Protein Kinase C θ Activation Induces Insulin-Mediated Constriction of Muscle Resistance Arteries

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OBJECTIVE—Protein kinase C (PKC) θ activation is associated with insulin resistance and obesity, but the underlying mechanisms have not been fully elucidated. Impairment of insulin-mediated vasoreactivity in muscle contributes to insulin resistance, but it is unknown whether PKCθ is involved. In this study, we investigated whether PKCθ activation impairs insulin-mediated vasoreactivity and insulin signaling in muscle resistance arteries.

RESEARCH DESIGN AND METHODS—Vasoreactivity of isolated resistance arteries of mouse gracilis muscles to insulin (0.02–20 nmol/l) was studied in a pressure myograph with or without PKCθ activation by palmitic acid (PA) (100 μmol/l).

RESULTS—In the absence of PKCθ activation, insulin did not alter arterial diameter, which was caused by a balance of nitric oxide–dependent vasodilator and endothelin-dependent vasoconstrictor effects. Using three-dimensional microscopy and Western blotting of muscle resistance arteries, we found that PKCθ is abundantly expressed in endothelium of muscle resistance arteries of both mice and humans and is activated by pathophysiological levels of PA, as indicated by phosphorylation at Thr^{538} in mouse resistance arteries. In the presence of PA, insulin induced vasoconstriction (21 ± 6% at 2 nmol/l insulin), which was abolished by pharmacological or genetic inactivation of PKCθ. Analysis of intracellular signaling in muscle resistance arteries showed that PKCθ activation reduced insulin-mediated Akt phosphorylation (Ser^{473}) and increased extracellular signal–related kinase (ERK) 1/2 phosphorylation. Inhibition of PKCθ restored insulin-mediated vasoreactivity and insulin-mediated activation of Akt and ERK1/2 in the presence of PA.

CONCLUSIONS—PKCθ activation induces insulin-mediated vasoconstriction by inhibition of Akt and stimulation of ERK1/2 in muscle resistance arteries. This provides a new mechanism linking PKCθ activation to insulin resistance. ***Diabetes* 57: 706–713, 2008**

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Received for publication 11 June 2007 and accepted in revised form 4 December 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on DOI: 10.2337/db07-0792.

Additional information for this article can be found in an online appendix at http://dx.doi.org/10.2337/db07-0792.

ACh, acetylcholine; ERK, extracellular signal–related kinase; ET-1, endothelin-1; IRS, insulin receptor substrate; L-NA, *N*-nitro-L-arginine; PA, palmitic acid; PKC, protein kinase C.

Obesity is associated with disturbed insulin signaling (1), leading to muscle insulin resistance (i.e., impaired insulin-mediated glucose uptake in muscle) (2). Insulin resistance increases the risk for development of type 2 diabetes and hypertension (3). The impairment of vasoactive responses to insulin in microcirculation (4) has been described to contribute to insulin resistance by reducing appropriate delivery of insulin and glucose to skeletal muscle myocytes. However, the exact mechanism behind impaired vascular insulin responses leading to insulin resistance remains to be elucidated.

Impairment of insulin-mediated vasoreactivity in the muscle microcirculation is characterized by an imbalance between insulin-mediated nitric oxide (NO) and endothelin-1 (ET-1) production. In microcirculation, insulin regulates vasoactive responses by stimulating both vasodilator and vasoconstrictor effects. Insulin has been shown to induce vasodilatation by activation of Akt, which enhances Ser^{1177} phosphorylation and activity of endothelial NO synthase (5). This vasodilator effect regulates nutritive muscle blood flow and, consequently, contributes to insulin-mediated glucose uptake in muscle (6). Vasoconstrictor effects of insulin are critically dependent on the activation of extracellular signal–related kinase (ERK) 1/2, which controls ET-1 release by the endothelium (7–9). Increased ET-1 activity, as observed in insulin-resistant states, has been shown to impair blood flow and glucose uptake (10). In microvessels of insulin-resistant animals, it has been observed that insulin-mediated Akt activation is selectively impaired, whereas ERK1/2 activation is not altered (11).

Protein kinase C (PKC) θ activation impairs insulin signaling and may be responsible for impaired capillary recruitment in muscle, leading to a decrease in glucose uptake. PKCθ, one of the novel Ca^{2+}-independent PKC isoforms (12), can be activated by lipid infusion (13,14), a high-fat diet (15), or direct stimulation with saturated fatty acids (16,17). PKCθ activation, induced by fatty acids, has been shown to impair insulin-mediated glucose uptake in skeletal muscle myocytes and in adipocytes by the inhibition of Akt in in vitro studies (16–18). As outlined above, however, impaired glucose uptake in muscle is also caused by impaired nutritive blood flow, which is critically dependent on activation of Akt (19). It has been reported that fatty acids directly impair insulin-mediated nutritive muscle blood flow and cause insulin resistance (20). Moreover, mice lacking PKCθ are protected from acute fatty acid–induced insulin resistance (21), and PKCθ activity is increased in skeletal muscle from obese diabetic patients (22). However, whether PKCθ activation impairs

TABLE 1
General characteristics of diameter from gracilis arteries of wild-type and PKCθ-KO mice

General characteristics	Diameter (wild type)	Diameter (PKCθ-KO)	*P* value
A. Passive diameter (μm)	127 ± 4	132 ± 4	0.48
B. Basal diameter (μm)	69 ± 4	69 ± 3	0.92
C. Active response (%)*	55 ± 3	49 ± 2	0.29
D. Diameter after ACh (μm)	99 ± 4	97 ± 5	0.86
E. Diameter change after ACh (%)†	57 ± 5	44 ± 5	0.17

Data are means ± SE. Basal diameter was determined after preconstriction with KCl (25 mmol/l). Wild type, $n = 20$; PKCθ = KO, $n = 6$. *Basal arterial tone was calculated by {[(A − B)/A] ∗ 100%}. †Diameter change after ACh was calculated by {[(D − B)/(A − B)] ∗ 100%}.

insulin's vasoactive effects and thereby contributes to the development of insulin resistance is unknown.

We hypothesize that PKCθ activation impairs insulin-mediated vasoreactivity in skeletal muscle resistance arteries by interfering in the insulin signaling pathway. To study the vasoactive effects of insulin and PKCθ activation on resistance arteries, we used resistance arteries of gracilis muscles from wild-type and PKCθ knockout (PKCθ-KO) mice.

RESEARCH DESIGN AND METHODS

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publ. no. 85 to 23, revised 1996). The local ethics committee for animal experiments approved the procedures. Male C57BL6 mice (Harlan, Zeist, the Netherlands) and male PKCθ-KO mice (stock: 005711; The Jackson Laboratories, Bar Harbor, ME) weighing between 25 and 30 g were killed by CO_2 inhalation, and first-order resistance arteries of the gracilis muscle were isolated. PKCθ-KO mice were generated by inactivation of gene-encoding PKCθ by replacing the exon encoding the ATP-binding site of its kinase domain (amino acid residues 396–451) with the neomycin resistance gene (23). PCR was used to confirm the inactive PKCθ in these mice with the following primers: 5′-TTGGTTCTCT TGAACTCTGC-3′, 5′-ACTGCATCTGCGTGTTCGAA-3′, and 5′-TAAGAGTA ATCTTCCAGAGC-3′.

Human skeletal muscle biopsies were kindly provided by Dr. HW Niessen. Participants gave informed consent for participation in the study. The study was undertaken with approval of the local ethics committee and performed in accordance with the Declaration of Helsinki.

Chemicals. MOPS buffer consisted of (in mmol/l) 145 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.2 NaH_2PO_4, 2.0 pyruvate, 0.02 EDTA, 3.0 MOPS (3-[N-morpholino] propanesulfonic acid), 5.5 glucose, and 0.1% BSA, at pH 7.4. Palmitic acid (PA) (10 mmol/l C16:0) was dissolved in 0.1 mol/l NaOH, coupled to 10% BSA, as described by Kim et al. (24), and diluted to a final concentration of 100 μmol/l PA/0.1% BSA in MOPS buffer (pH 7.4). PA, BSA, *N*-nitro-L-arginine [L-NA], papaverine, and acetylcholine (ACh) were obtained from Sigma (St. Louis, MO). The PKCθ inhibitor (Biosource, Camarillo, CA), an isoform-specific pseudosubstrate (H_2N-Leu-His-Gln-Arg-Arg-Gly-Ala-Ile-Lys-Gln-Ala-Lys-Val-His-His-Val-Lys-Cys-NH_2), inhibits the activity of PKCθ by binding to the substrate site in its regulatory C1-domain (12,25–28).

Vasoreactivity experiments. After dissection, the gracilis artery was placed in a pressure myograph and studied at a pressure of 80 mmHg and a temperature of 37°C in MOPS buffer as described (7). The arteries were preconstricted to ~50% of the maximal diameter with 25 mmol/l KCl. Endothelial integrity was determined by measuring vasodilator response to ACh (0.1 μmol/l) before and after experiments. Acute effects of insulin (Novo Nordisk, Alphen a/d Rijn, the Netherlands) on the diameter of the gracilis artery were studied at four concentrations of insulin (0.02, 0.2, 2, and 20 nmol/l), and diameter changes at each concentration were recorded for 30 min (7). The roles of NO and ET-1 in insulin-mediated vasoreactivity were assessed by pretreatment for 30 min with the nonselective ET-1 receptor antagonist (10 μmol/l PD142893; Kordia, Leiden, the Netherlands) or an inhibitor of NO synthase (0.1 mmol/l L-NA) before addition of insulin. To study the interaction between PKCθ and insulin in resistance arteries, artery segments were pretreated with PA (100 μmol/l) for 30 min to activate PKCθ and were thereafter subjected to insulin. To inhibit PKCθ activity, artery segments were either pretreated with PKCθ pseudosubstrate (1 μmol/l PKCθ pseudosubstrate; Biosource) before adding PA and insulin or by the isolation of gracilis artery segments from PKCθ-KO mice.

Western blot experiments. Western blot analysis was performed as described (8). Segments of gracilis arteries from the same mouse (3 mm in length, $n = 4$) were exposed to solvent, insulin, insulin with PA, or insulin with PA and PKCθ pseudosubstrate for 15 min at 37°C. The protein lysates of different arterial segments were stained with a specific primary antibody against phosphorylated Akt (60 kDa), phosphorylated ERK1/2 (44/42 kDa), phosphorylated PKCθ (79 kDa), total Akt, and total ERK1/2 (Cell Signaling Technology, Boston, MA) and were visualized with a chemiluminescence kit (Amersham). Differences in phosphorylated protein were adjusted for differences in the corresponding total protein staining or actin (50 kDa).

Immunohistochemistry. PKCθ was stained in endothelium and smooth muscle of arterial segments and in human arteries in skeletal muscle biopsies using a primary antibody against total PKCθ (1:50; New England Biosource) and a fluorescein isothiocyanate–labeled secondary antibody. DAPI was used as nuclear counterstain (29). Three-dimensional images of arterial segments were obtained using a ZEISS Axiovert 200 Marianas inverted digital imaging microscope workstation using Slidebook software (Slidebook version 4.1; 3I Intelligent Imaging Innovations).

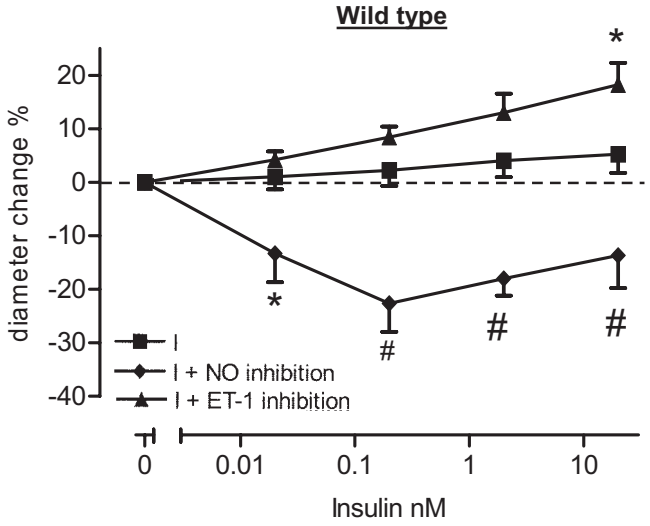
Statistics. Steady-state responses are reported as mean changes in diameter from baseline (in percent) ± SE. The basal diameter was defined as the arteriolar diameter just before addition of the first concentration insulin. Differences between means at each concentration and differences in phosphorylation by Western blot were assessed by one-way ANOVA with Bonferroni post hoc tests. Data were expressed as relative to unstimulated controls (C), assigning a value of 1 to the control. Differences were considered statistically significant if $P < 0.05$.

RESULTS

General characteristics of resistance arteries were similar in the PKCθ-KO and the wild-type mice (Table 1). Preconstriction with KCl (25 mmol/l) induced arterial tone, reducing the diameter of these arteries to 55 ± 2% and 49 ± 2% in wild-type and PKCθ-KO mice, respectively. All arterial segments dilated >25% in response to the endothelium-dependent vasodilator ACh (0.1 μmol/l) at the start of the experiment. PA was used to activate PKCθ in this study.

Insulin exerts NO-dependent vasodilator and ET-1–dependent vasoconstrictor effects. We first characterized, in wild-type mice, the vasoactive effects of insulin in muscle resistance arteries and the role of NO and ET-1 activity therein. Insulin alone had no acute effect on the diameter of these arteries due to a balance of vasoconstrictor and vasodilator effects (Fig. 1). This balance became apparent after inhibition of either insulin's vasodilator effects, by blocking the NO production (L-NA), or insulin's vasoconstrictor effects, by adding the ET-1 receptor blocker PD142893 (Fig. 1). In the absence of insulin, the inhibitors of NO and ET-1 had no significant effect on the arterial diameter (−4.2 ± 3.5% and 1.3 ± 2.1%, respectively). Thus, the vasodilator effects of insulin are NO dependent and the vasoconstrictor effects of insulin are ET-1–dependent in muscle resistance arteries of wild-type mice.

PA induces insulin-mediated vasoconstriction. PA was used to study the effects of activation of PKCθ in resistance arteries of wild-type mice. PA induced a slight dose-dependent vasoconstriction in the absence of insulin,



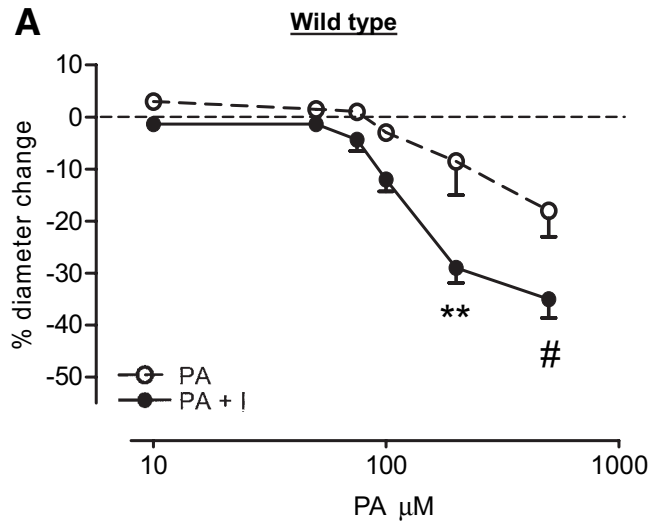


FIG. 1. Effects of insulin on the diameter of mouse resistance arteries. Vasoactive effects of insulin (I) alone (■) and during inhibition of NO (♦) (0.1 mmol/l L-NA) or ET-1 (▲) (10 μmol/l PD142893). Responses are given as percent change from the baseline diameter. *$P < 0.05$; #$P < 0.001$ vs. insulin ($n = 5$).

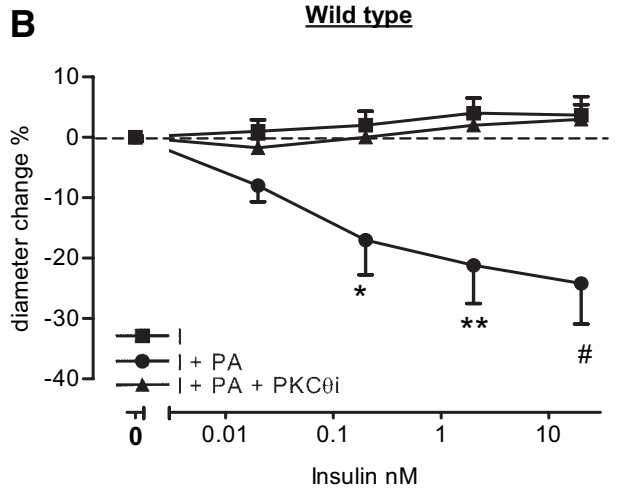


FIG. 2. Effects of PA on insulin responses in mouse resistance arteries. *A*: Effects of physiological concentrations of PA (10–600 μmol/l) on arterial diameter, in the absence (○) and presence (●) of insulin (I) (2 nmol/l). **$P < 0.01$; #$P < 0.001$ ($n = 3$). *B*: Vasoactive effects of insulin alone (■) with PA (●) (100 μmol/l) and with PA and PKCθ inhibition by a pseudosubstrate (▲) (1 μmol/l PKCθ pseudosubstrate). Responses are given as percent change from the baseline diameter. *$P < 0.05$; **$P < 0.01$; #$P < 0.001$ insulin vs. insulin plus PA, insulin plus PA vs. insulin plus PA plus PKCθ pseudosubstrate ($n = 5$).

and this vasoconstriction was enhanced in the presence of insulin (Fig. 2*A*). Physiological levels of PA (10–50 μmol/l) (30) induced no vasoconstriction in the presence of insulin (2 nmol/l), whereas pathophysiological levels of PA (100–600 μmol/l) (31) induced a dose-dependent vasoconstriction to insulin (2 nmol/l) (Fig. 2*A*).

Figure 2*B* shows that in the presence of PA, at a concentration that PA itself had no significant effect (100 μmol/l), insulin induced a dose-dependent vasoconstriction (for example, vasoconstriction of 21 ± 6% at 2 nmol/l insulin). Inhibition of PKCθ with a pseudosubstrate (1 μmol/l) abolished the insulin-mediated vasoconstriction in the presence of PA and restored the balance of insulin's vasodilator and its vasoconstrictor effect. In the absence of PA, the PKCθ inhibitor itself had no significant effect on arterial diameter (8 ± 6%). In contrast, 2 nmol/l insulin induced vasoconstriction (−16 ± 2%) during inhibition of PKCθ (data not shown). This suggests that basal activity of PKCθ is needed for normal insulin-mediated vasoreactivity in the absence of fatty acids.

PKCθ is present in endothelium of resistance arteries of both mice and humans and is activated by PA. To further clarify the effects of PKCθ on insulin responses, we first investigated whether PKCθ is present and whether PKCθ can be activated in muscle resistance arteries of the mouse. The presence of PKCθ was demonstrated by staining PKCθ in mouse arterial segments. Figure 3*A–C* shows that PKCθ is abundantly expressed in endothelial cells but is almost absent in smooth muscle (Fig. 3*D–F*) of mouse gracilis resistance arteries. PKCθ is also present in small arteries of human skeletal muscle. Figure 3*G–K* shows a costaining of PKCθ with the endothelium-specific marker CD31.

The activation of PKCθ by PA in the arterial segments was demonstrated by measuring the phosphorylation of PKCθ at Thr^{538}, which reflects catalytically active PKCθ (32,33). Figure 3*L* shows that pathophysiological levels of PA (100 μmol/l), alone or in combination with physiological levels insulin (2 nmol/l), increased the phosphorylation of PKCθ at Thr^{538} in these resistance arteries. Taken together, these data show that vascular PKCθ is present predominantly in the endothelium and is more than additionally activated by the combination of PA and insulin.

PKCθ-KO mice are protected from insulin-mediated vasoconstriction induced by PA. To further explore whether PKCθ activation impairs insulin responses, we subsequently studied insulin-mediated vasoreactivity in gracilis arteries of mice, in which PKCθ was functionally inactivated (further indicated as PKCθ-KO mice). Resistance arteries of PKCθ-KO mice had normal basal arterial tone and slightly attenuated vasodilator effects to ACh (0.1 μmol/l) (Table 1). Figure 4 shows that insulin induced a vasoconstriction, independent from PA, in PKCθ-KO mice. Furthermore, the addition of either PA (100 μmol/l) or the combination of PA and the PKCθ pseudosubstrate (1 μmol/l) did not induce additional effects.

PKCθ activation inhibits insulin-mediated Akt activation and enhances insulin-mediated ERK1/2 activation in muscle resistance arteries. The vasodilator and vasoconstrictor effects of insulin in muscle resistance

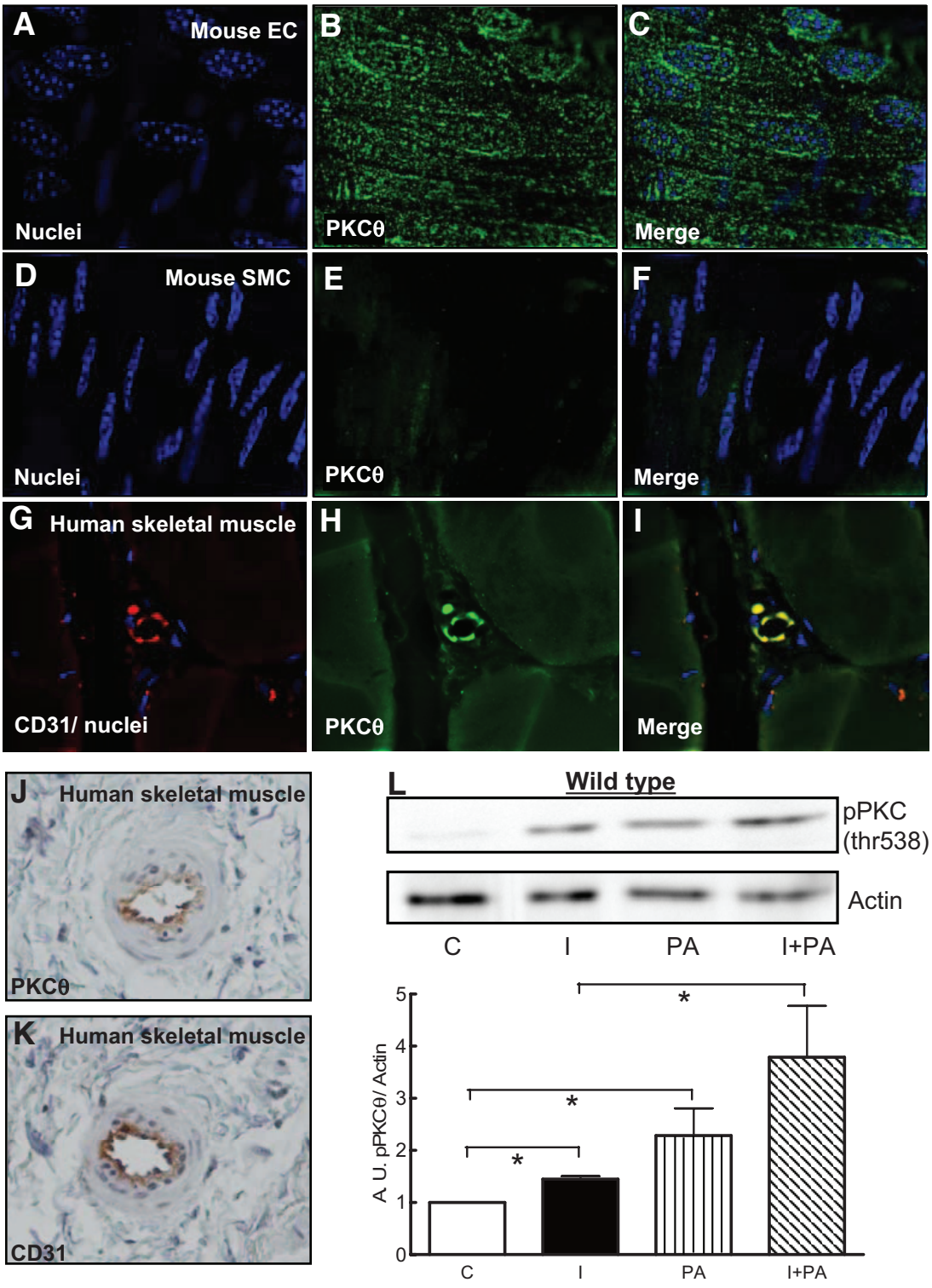


FIG. 3. PKCθ localization in mouse and human resistance arteries and PKCθ activation by PA. *A–F*: Presence of PKCθ in the endothelial cell layer (*A–C*) and not in the smooth muscle cell layer (*D–F*) of mouse resistance arteries with immunohistochemistry at ×63 magnification. EC, endothelial cell layer; SMC, smooth muscle cell layer. *G–K*: Presence of PKCθ in small arteries in human quadriceps muscle with costaining of endothelial marker CD31 with fluorescence (*G–I*) and with bright light (*J* and *K*) at ×40 magnification. *L*: PKCθ activation measured by the phosphorylation of PKCθ at Thr^{538} ($n = 4$). *$P < 0.05$. C, control; I, insulin (2 nmol/l); PA, 100 μmol/l PA. Western blots shown are representative of four independent experiments.

arteries require the activation of Akt and ERK1/2, respectively. To establish which of these signal transduction pathways of insulin were affected by PKCθ activation, Western blot analyses of Akt (Ser^{473}) and ERK1/2 (Thr^{202}/Tyr^{204}) phosphorylation were performed. Gracilis resistance arteries of wild-type and PKCθ-KO mice were exposed to insulin, insulin with PA, and insulin with PA with a PKCθ inhibitor. In wild-type mice, the exposure of gracilis resistance arteries to PA reduced the insulin-mediated Akt activation and caused an increase in insulin-

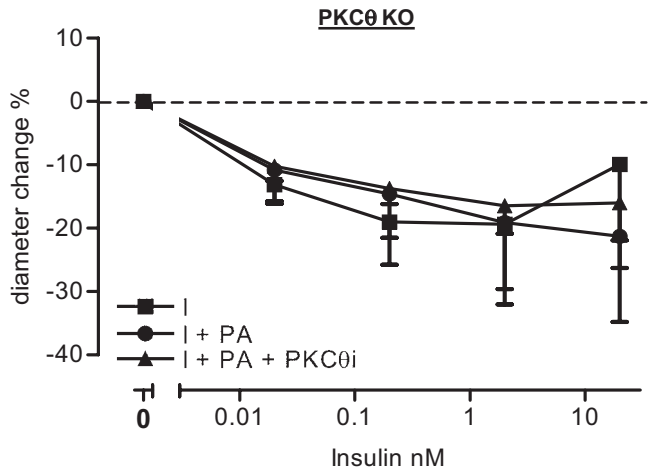


FIG. 4. PKCθ-KO mice induce insulin-mediated vasoconstriction independent from PA in resistance arteries. Vasoactive effects of insulin alone (■) with PA (●) (100 μmol/l) and with PA and PKCθ inhibition (▲) by a pseudosubstrate (1 μmol/l PKCθ pseudosubstrate) in PKCθ-KO mice. Responses are given as percent change (±SE) from the baseline diameter.

mediated ERK1/2 phosphorylation (Fig. 5*A* and *B*). PA alone had no significant effect on the phosphorylation of Akt and ERK1/2 (data not shown). Treatment with a PKCθ pseudosubstrate (1 μmol/l) restored the disrupted insulin-mediated activation of Akt and ERK1/2 in gracilis arteries of wild-type mice in the presence of PA (Fig. 5*A* and *B*). In PKCθ-KO mice, insulin-mediated activation of Akt and ERK1/2 was not affected by either PA (100 μmol/l) or the combination of PA with a PKCθ pseudosubstrate (1 μmol/l) (Fig. 5*C* and *D*). Taken together, these data show that activated PKCθ interferes in insulin signaling in muscle resistance arteries by inhibiting Akt activation and enhancing of ERK1/2 activation and shows that both vasodilator and vasoconstrictor effects of insulin are affected.

Insulin-mediated vasoconstriction during PKCθ activation is ET-1 dependent. PKCθ activation shifts the balance of insulin-mediated vasoreactivity toward vasoconstriction in wild-type mice (Fig. 2*B*), whereas in PKCθ-KO mice insulin also induces vasoconstriction (Fig. 4). To verify whether this vasoconstriction is indeed ET-1 dependent, arteries from wild-type and PKCθ-KO mice

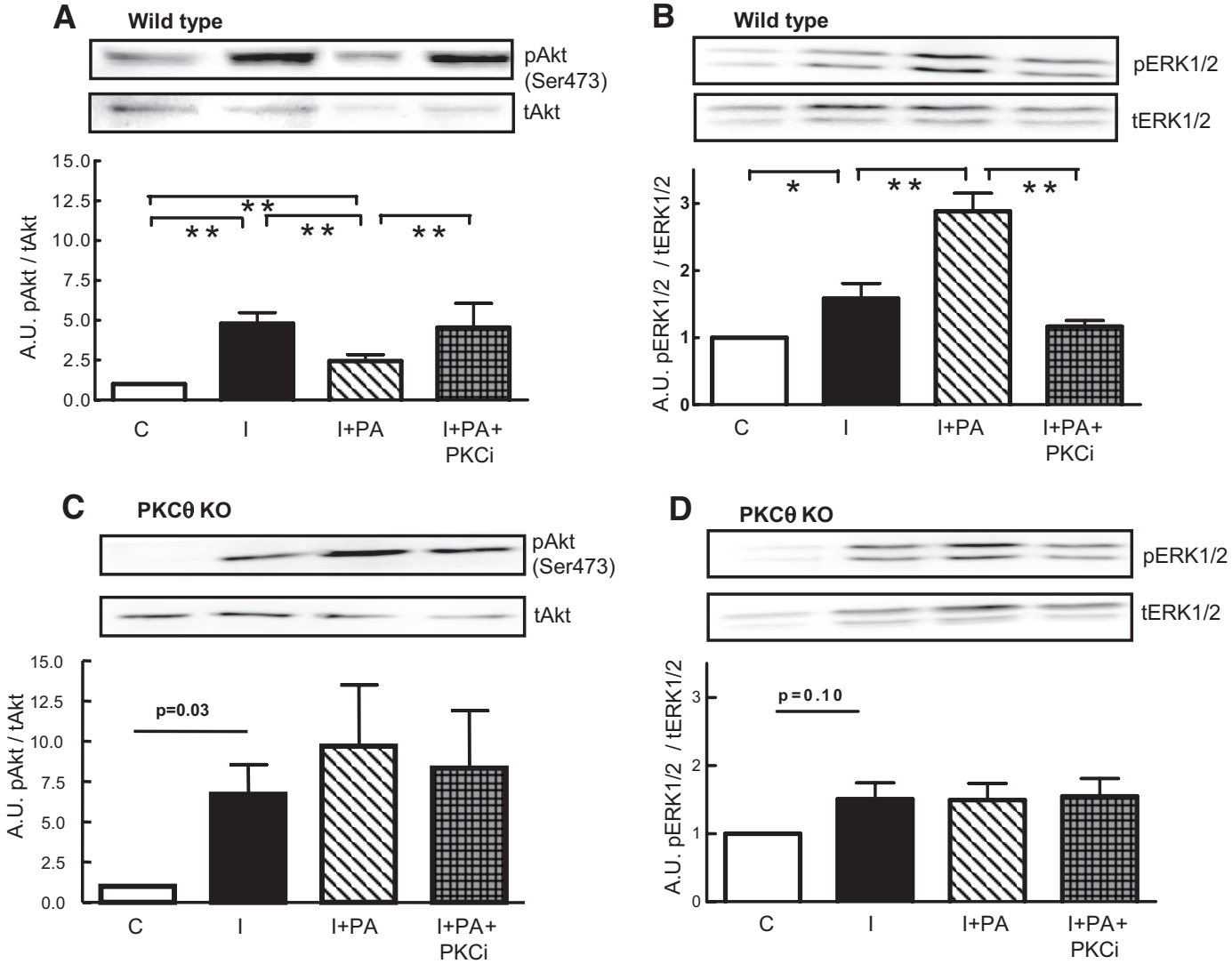
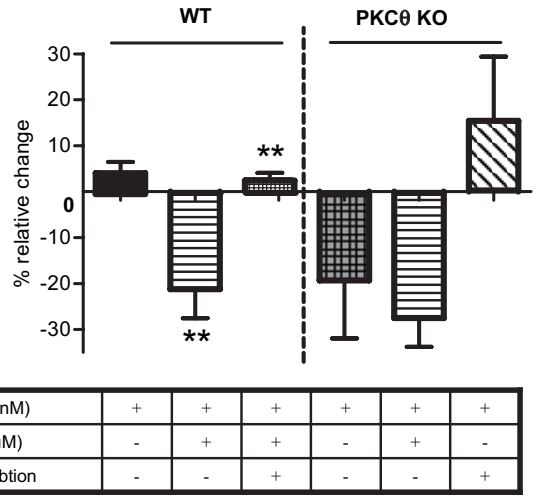


FIG. 5. Effect of PKCθ activation on intracellular signaling of insulin. *A*: Insulin-mediated Akt phosphorylation at Ser^{473} in wild-type mice (n = 5). *B*: Effect of ERK1/2 phosphorylation at Thr^{202}/Tyr^{204} in wild-type mice (n = 5). *C*: Effect of Akt phosphorylation in PKCθ-KO mice (n = 6). *D*: Effect of ERK1/2 phosphorylation in PKCθ-KO mice (n = 5). C, control; I, insulin (2 nmol/l); PA, 100 μmol/l PA; PKCθi, PKCθ pseudosubstrate (1 μmol/l). *P < 0.05; **P < 0.01.



Insulin (2nM)	+	+	+	+	+	+
PA (100μM)	-	+	+	-	+	-
ET-1 inhibtion	-	-	+	-	-	+

FIG. 6. Effect of ET-1 inhibition on insulin-mediated vasoreactivity in wild-type (WT) and PKCθ-KO mice. Insulin-mediated vasoreactivity was studied with or without PA or ET-1 inhibition. Responses are given as percent change from the baseline diameter and bars in the graph correspond to table below. **$P < 0.01$. WT: $n = 5$; PKCθ-KO: $n = 6$ (insulin plus PA) and $n = 2$ (insulin plus ET-1 inhibition).

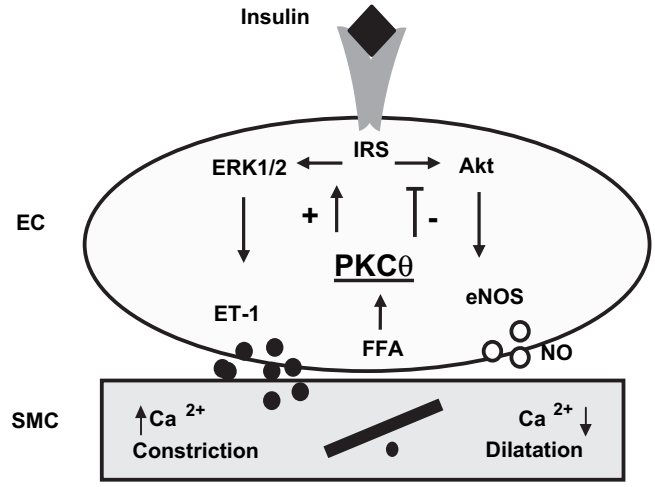


FIG. 7. Schematic overview of the effects of free fatty acids (FFAs) in insulin signaling in gracilis resistance arteries of the mouse. PKCθ activation impairs activation of Akt (*A*) and enhances activation of ERK1/2 (*B*), shifting the balance of insulin-mediated vasoreactivity to vasoconstriction. (*A* and *B* correspond with the numbers mentioned in first paragraph of the discussion.) EC, endothelial cell; SMC, smooth muscle cell.

were pretreated with an ET-1 receptor antagonist (10 μmol/l PD142893). In the presence of PA, ET-1 receptor antagonist abolished the insulin-mediated vasoconstriction induced by PA/PKCθ activation in wild-type mice (Fig. 6). In PKCθ-KO mice, ET-1 receptor antagonist also abolished insulin-mediated vasoconstriction and even caused vasodilation (Fig. 6), indicating that both vasodilator and vasoconstrictor effects of insulin are present.

DISCUSSION

This study demonstrates for the first time that PKCθ is present in the endothelium of muscle resistance arteries of both mice and humans and is activated by physiological levels of insulin and pathophysiological levels of PA. By genetic and pharmacological inhibition of PKCθ activity in mice, we demonstrated that activated PKCθ induces insulin-mediated vasoconstriction by the inhibition of insulin-mediated Akt activation, which results in a reduction of vasodilation (*A*), and by the stimulation of insulin-mediated ERK1/2 activation, resulting in enhanced ET-1–dependent vasoconstriction (*B*).

Insulin alone had no effect on the arterial diameter of muscle resistance arteries due to a balance of vasodilator and vasoconstrictor effects. Previously, others and we (5,8) have shown that insulin's vasodilator and vasoconstrictor effects require the activation of Akt and NO as well as ERK1/2 and ET-1, respectively. Inhibition of NO synthase in our study resulted in insulin-mediated vasoconstriction and inhibition of ET-1 activity resulted in insulin-mediated vasodilation. Our findings are in agreement with studies in rat resistance arteries and in the human forearm (7,34), which have shown that insulin-mediated production of NO and ET-1 and their effects are either balanced or result in net vasodilation. This suggests that the model used for this study is representative for the human microcirculation (34) with respect to studying vasoactive effects of insulin. Figure 7 shows a schematic overview of the main findings of this study in the context of Akt-mediated NO production and ERK1/2-mediated ET-1 release, which have been reported earlier (8,9).

In this study, we used isolated first-order gracilis resistance arteries as a model for skeletal muscle resistance arteries during preconstriction with KCl. All experiments in the present study were performed during preconstriction with KCl. Potassium influences the Ca^{2+} handling by depolarization of the cell membrane of smooth muscle cells in the arterial wall, resulting in vasoconstriction. This simulation of arterial tone is commonly used in other studies on arterial vasoregulation (35–37). It is unlikely that the induced state of tone will bear impact on the results of insulin-mediated vasoreactivity and PKCθ activation because we found that insulin-mediated vasodilation is NO dependent and insulin-mediated vasoconstriction is ET-1 dependent. Another difference between studies on insulin-mediated vasoreactivity in vivo and ex vivo is the presence of shear forces in vivo. As shear is a well-known stimulator of endothelial NO production (38), and NO inhibits insulin's vasoconstrictor effects (7), this may explain the predominance of insulin's vasodilator effects in vivo. Despite this, vasoconstrictor effects of insulin have been demonstrated in a number of in vivo studies (34,39).

An interesting novel observation of our study is that PKCθ is abundantly expressed in the endothelium of muscle resistance arteries of both mouse and humans. PKCθ was, until now, mainly described in skeletal muscle samples (13–15), fibroblasts (17), or cultured skeletal muscle myocytes (16) and T-cells (12). Moreover, PKCθ was encountered in cultured aortic endothelial cells (40) and cultured human umbilical vein endothelial cells (41,42), and small amounts have occasionally been found in homogenates of tracheal (43) and aortic (44) smooth muscle cells of rodents. However, the present study shows, for the first time, the presence and localization of PKCθ in situ in the vascular system of skeletal muscle of both mice and humans and particularly in the endothelial cell layer, in which the insulin-mediated production of NO and endothelin-1 occurs.

PKCθ activation interferes in insulin-mediated vasoreactivity by the inhibition of Akt and stimulation of ERK, resulting in insulin-mediated vasoconstriction (Fig. 2*B*). In the present study, PKCθ activation by PA and insulin in

muscle resistance arteries was determined by the phosphorylation of PKCθ at Thr^{538}. This phosphorylation site is most important in PKCθ activation, and mutations in this site inhibit the catalytical activity of PKCθ (13). We showed that activated PKCθ inhibits insulin-mediated Akt activation, thereby reducing vasodilation. Simultaneously, it stimulates insulin-mediated ERK1/2, thereby enhancing vasoconstrictor effects of insulin. In cultured fibroblasts, PKCθ activation is associated with the inhibition of Akt activation (17) and PKCθ was described as an upstream activator of the mitogen-activated protein kinase/ERK cascade (16,17). Furthermore, PKCθ can directly phosphorylate insulin receptor substrate (IRS)-1 at Ser^{307} (17) and Ser^{1101} (18), and IRS-1 in turn is described to be involved in both the activation of Akt (18,45) as well as the stimulation of the mitogen-activated protein kinase/ERK pathway by insulin (46). It is possible that PKCθ can also influence IRS-1 function in muscle resistance arteries directly, by specific phosphorylation, and thereby influence insulin signaling. This results in reduced Akt activation, which causes reduced vasodilation, and increased ERK1/2 activation, resulting in enhanced vasoconstriction. Thus, PKCθ can be a key player in shifting insulin-mediated vasoreactivity toward vasoconstriction by modulating IRS-1 phosphorylation.

Surprisingly, insulin induced vasoconstriction in wild-type mice during inhibition of PKCθ in the absence of PA and in PKCθ-KO mice (Fig. 4). As ACh-mediated vasodilatation was also slightly attenuated in PKCθ-KO mice (Table 1), this effect may be caused by a positive effect of constitutional PKCθ activity on endothelial NO synthase activity in the absence of fatty acids. This may involve direct phosphorylation of endothelial NO synthase by PKCθ at a serine residue that positively regulates its activation, such as Ser^{114}, Ser^{615}, or Ser^{633} (47). This effect was absent in wild-type resistance arteries in the presence of PA (Fig. 2*B*), suggesting that PA-induced hyperactivity of PKCθ has predominantly unfavorable vasoconstrictive effects. More studies are required to unravel this possible dual role of PKCθ.

Our data support the hypothesis that PKCθ activation in muscle resistance arteries contributes to fatty acid–induced insulin resistance. Clerk et al. (20) described that fatty acid–induced insulin resistance in muscle is partially caused by impairment of insulin-mediated nutritive muscle blood flow (20), which is dependent on activation of phosphatidylinositol 3-kinase/Akt in muscle resistance arteries (19). These authors suggest a possible role for PKCθ in the impairment of insulin signaling in muscle resistance arteries (20). This suggestion is supported by the observation that mice lacking PKCθ are protected from acute fatty acid–induced insulin resistance (21). Indeed, this can be explained with our data, which show that fatty acids activate PKCθ in endothelium of muscle resistance arteries, resulting in impaired insulin-mediated activation of Akt and a shift in insulin-mediated vasoreactivity to vasoconstriction. We propose that fatty acids, in addition to other metabolic effects, induce muscle insulin resistance by activation of PKCθ in endothelium of muscle resistance arteries, which leads to reduction of insulin-mediated nutritive muscle blood flow. The findings in studies on muscle-specific PKCθ-KO are consistent with this hypothesis. Serra et al. (48) have recently shown that specific expression of dominant-negative PKCθ in skeletal muscle myocytes reduces, rather than enhances, insulin sensitivity. Therefore, the insulin-sensitizing effect of blocking PKCθ, shown by Kim et al. (21), cannot be explained by a direct effect on myocellular glucose uptake but must be caused by other mechanisms, such as improved nutritive blood flow.

In summary, PKCθ activation by PA induces insulin-mediated vasoconstriction in muscle resistance arteries, which can explain how fatty acids cause a decrease in nutritive blood flow and impaired glucose uptake in muscle. This provides new mechanistic evidence of how PKCθ activation results in insulin resistance and suggests that PKCθ is a promising novel target for improvement of vascular function in obesity.

ACKNOWLEDGMENTS

This study was supported by the Dutch Diabetes Foundation (grant no. 2003.00.030), the Dutch Kidney foundation (grant no. C03.2046), the Dutch Organization for Scientific Research (grant no. 916.76.179), and the European Vascular Genomics Network (grant no. LSHM-CT-2003-503254).

We thank Ing. Iolente Korstjens for technical support.

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