduction of IKKβ activity by salicylate inhibition or by decreased IKKβ expression improves insulin sensitivity by decreasing the IKKβ-mediated serine phosphorylation of IRS-1. IKKβ is a serine kinase that controls the activation of NF-κB, a transcription factor associated with inflammation. Recent data suggest that IRS-1 may be directly phosphorylated by IKKβ at serine residues, and that IRS-1 may represent a novel class of substrates for IKKβ. These findings may represent a mechanism by which the inflammatory pathways contribute to impaired insulin signaling.

In this report, we test the hypothesis that FFA could decrease IRS-1 tyrosine phosphorylation and subsequent serine phosphorylation of Akt, eNOS, and NO production in response to insulin through an IKKβ-mediated effect on the IRS-1/Akt/eNOS pathway. For these studies, we used palmitic, oleic, and linoleic acids complexed with BSA because these FFA comprise the majority of FFA found in serum. In support of our hypothesis, we demonstrate that a dominant-negative construct of IKKβ is able to reverse the FFA impairment of insulin-dependent IRS-1 tyrosine phosphorylation, Akt, and eNOS serine phosphorylation and NO production. Overexpression of wild-type IKKβ recapitulates the effects of FFA on signaling via IRS-1/Akt/eNOS. Taken together, these results support the conclusion that IKKβ, an important regulator in the inflammation pathway, also mediates the negative FFA effects on endothelial NO production.

### Experimental Procedures

#### Materials

The monoclonal antibody (H32) to eNOS was purchased from BIOMOL Research Laboratories, Inc (Plymouth Meeting, Mass); anti-phospho (serine-473)-Akt, anti-Akt, anti-IRS-1, anti–phospho (serine 1179)-eNOS polyclonal antibodies, anti–phospho (threonine 495)-eNOS polyclonal antibody, anti–phospho-InsBo, and anti-phosphotyrosine monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, Mass). DAF-2 DA was purchased from Biotum (Beverly, Mass). DAF-2 DA was purchased from Alexis Corp (San Diego, Calif). Palmitic (C 16:0), oleic (C 18:1), and linoleic (C 18:2) fatty acids were obtained from Altech Associates, Inc (Deerfield, Ill). BSA (FFA-free) was purchased from Sigma (St. Louis, Mo). FFA-free were dissolved in 0.1 mol/L NaOH at 70°C and then complexed with 10% BSA at 55°C for 10 minutes. Cells were washed twice with ice-cold phosphate-buffered saline and then fixed on ice with 2% paraformaldehyde (Electron Microscopy Sciences, Ft Washington, Pa) for 3 minutes. Cells were washed twice with cold phosphate-buffered saline and cover slips were mounted onto slides with Gel Mount (Sigma, St. Louis, Mo).

#### Expression Constructs and Retroviral Infection

All constructs were generated using standard molecular biology techniques. Kinase-inactive IKKβ (K44A) was a generous gift from Tulark Inc (South San Francisco, Calif). Retroviral expression plasmids were constructed using the internal ribosomal entry site (IRES) vector pBMN-ires-eGFP (G. P. Nolan, Stanford University, Stanford, Calif). High-titer retrovirus was prepared as previously described. For infection, BAECS were seeded into 6-well plates (1.5×10⁴ cells per well) and incubated in EBM with 10% fetal bovine serum. After 24 hours, the media was replaced with 5 mL viral stock in the presence of 8 μg/mL of polybrene (Sigma) and the 6-well plates were centrifuged at 2400 rpm for 2 hours at 27°C. After a further 8-hour incubation at 27°C, the viral media was then removed and replaced with fresh EBM supplemented with 10% fetal bovine serum. After 24 hours, the BAECS were trypsinized and plated in 60-mm tissue culture plates and experiments were performed 48 hours after plating. Stable overexpression of IKKβ protein in BAECS was confirmed by Western blot analysis.

#### Statistics

Differences between specific group mean values were compared using Bonferroni adjusted t test and considered significant if P<0.05.

#### Results

### Palmitic Acid Impairs Insulin-Mediated Phosphorylation of IRS-1, Akt, eNOS, and NO Production

Excess FFA has been shown to decrease eNOS activity in an endothelial cell culture model, and to also impair insulin-mediated vasodilation and NO production in human studies. However, the mechanism through which FFA impairs NO production in endothelial cells has not been defined. We hypothesized that FFA could inhibit endothelial insulin-dependent tyrosine phosphorylation of IRS-1, insulin-dependent serine phosphorylation of Akt and eNOS, and ultimately insulin-dependent NO production. In these studies, we used palmitic (C16:0) acid complexed with albumin as a...
model for FFA. We chose palmitic acid as the model FFA because it is a major component of dietary saturated fat, represents up to 20% of the total FFA serum concentration, and has been shown to be present in a high percentage of atherosclerotic lesions.

Insulin addition to BAECs rapidly increases IRS-1 tyrosine phosphorylation levels (Figure 1A), but pretreatment with 100 μmol/L FFA for 3 hours inhibits this insulin-dependent increase. No variations were seen in IRS-1 protein levels in the different treatment groups. Although insulin stimulation increases phosphorylation of Akt serine 473 and eNOS serine 1179, FFA pretreatment also impairs this insulin-mediated phosphorylation of Akt (pAkt) and phosphorylation of eNOS (peNOS) in the absence of any change in Akt or eNOS protein levels (Figure 1B and 1C). The FFA impairment of pAkt and peNOS are not equivalent, with the reduction of pAkt being greater. This suggests that another serine kinase may also be important in the phosphorylation of eNOS ser 1179.

To determine whether FFA would phosphorylate eNOS threonine T497 (bovine amino acid), a negative regulatory site of eNOS, FFA-treated lysates were analyzed by Western blot analysis using an anti-phospho (T495) (human amino acid) eNOS antibody. FFA treatment for 3 hours did not result in activation of T497 in BAECs (data not shown).

The 3-fold increase in NO production induced by insulin is also inhibited by >90% by pretreatment with FFA (Figure 1D). To allow comparison of endothelial cell changes in phosphorylation of candidate pathways and NO production under identical conditions (Figure 1), insulin concentrations of 100 nM were used for all experiments. Although alterations in Akt and eNOS phosphorylation can be seen with insulin concentrations as low as 10 nM, changes in NO production as measured by DAF-2 DA fluorescence can only be detected at insulin concentrations of 100 nM. To confirm the specificity of the DAF-2 DA assay, insulin-mediated NO production was also measured using the Nitric Oxide Detection Kit (Assay Design, Inc). This colorimetric-based assay confirmed the NO data obtained using the DAF-2 DA method. These data demonstrate that FFA inhibits insulin-dependent IRS-1 tyrosine phosphorylation with subsequent impairment of insulin-dependent phosphorylation of Akt serine 473, eNOS serine 1179, and NO production.

**Palmitic Acid Dose-Dependently Increases IKKβ Activity in Endothelial Cells and Other FFAs Abundant in Human Serum Also Induce IKKβ Activity**

IKKβ is one of several potential serine kinases that increase the serine phosphorylation of IRS-1 resulting in impairment of insulin-dependent IRS-1 tyrosine phosphorylation. Elevation of FFA in skeletal muscle cells activates IKKβ, leading to increased serine phosphorylation of IRS-1 and inhibition of its tyrosine phosphorylation in response to insulin. We therefore asked whether palmitic acid could alter IKKβ kinase activity in BAECs (Figure 2). Palmitic acid dose-dependently increases IKKβ kinase activity assessed using a phospho-specific IκBα antibody, which detects phosphorylation of serine 32 of IκBα by IKKβ. The FFA-dependent induction of IKKβ activity was also confirmed in an immune-complex kinase activity assay with recombinant IκBα as the substrate (data not shown).

We next determined whether other common FFAs could activate IKKβ. Previous investigators have shown that oleic acid and linoleic acid inhibit eNOS activity. We therefore hypothesized that these FFAs would also induce IKKβ activation. Both oleic acid and linoleic acid (100 μmol/L) treatments for 3 hours increase IKKβ activity, although to a lesser extent than seen with palmitic acid (Figure 2B). Oleic and linoleic acid treatment also inhibit insulin-mediated eNOS activation (data not shown).

**Dominant-Negative IKKβ Reverses FFA Impairment of Insulin-Mediated IRS-1, Akt, and eNOS Phosphorylation and NO Production in Endothelial Cells**

To further elucidate the role of IKKβ in mediating FFA impairment of insulin-dependent NO production, we asked...
whether the expression of a dominant-negative IKKβ protein could block FFA impairment of insulin-dependent IRS-1, Akt, and eNOS phosphorylation. BAECs were transduced with an IRES-based retroviral construct encoding either a kinase inactive form of IKKβ (K44A) with an EGFP reporter in the second cistron or IRES–EGFP alone as a vector control. As shown by Western analysis, a substantial increase in IKKβ protein expression is observed compared with EGFP control (Figure 3A), and overexpression of the kinase inactive IKKβ is sufficient to reverse the FFA impairment (palmitic acid mediated) of insulin-dependent IRS-1 tyrosine phosphorylation when compared with the EGFP controls (Figure 3B). Similarly, when the kinase inactive IKKβ was overexpressed in BAECs, FFA pretreatment did not inhibit insulin-dependent pAkt or peNOS (Figure 3C and 3D), and the attenuation of NO production induced by FFA was prevented (Figure 4).

Wild-Type IKKβ Recapitulates the Effect of FFA on Insulin Signaling and NO Production in Endothelial Cells
To test whether increased IKKβ signaling is sufficient to explain the deleterious effects of FFA on endothelial cell function, we determined if increased IKKβ activity diminishes insulin-mediated activation of pAkt, peNOS, and NO production. This was accomplished by transducing BAECs using an IRES-based retroviral construct encoding wild-type IKKβ. As predicted, overexpression of IKKβ results in nearly complete inhibition of insulin-mediated pAkt, peNOS activation, and NO production in both the control and FFA conditions (Figure 5). Increased signaling via IKKβ is therefore sufficient to mimic the deleterious effects of FFA on pAkt, peNOS, and NO production in endothelial cells.

Discussion
High plasma concentrations of FFA impair endothelial function and are associated with a number of cardiovascular risk factors linked to insulin resistance, including hypertension, dyslipidemia, and abnormal fibrinolysis. In view of recent data in rodents and humans implicating an important role for IKKβ in mediating FFA-induced insulin resistance,4,17 we decided to test the hypothesis that IKKβ could mediate FFA-dependent endothelial dysfunction. Here, we demonstrate that FFA impairment of insulin-mediated NO production is critically dependent on activation of IKKβ, a cellular response that disrupts signaling via the IRS-1/pAkt/peNOS pathway. This conclusion is supported by the observation that inhibition of IKKβ using a dominant-negative construct of IKKβ can reverse or attenuate the FFA-induced inhibition of endothelial NO production. Conversely, overexpression of wild-type IKKβ recapitulates the
effect of FFA to impair insulin-mediated NO production in BAECs. Together, these findings establish that IKKβ signaling is a critical mediator of the negative effects of FFA on NO production by endothelial cells.

Clinical studies have demonstrated a profound effect of FFA on NO production. In normal patient volunteers, the ingestion of a single high-fat meal transiently impairs endothelial function as measured by flow-mediated brachial artery vasodilation.18 Infusion of high doses of intralipid plus heparin into normal volunteers raises circulating FFA concentrations from a starting concentration of 350 μmol/L to a peak of 3800 μmol/L.19 Methacholine-induced vasodilation was reduced by as much as 20%, indicating that elevated FFA levels induce endothelial dysfunction.19 In a separate study, raising FFA levels resulted in impairment of basal and insulin-mediated NO production.14 These human studies suggest that the production of NO is impaired in the presence of high circulating levels of FFA.

Our studies examined 3 of the most abundant FFA in human serum: palmitic, oleic, and linoleic acids. Together they constitute up to 70% of the total FFA and are individually found in concentrations between 10 to 50 μmol/L.15,20 Although the concentrations of palmitic, oleic, and linoleic acids used in these in vitro studies are slightly higher than found in humans, palmitic/BSA did activate IKKβ at concentrations as low as 1 to 10 μmol/L, which support the clinical relevance of these studies.

There are >40 measurable FFA in serum and it is beyond the scope of this study to examine the effect of each FFA. However, it is likely that different FFAs have a differential effect on eNOS function. For example, high doses of eicosapentaenoic (EPA) (C20:5) actually increase insulin-mediated NO production,21 suggesting that certain FFAs could improve endothelial function. Epidemiological studies have shown that increased consumption of (n-3) fatty acids from fish is associated with improved endothelial function,22 suggesting that certain FFAs are beneficial.

The cellular mechanism responsible for FFA impairment of NO production has not been studied in detail. However, previous endothelial cell culture studies have demonstrated that short-term exposure to 100 μmol/L oleic acid (C 18:1) or linoleic (C 18:2) can inhibit eNOS activity through a PKC-independent pathway.13 Long-term exposure to FFA (100 to 300 μmol/L) for 24 hours has been shown to trigger apoptosis in human endothelial cells and also alter total eNOS protein levels.23 In our studies, we chose conditions that would not induce apoptosis because we were primarily interested in determining a mechanism by which FFA might alter eNOS function.

Previous work has demonstrated that the IRS-1/PI3-kinase/pAkt/pE0S pathway is important for endothelial NO production. Transfection with mutated IRS-1, inhibitory PI3-kinase mutants, or dominant-negative Akt abrogate insulin-dependent NO production.2,24 Many of the metabolic abnormalities found in diabetes and obesity, which include elevation of TNF-α and metabolites such as FFAs, have been shown to have negative effects on the IRS-1/PI3-kinase signaling pathway in myocytes and hepatocytes. Similarly, we have shown that endothelial cell exposure to TNF-α...
impairs insulin and fluid shear-dependent NO production. The current studies establish that FFA also impairs NO production through its negative effects on the IRS-1/pAkt/peNOS pathway in much the same way as shown for TNF-α.

It thus is reasonable to hypothesize that other mediators of impaired insulin signaling, such as hyperglycemia or excess angiotensin II, could also impair NO production through alteration of the IRS-1/pAkt/peNOS pathway. Additional experiments demonstrate that FFA pretreatment also results in impaired fluid shear stress-induced Akt and eNOS phosphorylation in BAECs (data not shown). These findings suggest that the effect of FFA on NO production is not dependent on insulin as the agonist. Other studies with vascular endothelial growth factor as an eNOS agonist suggest that eNOS is still functional because FFA pretreatment did not inhibit eNOS phosphorylation by vascular endothelial growth factor (unpublished observation).

In conclusion, the present studies demonstrate that the common FFAs found in serum impair insulin-dependent NO production through downregulation of cell signaling via the IRS-1/pAkt/peNOS pathway. Furthermore, this FFA-induced decrease of NO production is dependent on IKKβ, and IKKβ activation is sufficient to recapitulate the deleterious effects of FFA on endothelial function. Taken together, these results indicate that in addition to its central role in cellular inflammatory responses, increased IKKβ signaling plays a critical role in FFA-induced impairment of NO production in endothelial cells.

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