Epooxyeicosatrienoic acids mediate insulin-mediated augmentation in skeletal muscle perfusion and blood volume

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Shim CY, Kim S, Chadderdon S, Wu M, Qi Y, Xie A, Alkayed NJ, Davidson BP, Lindner JR. Epooxyeicosatrienoic acids mediate insulin-mediated augmentation in skeletal muscle perfusion and blood volume. Am J Physiol Endocrinol Metab 307: E1097–E1104, 2014. First published October 21, 2014; doi:10.1152/ajpendo.00216.2014.—Skeletal muscle microvascular blood flow (MBF) increases in response to physiological hyperinsulinemia. This vascular action of insulin may facilitate glucose uptake. We hypothesized that epoxyeicosatrienoic acids (EETs), a family of arachadonic acid metabolites. EETs are mediators of insulin’s microvascular effects. Contrast-enhanced ultrasound (CEU) was performed to quantify skeletal muscle capillary blood volume (CBV) and MBF in wild-type and obese insulin-resistant (db/db) mice after administration of vehicle or trans-4-[3-adamantan-1-ylureido)cyclohexyloxy]benzoic acid (t-AUCB), an inhibitor of soluble epoxide hydrolase that converts EETs to less active dihydroxyeicosatrienoic acids. Similar studies were performed in rats pretreated with L-NAME. CEU was also performed in rats undergoing a euglycemic hyperinsulinemic clamp, half of which were pretreated with the epoxygenase inhibitor MS-PPOH to inhibit EET synthesis. In both wild-type and db/db mice, intravenous t-AUCB produced an increase in CBV (65–100% increase at 30 min, P < 0.05) and in MBF. In db/db mice, t-AUCB also reduced plasma glucose by ~15%. In rats pretreated with L-NAME, t-AUCB after produced a significant 20% increase in CBV, indicating a component of vascular response independent of nitric oxide (NO) production. Hyperinsulinemic clamp produced a time-dependent increase in MBF (19 ± 36 and 76 ± 49% at 90 min, P = 0.026) that was mediated in part by an increase in CBV. Insulin-mediated changes in both CBV and MBF during the clamp were blocked entirely by MS-PPOH. We conclude that EETs are a mediator of insulin-mediated augmentation in skeletal muscle perfusion and are involved in regulating changes in CBV during hyperinsulinemia.

epoxyeicosatrienoic acids; contrast ultrasound; insulin; muscle blood flow

ACUTE INCREASES IN PLASMA INSULIN within and above the normal physiological range produce a dose-dependent increase limb skeletal muscle blood flow (4, 5, 15, 40). This vascular action of insulin is thought to potentiate delivery of glucose to muscle. Techniques that can quantify not only microvascular blood flow (MBF) but also capillary blood volume (CBV) in vivo have demonstrated that capillary recruitment is an important component of microvascular response to insulin (15, 21, 43). A functional increase in CBV increases effective vascular surface area for not only glucose uptake but possibly also insulin transport into tissue (34). Augmentation of skeletal muscle perfusion in response to either insulin or glucose challenge is blunted in insulin resistance (IR) (10, 13, 14, 44), suggesting that microvascular dysfunction contributes to impaired glucose homeostasis. Insulin-mediated capillary recruitment has been shown to involve production of nitric oxide (NO) (40, 41), thereby establishing a potential link between endothelial IR, which is manifested by reduced phosphorylation of endothelial NO synthase (eNOS), and impaired flow response to insulin (24). However, the contributions of other endothelial-derived vasodilators have not been evaluated in detail and may represent a new therapeutic target for treating IR.

Epoxyeicosatrienoic acids (EETs) are a family of regioisomers that are metabolites of arachadonic acid and are formed by 2C and 2J cytochrome P450 epoxygenases (28). There is evidence that EETs are endothelial-derived hyperpolarizing factors (EDHFs) and produce vasodilation directly through calcium-activated potassium channels or the TRPV4 channel and indirectly through NO production (16, 17, 23, 33). This knowledge has led to the development of inhibitors of soluble epoxide hydrolase (sEH), an enzyme responsible for breakdown of EETs to less active dihydroxyeicosatrienoic acids, for a variety of purposes, including as antihypertensive therapy (12, 20). It has been shown that pharmacological interventions that increase EETs also improve insulin-mediated glucose uptake (22, 29, 38). Whether improved insulin-mediated glucose storage is related to the microvascular effects of EETs is untested. We hypothesized that EETs participate in regulating skeletal muscle perfusion and specifically in insulin-mediated augmentation in MBF. To test these hypotheses, we used contrast-enhanced ultrasound (CEU) perfusion imaging of the skeletal muscle microcirculation after inhibition of sEH and during euglycemic hyperinsulinemia after inhibiting production of EETs.

METHODS

Animals. The study protocol was approved by the Animal Care and Use Committee of the Oregon Health and Science University. We studied 21 male Sprague-Dawley rats weighing 230–270 g (Hilltop Laboratory Animals, Scottdale, PA), 14 wild-type C57Bl/6 mice, and eight insulin-resistant obese db/db mice genetically deficient for the leptin receptor (B6.Cq-m/+Lepr<sup>db/db</sup>). The Jackson Laboratory, Bar Harbor, ME) aged 8–13 wk. Animals were housed with a 12:12-h light-dark cycle and provided with food and water ad libitum. Rats undergoing hyperinsulinemic clamp were studied in the fasting condition.

Animal preparation. Animals were anesthetized with inhaled isoflurane (1.0–1.5% for mice, 1.5–2.0% for rats), and euthermia was maintained by a heating pad and lamp. In mice, a jugular vein was cannulated for administration of microbubbles and drugs, and a 1.4 French micromanometer-tipped catheter (SPR-671; Millar Instu-
ments) was placed in the right carotid artery for blood pressure measurement in all but three mice. For rats undergoing euglycemic hyperinsulinemic clamp, catheters were placed in the carotid artery for blood sampling and in a jugular vein and the right femoral vein for intravenous infusion of microbubbles, glucose, and insulin. An ultrasound flow probe (T106; Transonics) was placed on the exposed right femoral artery. In select animals, the micrometeromocatheter was placed in the left carotid artery for pressure measurement.

**Experimental protocols.** Protocol 1 was designed to characterize changes in perfusion that occur with pharmacological increase in EETs in wild-type and db/db mice. CBV and MFB in the proximal hindlimb skeletal muscle were measured by CEU at baseline and at 15-min intervals for 45 min after administration of the sEH-inhibitor trans-4-[4-(3-adamantyl-1-ylureido)cyclohexyloxy]benzoic acid (t-AUCB; 1 mg/kg iv) (provided as a gift from Dr. Bruce Hammock, University of California Davis) in seven wild-type and eight db/db mice or after administration of vehicle [1% dimethylsulfoxide (DMSO) in saline] in six wild-type mice. The dose of t-AUCB was chosen based on pharmacokinetic optimization studies showing that this dose results in plasma concentrations of >100 nM sustained over 1 h without production of hypotension (26). Heart rate (HR), blood pressure (BP), and venous glucose concentration were measured at each interval.

**Protocol 2** was designed to characterize changes in perfusion that occur with the pharmacological increase in EETs independent of secondary NO production. In 10 rats, CBV and MFB were measured by hindlimb skeletal muscle CEU at baseline and then 30 min after inhibition of NOS with L-N^5^-nitroarginine methyl ester (L-NAME; 3 mg/kg, then 50 μg·kg^{-1}·min^{-1} iv). CEU was then repeated at 30-min intervals for 2 h after administration of the sEH-inhibitor t-AUCB (1 mg/kg iv) or sham controls (n = 5 for each). In an additional four rats, HR and BP were measured continuously after administration of L-NAME and t-AUCB.

**Protocol 3** was designed to test the role of EETs in insulin-mediated capillary recruitment. In 11 rats, baseline CEU and femoral artery blood flow measurement and analysis of arterial blood plasma insulin concentration by radioimmunoassay were performed 1 h after surgical preparation to allow steady-state conditions. A euglycemic hyperinsulinemic clamp was then performed by administration of insulin (10 mU·min^{-1}·kg^{-1}) for 90 min. Arterial glucose was measured at baseline and at 10-min intervals for the first hour of the clamp and then every 15 min for the remaining hour. Dextrose (30%) was infused at a variable rate to maintain glucose concentration at fasting basal levels. Total body glucose utilization was determined from the dextrose infusion rate required to maintain euglycemia and was expressed normalized to body weight. Hindlimb skeletal muscle CEU, femoral blood flow recording, and arterial plasma insulin measurement were performed at 30 and 90 min after the initiation of the hyperinsulinemic clamp. Upon completion of the clamp, hindlimb muscle samples were obtained for measurement of capillary density by immunohistochemistry. In six of the rats undergoing hyperinsulinemic clamp, EET synthesis was inhibited by N-methylsulfonyl-1,2-(2-propynyloxy)-benzenehexanamide (MS-PPOH), which was administered by intraperitoneal placement of an osmotic pump (2001D; Durect, Cupertino, CA) 24 h before the clamp. The pump and concentration were designed for a MS-PPOH release rate of 0.21 mg/h, which was similar to doses used to block beneficial effects of sEH inhibitors on rat muscle perfusion (30).

**Contrast-enhanced ultrasound.** Lipid-shelled decafluorobutane microbubbles were prepared by sonication of a gas-saturated aqueous suspension of distearoylphosphatidylcholine (2 mg/ml) and polyoxyethylene-40-stearate (1 mg/ml). Microbubble concentration and size distribution were measured by electrozone sensing (MultiSizer III; Beckman Coulter, Fullerton, CA). The average diameter of these microbubbles was 1.8–2.0 μm. CEU was performed in the transaxial plane using a linear-array transducer interfaced with an ultrasound imaging system (15L8 transducer, Sequoia 512; Siemens Medical Systems, Mountain View, CA). A contrast-specific multipulse algorithm was used at a transmit frequency of 7 MHz, a mechanical index of 0.18, and a 55-dB dynamic range. Gains were optimized at the beginning of each study to levels that just eliminated background tissue speckle and were kept constant. Blood pool signal (IB) was measured first from the left ventricular cavity at end diastole during an intravenous microbubble infusion rate of 1 × 10^6 min for mice or 1 × 10^7 min for rats. The infusion rate was then increased 10-fold, and the proximal hindlimb adductor muscles (adductor magnus and semimembranosus) were imaged midway between the inguinal fold and the knee. Images were acquired at a frame rate of 2 Hz immediately after a brief high-power (MI 1.9) destructive pulse sequence, and time intensity data were fit to the function $y = A(1 - e^{-t/B})$, where $y$ is intensity at time $t$, $A$ is the plateau intensity, and the rate constant $B$ represents the microvascular flux rate (15, 45). Skeletal muscle CBV was quantified by scaled comparison of plateau intensity to blood pool and calculated by $A/(1.06 × IB × F × C)$, where 1.06 is tissue density (g/cm³), $F$ is the scaling factor that corrected for the different infusion rate for measuring $IB$ to avoid dynamic range saturation, and $C$ is a coefficient to correct for sternal attenuation measured a priori (1.1 for mice, 1.2 for rats) (9). MFB was quantified by the product of CBV and $B$ (15, 45).

**Capillary density.** Immunohistochemistry was performed on fixed, paraffin-embedded sections of hindlimb skeletal muscle. For endothelial cell staining, biotinylated griffonia simplicifolia (Vector Laboratories) was used with diaminobenzidine secondary staining. At least 15 random optical fields for each animal were analyzed, blinded to animal identity. Capillary density was determined in transverse muscle sections. Data were expressed as a percentage of the total muscle area.

**Statistical analysis.** Comparisons were made by repeated-measures ANOVA for time-dependent data. Post hoc comparisons were made with Bonferroni’s corrections for multiple comparisons using either paired Student’s t-tests for differences within a subject or non-paired Student’s t-tests for comparisons between cohorts. Nonnormally distributed data were compared using either Mann-Whitney or Kruskal-Wallis tests. Changes in blood pressure and heart rate after L-NAME were made using a paired Student’s t-test. Correlations were made using linear regression analysis and Pearson product moment. Tests for linear trends for ordinal data were made by a Spearman’s correlation coefficient. Data were considered significant at $P < 0.05$ (two sided).

**RESULTS**

**Functional capillary recruitment mediated by EETs.** In protocol 1, there were no significant changes in either HR or BP after administration of the sEH inhibitor t-AUCB in either wild-type or db/db mice (age 8–13 wk) (Table 1). Baseline venous glucose concentration was on average threefold higher ($P < 0.01$) for db/db compared with wild-type mice (Table 1). In db/db mice, treatment with t-AUCB resulted in a progressive reduction in venous glucose over 45 min ($P = 0.025$ for linear trend). On CEU imaging in wild-type mice, t-AUCB produced a rapid increase in CBV and MFB, whereas perfusion did not change in vehicle-treated controls (Figure 1, A and B). Treatment with t-AUCB had no significant effect on microvascular flux rate ($B$) in wild-type mice. In db/db mice, t-AUCB also produced a significant increase in skeletal muscle CBV and MFB (Fig. 1C). The time to effect tended to be slightly delayed for db/db compared with wild-type mice. In the db/db strain, mouse age was inversely related to baseline MFB (Fig. 1D). However, the peak MFB and CBV achieved after administration of t-AUCB was similar irrespective of age. There was...
also an anticipated linear relation between age and body mass in db/db mice (range 40–55 g; $r^2 = 0.74$, $P = 0.003$).

**Direct vs. NO-mediated effects of EETs.** In sEH studies performed in rats pretreated with L-NAME, hemodynamic measurements indicated that L-NAME produced an increase in systolic and diastolic BP and a decrease in HR (Table 2). Administration of t-AUCB 30 min after L-NAME produced only a mild, gradual decrease in blood pressure that was not statistically significant. On CEU imaging, pretreatment of rats with L-NAME produced a mild reduction in CBV (Fig. 2, top). However, MBF did not change because of a concomitant increase in microvascular flux rate (Fig. 2, bottom). Administration of t-AUCB 30 min after L-NAME produced a significant ($P < 0.05$) increase in CBV that did not occur for L-NAME-treated rats not treated with t-AUCB. However, there were no major changes in MBF between the two groups.

**EETs in insulin-mediated microvascular response.** In protocol 3, there were no significant differences in basal insulin concentration, arterial or venous glucose concentration, femoral artery blood flow, or limb glucose uptake between control rats and those treated with the EET-inhibitor MS-PPOH (Table 3). Euglycemic hyperinsulinemic clamp produced significant increases in plasma insulin concentration in both groups, although there was a trend toward higher insulin concentrations in the MS-PPOH-treated group at both 30 and 90 min after insulin infusion. The increase in femoral artery blood flow with stable arterial and venous glucose concentrations during hyperinsulinemia indicated that limb glucose uptake increased in both groups. The glucose infusion rate required to maintain euglycemia during the clamp was used as an indicator of total body glucose uptake and was similar between groups (Table 3 and Fig. 3). The glucose infusion rate normalized to plasma insulin concentration, which serves as a measure of insulin sensitivity, was less for MS-PPOH-treated mice, but this difference did not reach statistical significance after correction for multiple comparisons.

Examples of CEU images and time intensity data at baseline and after 90 min of euglycemic hyperinsulinemia for a control rat and an MS-PPOH-treated rat are shown in Fig. 4. In the control rat (Fig. 4A), an increase in MBF with insulin is manifested by both an increase in the microvascular flux rate ($\beta$ or rate constant of the exponential function) and an increase in the plateau intensity that when normalized to blood pool represents CBV. For the MS-PPOH-treated rat, insulin did not produce any changes in either the rate constant or plateau intensity. When all animals were analyzed, hyperinsulinemic changes in CBV were no major changes in MBF between the two groups.
Our studies indicate that treatment with sEH inhibitors produces an increase in skeletal muscle CBV and MBF that is in part independent of NO production. We have also demonstrated that EETs play a role in insulin-mediated augmentation of MBF in skeletal muscle.

There is a growing foundation of evidence supporting the notion that insulin’s microvascular effects are permissive for facilitating glucose uptake (5, 15, 40, 42, 43). Animal and human studies have also demonstrated an association between impaired CBV response to insulin and impaired glucose homeostasis, thereby suggesting that impaired vascular responses contribute to IR (13, 14, 39, 44). It has been shown that abnormal capillary responses occur very early in the course of IR in nonhuman primates produced by inactivity and in humans at the stage of obesity and mild to moderate IR (10, 14). These observations have helped to understand how tissue perfusion influences glucose regulation, but they have not yet been translated into new therapies for IR or diabetes mellitus.

Previous studies evaluating the vascular actions of insulin have implicated NO-dependent pathways (11, 35, 40). Insulin at physiological concentrations (100–500 pm) promotes NO production through insulin receptor phosphatidylinositol 3-kinase/Akt signaling of eNOS (32, 42, 47). Skeletal muscle CEU and other nonimaging techniques for assessing the status of the peripheral microcirculation have demonstrated that inhibition of eNOS with l-NAME blunts insulin-mediated augmentation in effective CBV (41, 42). There is also evidence that NO may be exerting an effect through a central nervous system mechanism (6). However, in many studies, inhibition of eNOS does not entirely eliminate insulin-mediated arteriolar vasodilation, suggesting the presence of other mechanisms that can augment glucose uptake through vascular recruitment (6).

In this study, we focused on EETs, which are metabolites of arachadonic acid formed by cytochrome P450 epoxygenases of the 2C and 2J subclasses. These compounds are metabolized to less active dihydroxy eicosanoids by sEH (20, 28, 33). There is strong evidence that, among their varied biochemical effects, EETs are endothelial-derived hyperpolarizing factors and produce vasodilation in many organs, including heart, brain, and skeletal muscle either directly or indirectly through NO (2, 16, 17, 23, 25, 33). Recently, it has been shown that interventions that increase EETs improve insulin sensitivity. In obese and IR hemeoxygenase-deficient mice, all EET isomers were noted to be severely reduced, and inhibition of sEH improved both BP and glucose disposal during insulin challenge (38). In rat and murine models of diet-induced IR, treatment with an sEH inhibitor improved glucose storage after insulin administration or after glucose challenge (1, 22). There have been several other studies confirming that either sEH inhibition or gene-targeted therapies that increase EETs (either CYP-2J3 gene therapy or sEH deletion)
lead to improvements in insulin sensitivity (27, 29, 46). Several explanations have been offered for these effects. EETs increase pancreatic β-cell production of insulin (18, 27), and they have been shown to potentiate insulin’s suppressive actions on hepatic gluconeogenesis (37). However, these observations do not explain why peripheral insulin sensitivity is improved by an increase in plasma EETs.

Our results suggest that EETs mediate insulin-mediated capillary recruitment and augmentation of MBF in skeletal muscle. The sEH inhibitor t-AUCB, which selectively increases EETs (36), was used in doses that produce blood levels that have been demonstrated to produce vasodilation (7) and improve fasting blood glucose levels in diabetic mice (49). Using CEU, we showed that t-AUCB produced increased in muscle CBV and MBF in both wild-type and db/db mice. Rat studies were used to evaluate the effect of t-AUCB in the presence of the eNOS inhibitor L-NAME since hemodynamic effects of L-NAME tend to be more stable in rats than mice and because rats were used for euglycemic hyperinsulinemic clamp studies. In these experiments, t-AUCB still increased CBV after pretreatment with L-NAME. However, MBF was not different between animals treated with L-NAME alone and L-NAME and t-AUCB. When placed in context with the mouse studies, these findings suggest NO-dependent and independent components of the microvascular response to t-AUCB, which is consistent with previous findings that mesenteric arterial dilation to 11,12-EET and 14,15-EET or to sEH inhibitors is only partially inhibited by L-NAME (31, 48). Studies were performed with L-NAME and not in eNOS-deficient mice since these mice have been shown to have a compensatory upregulation of EDHF vasoregulatory response that may have overestimated the vascular response to t-AUCB (19).

Our finding that t-AUCB increased CBV and MBF in obese db/db mice is important since previous studies have suggested that the vasodilatory effects of EETs could be blunted in states of IR (48). Although basal CBV and MBF were substantially lower in db/db than in wild-type mice, the peak CBV and MBF achieved after inhibiting soluble epoxide hydrolase were nearly equal between strains. We chose to study db/db mice over a modest range of age to study a spectrum of mild to moderate IR. There was an inverse relationship between age and either CBV or MBF; however, the peak flow response to t-AUCB was not influenced by age. The increase in muscle perfusion with t-AUCB in db/db mice was also associated with a decreased in plasma glucose, although we cannot necessarily infer that the two were causatively linked.

In our insulin clamp experiments, the selective epoxygenase inhibitor MS-PPOH prevented insulin-mediated increases in CBV and MBF. These data strongly suggest that epoxygenase metabolites that include EETs are involved in insulin-mediated microvascular recruitment in muscle. Although the t-AUCB studies suggested that EETs can mediate flow augmentation, the finding that preinsulin clamp perfusion was not affected by MS-PPOH suggests that EETs may not play a major role in basal control of skeletal muscle perfusion.

There are several limitations of the study that should be mentioned. Although capillary recruitment and MBF augmentation during hyperinsulinemia were prevented by MS-PPOH, measurements of glucose homeostasis were not significantly different between groups. The ratio of glucose infusion to insulin concentration was used as a measurement of IR and was only mildly reduced in the MS-PPOH group. This finding does not necessarily imply that augmentation of muscle perfusion with sEH therapy in IR patients would not be helpful for improving glucose storage, and further studies will be needed to determine whether this is the case. We also have not evaluated the dose response for the sEH inhibitor and instead used concentrations that have been found to have vasodilatory effects. Finally, we did not perform specific assays for EETs and their metabolites because these studies have been performed previously and because of concern that the amount of blood volume required would alter hemodynamics. Finally,
although our resting MBF values in mice and rats were similar to previous studies that used microspheres (3, 8), these flows are substantially higher than in humans, which limits extrapolation to human skeletal muscle.

In conclusion, our data suggest that EETs have direct effects on augmenting skeletal muscle perfusion and CBV and that this effect of EETs is an important contributor to insulin's microvascular effects. These findings are important for understanding how drugs that modulate EET metabolism may influence glucose homeostasis through substrate delivery.

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