Elevated Epidermal Growth Factor Receptor Phosphorylation Induces Resistance Artery Dysfunction in Diabetic *db/db* Mice

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OBJECTIVE—We previously showed epidermal growth factor receptor (EGFR) transactivation to be key mechanism in the regulation of resistance artery myogenic tone. Type 2 diabetes is associated with microvascular complications. We hypothesized that elevated EGFR phosphorylation contributes to resistance artery dysfunction in type 2 diabetes.

RESEARCH DESIGN AND METHODS AND RESULTS—Diabetic *db/db* and nondiabetic (control) mice were treated with EGFR inhibitor (AG1478; 10 mg · kg^{-1} · day^{-1}) for 2 weeks. Isolated coronary artery and mesenteric resistance artery (MRA) were mounted in an arteriograph. Pressure-induced myogenic tone was increased in MRA and coronary artery from diabetic mice and normalized by AG1478. Phenylephrine-induced contraction and nitric oxide donor–induced relaxation were similar in all groups. Endothelium-dependent relaxation in response to shear stress and acetylcholine of MRA and coronary artery from diabetic mice was altered and associated with reduced endothelial nitric oxide synthase (eNOS) expression and phosphorylation. Treated diabetic mice with AG1478 improved coronary artery and MRA endothelial function and restored eNOS expression. Immunostaining and Western blot analysis showed increased endothelial and smooth muscle cell EGFR phosphorylation of MRA and coronary artery from diabetic mouse, which was reduced by AG1478. Primary cultured endothelial cells from resistance arteries treated with high glucose for 48 h showed an increase of EGFR phosphorylation associated with eNOS expression and phosphorylation decrease in response to calcium ionophore. Pretreatment of endothelial cells with AG1478 prevented the effect of high glucose.

CONCLUSIONS—This study provides evidence of the role of elevated EGFR phosphorylation in coronary artery and MRA dysfunction in diabetic *db/db* mice. Therefore, EGFR should be a potential target for overcoming diabetic small artery complications. ***Diabetes*** **57:1629–1637, 2008**

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AGE, advanced glycation end product; EEL, external elastic lamina; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; IEL, internal elastic lamina; MMP, metalloproteinase; MRA, mesenteric resistance artery; SMC, smooth muscle cell.

Resistance artery tone is mainly regulated by mechanical factors (pressure and flow) and vasoactive agents (1). In general, flow induces vasodilation endothelium dependently (2). On the other hand, pressure-induced contraction (myogenic tone) is generally endothelium independent and is mediated by direct effect of intraluminal pressure on vascular smooth muscle cells (SMCs) (1). Although the multifactorial effects of type 2 diabetes on the regulatory mechanisms that dictate altered microvessel function are not well understood, it is likely that altered vascular reactivity is involved. Limited studies of the relationship between diabetes and altered vascular responsiveness have been conducted in the microvasculature from diabetic models, and conflicting results have been obtained. For instance, skeletal muscle arterioles of streptozotocin-injected rats exhibit enhanced pressure-induced myogenic responsiveness that is endothelium independent but requires increased activation of L-type Ca^{2+} channels and protein kinase C (3–5). Similarly, Bagi et al. (6) observed an increased basal tone of gracilis muscle arterioles from diabetic mice. On the other hand, small arteries (65–230 μm) from gluteal fat biopsies from patients with type 2 diabetes demonstrated decreased myogenic tone responsiveness (7).

Epidermal growth factor receptor (EGFR) is an 1,186–amino acid glycoprotein containing a single transmembrane domain with intracellular portion harboring the tyrosine kinase domain. Transactivation of EGFR by some G-protein–coupled receptor agonists was originally reported by Daub et al. (8) in 1996. Importantly, we have shown in a previous study the importance of EGFR transactivation by mechanical factors in the development of myogenic tone (9). Although there is a plethora of information concerning the growth-promoting effects of EGFR, its role in the complications of resistance arteries in type 2 diabetes is unknown. Additionally, it has been shown that EGFR is regulated by high glucose through EGFR–*N*-glycosylation (10) and increased mitogenic response to heparin-binding epidermal growth factor (11).

Type 2 diabetes is characterized by a chronic hyperglycemia due to inadequate insulin secretion in the setting of insulin resistance and is often associated with obesity, hypercholesterolemia, and hyperlipidemia (12–14). The morbidity and mortality of diabetes are principally due to the development of both macrovascular and microvascular complications (15–17). Recently, it was reported that downregulation of EGFR expression in the pancreas islet cells (altered β-cell proliferation) was responsible for the induction of diabetes in 1- to 8-week-old mice (18), indi-

cating the importance of EGFR as a protecting factor against the initiation of diabetes in the early stage of development. On the other hand, different studies stated the beneficial effect of EGFR inhibition in mature adult diabetic animals. For instance, it has been shown that diabetes is associated with an increase in advanced glycation end products (AGEs) formation. It is noteworthy that AGE-induced oxidative stress increases through EGFR transactivation, which is suppressed in cells overexpressing AGEs receptor 1 (19), providing evidence for a relationship between diabetes, AGEs, EGFR, and oxidative stress.

Thus, in the present study, we determined the role of elevated EGFR phosphorylation in the dysfunction of resistance arteries observed in type 2 diabetes. Mesenteric resistance arteries and coronary arterioles function were studied in type 2 diabetic (db^-/db^-) mice with or without EGFR phosphorylation inhibitor.

RESEARCH DESIGN AND METHODS

Obese homozygote (db^-/db^-) type 2 diabetic mice lacking the gene encoding for leptin receptor $Lepr^{db-/db-}$ (diabetic, 10–14 weeks old) and heterozygote $Lepr^{db-/db+}$ (db^-/db^+) nondiabetic (control, 10–14 weeks old) adult male mice were obtained from The Jackson Laboratories and maintained on a normal rodent chow diet. Mice were divided into four groups: *1*) control mice with no treatment ($n = 6$); *2*) control mice who received 10 mg · kg^{-1} · day^{-1} AG1478 from mini-osmotic pumps ($n = 7$); *3*) diabetic mice with no treatment ($n = 6$); and *4*) diabetic mice that received 10 mg · kg^{-1} · day^{-1} AG1478 from mini-osmotic pumps ($n = 8$).

These studies conformed to the principles of the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" and were approved by the Louisiana State University Institutional Animal Care and Use Committee.

Blood glucose. Blood glucose measurements were obtained from tail vein samples using a blood glucose meter (Prestige Smart System HDI; Home Diagnostic) in all groups after a 5-h fast ($n = 6$).

Insulin resistance. One milliliter of blood was obtained by cardiac puncture with a syringe containing heparin from mice after a 5-h fast. Insulin resistance was determined by using the Mercodia Ultrasensitive Mouse Insulin ELISA protocol (enzyme immunoassay), which estimates steady-state insulin resistance ($n = 6$) (20).

Mean arterial pressure measurement. After 2 weeks of treatment, mice were anesthetized with ketamine/xylazine (45/2.5 mg/kg i.p., respectively), and a catheter (connected to a pressure transducer and recording system; www.livingsys.com) was placed in the left carotid. Mice were then subjected to a 15-min equilibration period before mean arterial pressure was measured.

For telemetry, in brief, mice were subjected to isofluorane anesthesia and a catheter connected to a radiotelemetry device was inserted in the left carotid artery to monitor heart rate and blood pressure by telemetry in conscious unrestrained conditions (PA-C10; Data Sciences International, St. Paul, MN). The body of the probe was placed in a subcutaneous pocket created in the subcutaneous abdominal area, and the wound was closed and sutured. After a recovery phase (24–48 h), mean arterial blood pressures were collected for 10-s intervals every 10 min for 5 days. Data were stored and analyzed using Dataquest A.R.T 4.0 software (Data Sciences International).

Isolated coronary and mesenteric resistance artery. For mechanotransduction, freshly isolated mesenteric resistance artery (MRA) and coronary artery (septal) were mounted onto two glass micropipettes in a vessel chamber and slowly pressurized to 100 mmHg using a pressure-servo-control perfusion (Living Systems Instruments; www.livingsys.com) to stretch the artery and set a constant artery length (9). Intraluminal pressure was then set at 50 mmHg for equilibration time (30–45 min). Vessel diameter was continuously monitored by a video image analyzer as previously described (3,9). Cannulated arterial segments were submerged in 2 ml physiological salt solution (pH 7.4), oxygenated with 10% O_2–5% CO_2 and 85% N_2. The functional integrity of the endothelial and SMC layer was assessed by endothelium-dependent relaxation and contraction in response to 1 μmol/l acetylcholine and 1 μmol/l phenylephrine, respectively. Next, pressure and shear stress diameter (active diameter) relationships were performed to determine myogenic tone and flow-induced dilation. At the end of each experiment, MRA and coronary artery were perfused and superfused with physiologic salt solution (PSS) containing 100 μmol/l exogenous nitric oxide donor (sodium nitroprusside) and 2 mmol/l EGTA to determine the maximum relaxation of artery (passive diameter). Myogenic tone is calculated as the percentage between active and passive diameter. Flow-induced dilation was represented as percentage of diameter in response to shear stress step increase.

Pharmacology studies. Freshly isolated and mounted MRA and coronary artery were equilibrated at 50 mmHg of intraluminal pressure for 30–45 min. Dose-response (10^{-10} to 10^{-5} mol/l) curves phenylephrine, acetylcholine, and nitric oxide donor diameter-change relationships were then performed.

Western blot analysis. Freshly isolated MRA from all groups was immediately snap-frozen in liquid nitrogen. Frozen vessel segments were pulverized and resuspended in ice-cold lysis buffer as described previously (21). Each sample was then subjected to immunoblotting with endothelial nitric oxide synthase (eNOS) or EGFR antibodies (1:1,000 dilution; Cell Signaling). Blots were stripped and reprobed with the β-actin antibody to verify the equal loading between the samples.

Primary cultured endothelial cells from resistance arteries. After 80% confluency, endothelial cells were starved for 48 h in culture medium containing 1% serum. Cultured cells were then stimulated with high glucose (22 mmol/l) with and without dose-response AG1478 (1, 0.1, and 0.01 μmol/l). Next, cells were stimulated for 5 min with calcium ionophore (eNOS activator), harvested, and subjected to Western blot analysis using phosphorylated (Ser1177 activator site and Thr495 negative regulatory site) and total eNOS and EGFR antibodies (Tyr1173). Membranes were stripped and reprobed with total β-actin antibody to verify the loading of samples.

Immunohistochemistry. Freshly isolated MRA placed in mold tissue Teck and Formalin-fixed heart embedded in paraffin were sectioned at 5 μm. Heart sections were heated at 58°C for 1 h for antigen retrieval. Endogenous peroxidase activity was quenched in heart and MRA section by 10-min incubation with 3% H_2O_2 in H_2O. Heart sections were treated with 10 mmol/l sodium citrate buffer (pH 6.0) at 100°C for 10 min. After blocking with 5% BSA in Tris-buffered saline (TBS), sections (heart and MRA) were incubated overnight at 4°C with mouse monoclonal antibody against phosphorylated EGFR (Tyr1173; 1:200 dilution). For every section, a negative control without first antibody was processed simultaneously. After three 5-min washes in TBS with Tween (TBST; 10 mmol/l Tris-HCl, 0.15 mol/l NaCl, 8 mmol/l sodium azide, and 0.05% Tween-20, pH 8.0), a secondary biotinylated antibody was added for 45 min at room temperature. After three 5-min washes in TBST, the avidin biotin-peroxidase complex (Vector Labs) was applied for 30 min at room temperature. The color reaction was developed with the diaminobenzidine detection kit (1 min incubation with the substrate for all the samples; Vector Labs) and counterstained with hematoxylin. Staining was evaluated using fluorescent microscope.

Immunocytochemistry. Cultured endothelial cells were stimulated with high glucose for 48 h with and without EGFR tyrosine kinase inhibitor. Briefly, cells were fixed with methanol and incubated in PBS solution with 5% of BSA for 1 h. Cells were then incubated with first antibody (phosphorylated EGFR tyrosine kinase antibody Tyr1173, dilution 1:200) overnight at 4°C. The next day, cells were washed and incubated for 1 h with fluorescent secondary antibody at room temperature. Staining was evaluated using fluorescent microscope.

In other slides of MRA, Verhoff Van Gieson (22) staining was performed to determine the internal elastic lamina (IEL) and external elastic lamina (EEL). Briefly, the tissue is stained with a regressive hematoxylin, consisting of ferric chloride and iodine. The differentiating is accomplished by using excess mordant (ferric chloride) to break the tissue-mordant dye complex. The dye will be attracted to the larger amount of mordant in the differentiating solution and will be removed from the tissue. The elastic tissue has the strongest affinity of the iron-hematoxylin complex and will retain the dye longer than the other tissue elements.

Statistical analysis. Results are expressed as means ± SE, where n is the number of arterial segments and cultured cells studied. Significance of the differences between groups was determined by one-repeated or two-factor ANOVA, where appropriate. Differences were considered significant at $P < 0.05$.

RESULTS

Blood pressure, measured under anesthesia and with telemetry (Table 1; Fig. 1*A*), was similar in all groups of mice (Table 1), indicating that db^-/db^- mice are normotensive. Blood glucose level was higher in diabetic mice compared with control mice (Table 1). Higher values of insulin resistance were found in plasma from type 2

TABLE 1
Blood pressure, glucose, insulin, and body weight measurements

	Control	Diabetic	Diabetic + AG1478
Blood pressure in carotid under anesthesia (mmHg)	97 ± 3.2	96.5 ± 4.4	95 ± 5.1
Blood pressure using telemetry (mmHg)	110 ± 6.4	112 ± 6.3	—
Plasma glucose concentration (mg/dl)	121 ± 15	321 ± 22*	291 ± 8.7*
Serum insulin concentration (μg/l)	1.21 ± 0.3	4.35 ± 0.4*	4.15 ± 0.5*
Body weight (g)	26 ± 3	45 ± 2.25*	—

Data are means ± SE. $P < 0.05$, statistically significant between diabetic or diabetic + AG1478 vs. control mice.

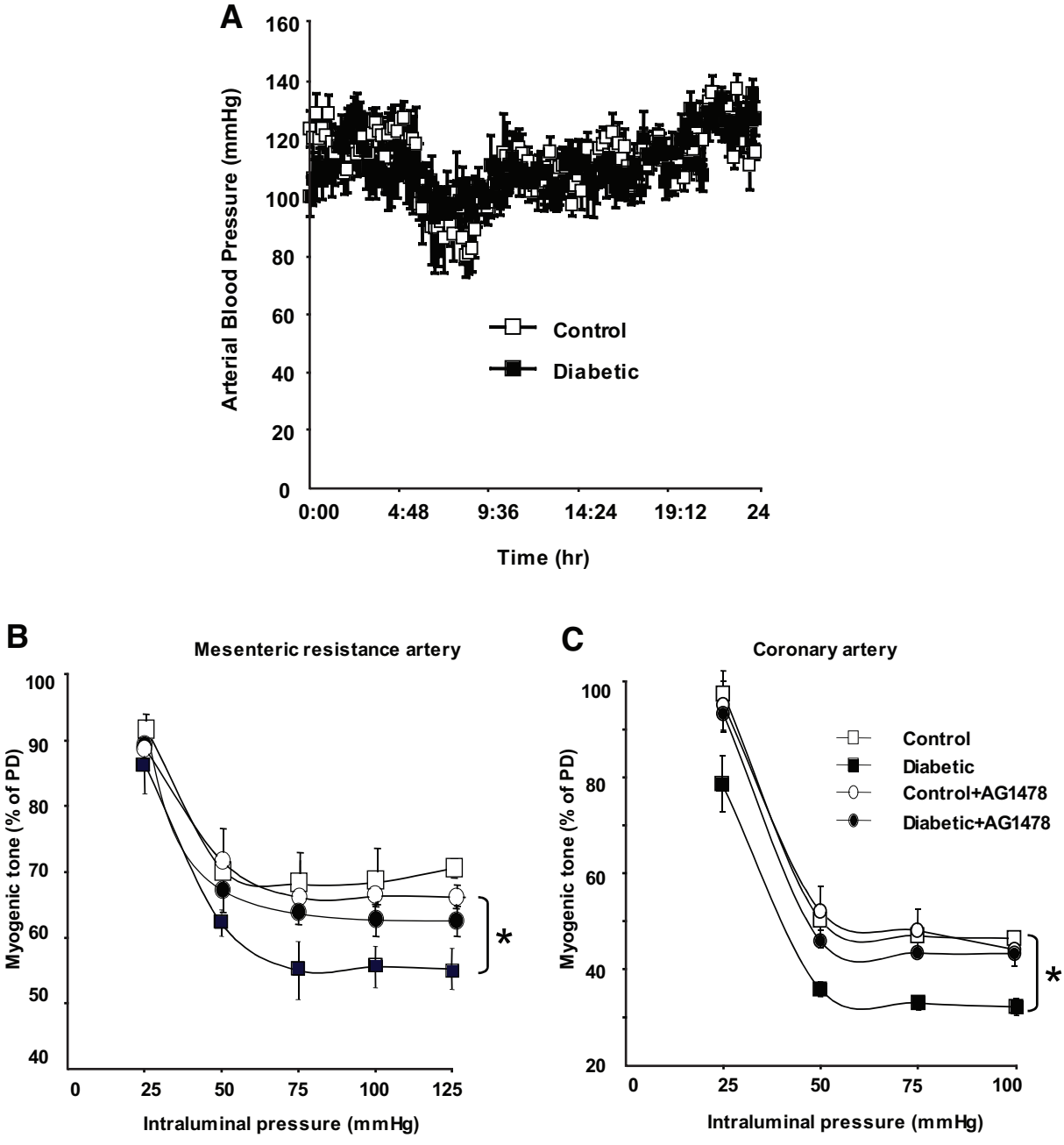

FIG. 1. Blood pressure measurements with telemetry and pressure-induced myogenic tone. In vivo blood pressure measurement with telemetry from diabetic and control mice; $P > 0.05$, not statistically different; $n = 5$ (*A*). Pressure-induced myogenic tone relationship of MRA (*B*) and coronary artery (*C*) from all groups treated with and without EGFR tyrosine kinase inhibitor (AG1478, 10 mg · kg^{-1} · day^{-1}); $n = 7$. *$P < 0.05$, statistically significant diabetic vs. control mice; diabetic + AG1478 vs. diabetic mice.

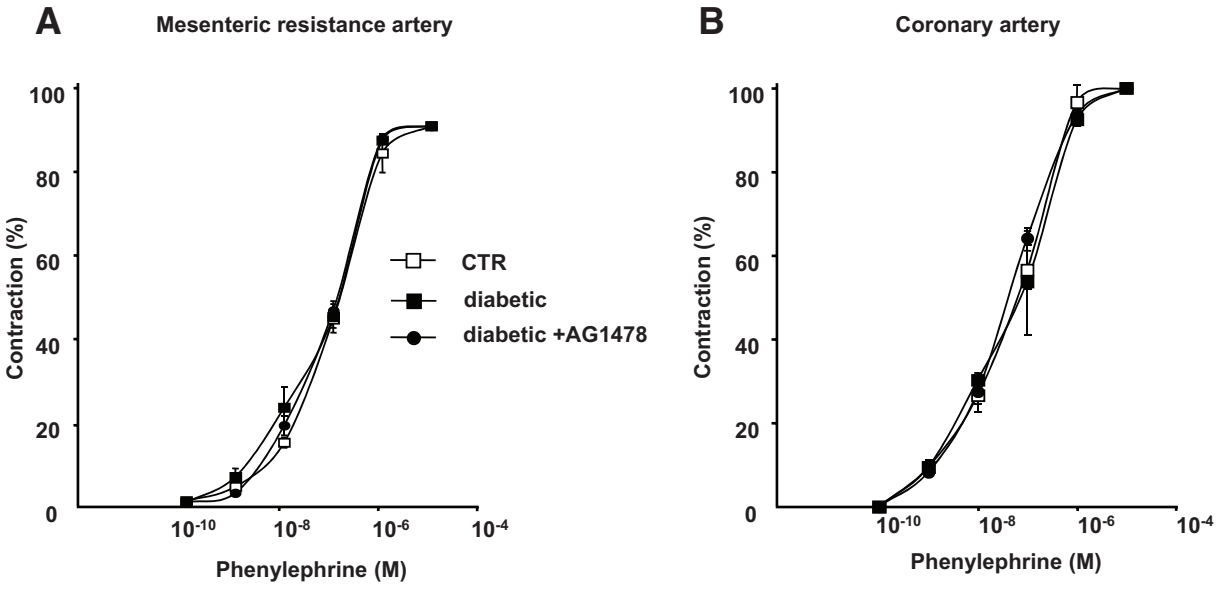

FIG. 2. Pharmacology approach. Phenylephrine dose-response (10^{-10} to 10^{-5} μmol/l)-induced contraction of MRA (*A*) and coronary artery (*B*) from control mice (CTR), diabetic mice, and diabetic mice treated with EGFR tyrosine kinase inhibitor (AG1478, 10 mg · kg^{-1} · day^{-1}); $n = 7$; $P > 0.05$, not statistically different.

diabetic mice compared with control mice (Table 1). The treatment of type 2 diabetic mice with AG1478 for 2 weeks had no effect on blood pressure, glucose level, and insulin resistance (Table 1).

Fresh MRA and coronary artery were isolated and mounted in an arteriograph. Stepwise increases in intraluminal pressure induced myogenic tone development, which was significantly enhanced in MRA and coronary artery from diabetic mice compared with control mice (Fig. 1*B* and *C*). The AG1478 treatment for 2 weeks reduced the myogenic tone potentiation in MRA and coronary artery from diabetic mice (Fig. 1*B* and *C*), indicating that the exacerbation of EGFR phosphorylation is involved in the enhanced myogenic tone. Increasing doses of phenylephrine induced similar contractions of MRA and coronary artery from all groups (Fig. 2). On the other hand, endothelium-dependent relaxation was significantly altered in MRA and coronary artery from diabetic mice compared with control mice as assessed with acetylcholine dose-response relaxation relationship (Fig. 3) and flow-induced dilation (Fig. 4*A*). Dysfunction of MRA from diabetic mice was associated with a decrease of eNOS phosphorylation and expression (Fig. 4*B*). Phosphorylation at the negatively regulatory eNOS site Thr495 was similar in MRA from control and type 2 diabetic mice (data not shown).

The treatment of diabetic mice with AG1478 for 2 weeks significantly improved the endothelium-dependent relaxation in response to shear stress– and acetylcholine (dose-response)-induced relaxation in MRA and coronary artery (Figs. 3 and 4*A*) and normalized the eNOS phosphorylation and expression (Fig. 4*B*).

The endothelium-independent relaxation of MRA and coronary artery from diabetic mice to exogenous nitric oxide donor was similar in all groups, indicating that the

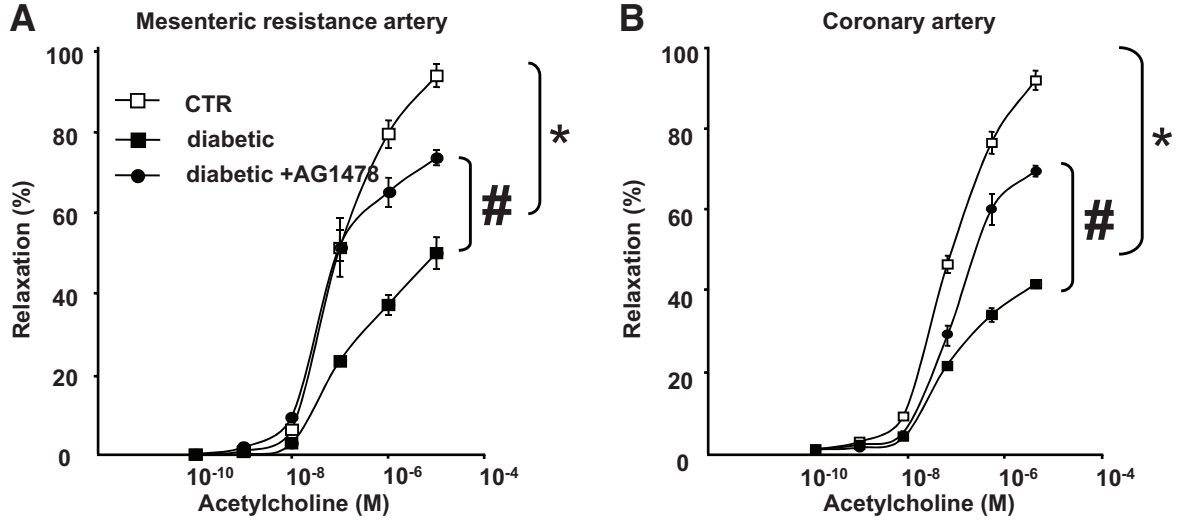

FIG. 3. Changes in diameter in response to dose response (10^{-10} to 10^{-5} mol/l) of acetylcholine in MRA (*A*) and coronary artery (*B*) from control mice (CTR), diabetic mice, and diabetic mice with EGFR tyrosine kinase inhibitor (AG1478, 10 mg · kg^{-1} · day^{-1}); $n = 6$. *$P < 0.001$ diabetic vs. control mice; diabetic vs. diabetic mice.

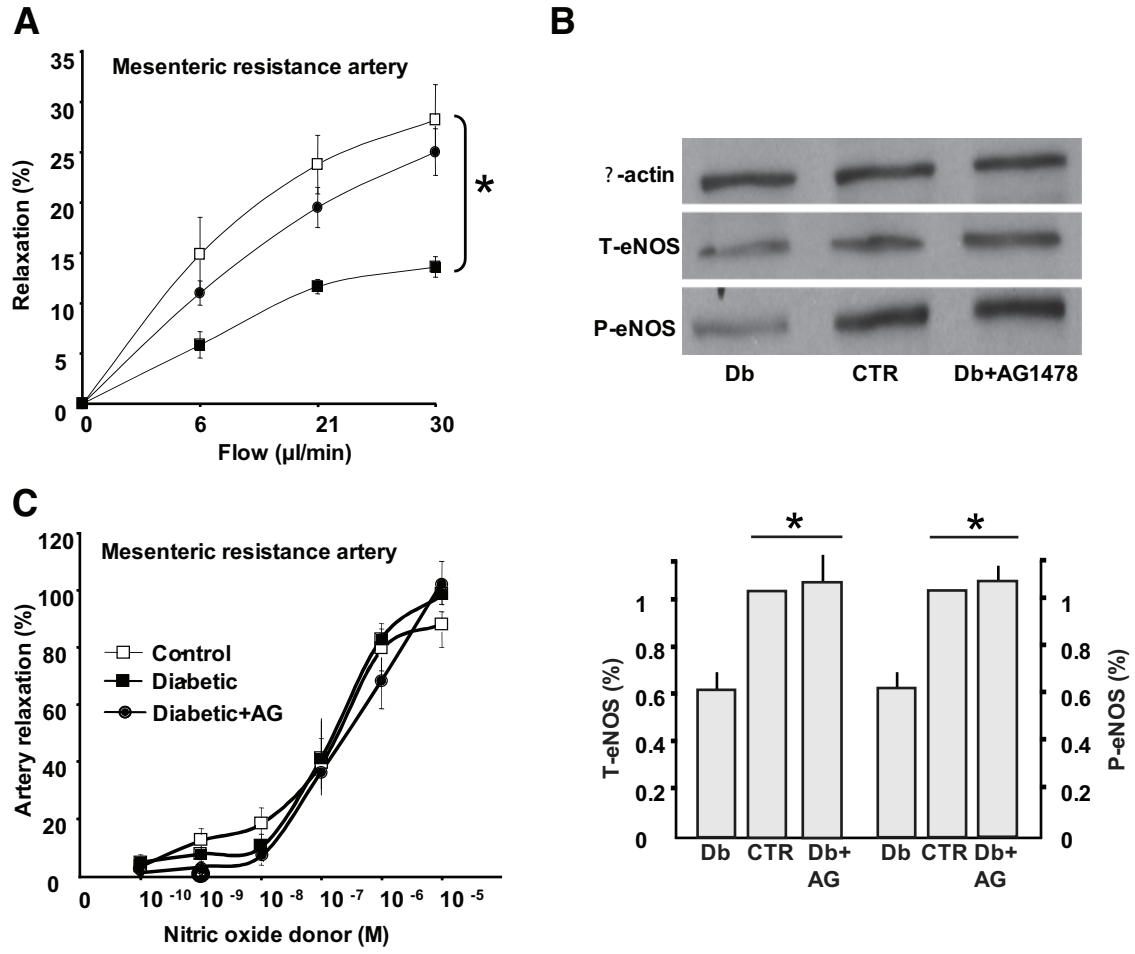

FIG. 4. ***A*: Changes in diameter in response to step increases of flow in MRA isolated from control mice (CTR), diabetic mice (Db), and diabetic mice treated with EGFR tyrosine kinase inhibitor (AG, AG1478; 10 mg · kg^{-1} · day^{-1}). *P < 0.001 diabetic vs. control mice, diabetic vs. diabetic + AG1478 mice. *B*: In vivo eNOS phosphorylation and expression in freshly isolated MRA from control mice, diabetic mice, and diabetic mice treated with AG1478. *P < 0.001 diabetic vs. control mice and diabetic + AG mice. *C*: Changes in diameter in response to dose response (10^{-10} to 10^{-5} μmol/l) of exogenous nitric oxide donor (sodium nitroprusside) in MRA from control mice, diabetic mice, and diabetic mice treated with EGFR tyrosine kinase inhibitor (AG, AG1478; 10 mg · kg^{-1} · day^{-1}); n = 6–8; P > 0.05 not statistically different.**

sensitivity of SMC to nitric oxide was not altered in type 2 diabetic mice (Fig. 4*C*).

Immunohistochemistry revealed an increase of EGFR phosphorylation of MRA and coronary artery from diabetic mice compared with control mice and diabetic mice treated with AG1478 (Figs. 5 and 6). In other MRA slides, IEL and EEL staining was performed (Fig. 5*D–F*).

To strengthen our approach, we used resistance artery primary cultured endothelial cells treated with high glucose (20 mmol/l) with or without dose response of AG1478 (1, 0.1, and 0.01 μmol/l) for 48 h. The treatment of endothelial cells with high glucose induced an increase in EGFR phosphorylation and an alteration in eNOS expression and phosphorylation in response to calcium ionophore (Fig. 7*A*), which were restored when endothelial cells were pretreated with AG1478 (Fig. 7*A*). Western blot analysis and immunostaining show an increase in phosphorylated EGFR on endothelial cells stimulated with high glucose for 48 h (Fig. 7*B* and *C*). The specificity of the primary antibody was confirmed by subjecting tissue section and primary cultured cells to immunostaining without the primary antibody because no signal was observed (data not shown). The control and pretreated endothelial cells with AG1478 showed low background staining of EGFR phosphorylation in response to high glucose (Fig. 7*C*).

DISCUSSION

In the present study, we provide strong evidence supporting the involvement of elevated EGFR phosphorylation in dysfunction of MRA and coronary arteriole (alteration of endothelial cells and SMCs) from the db^-/db^- mouse, which is a widely accepted model for obesity and type 2 diabetes. Endothelial cell dysfunction is associated with a decrease in eNOS expression and phosphorylation, whereas SMCs are hypersensitive to pressure changes, leading to the potentiation of myogenic tone in MRA and coronary artery from diabetic mice. Interestingly, endothelial and SMC function was significantly improved in MRA and coronary artery from diabetic mice treated with EGFR phosphorylation inhibitor.

Blood pressure, measured under anesthesia and with telemetry, was normal and similar in all groups, indicating that db^-/db^- mice are not hypertensive. Our data are not in agreement with the Bagi et al. (6) study showing an increase of systolic blood pressure in diabetic mice measured by tail-cuff method. However, our data are in agreement with a previous study showing a normal blood pressure in diabetic mice compared with control mice (20,23). Blood glucose and serum insulin levels were increased in type 2 diabetic mice compared with control mice and were not affected by AG1478 treatment, indicat-

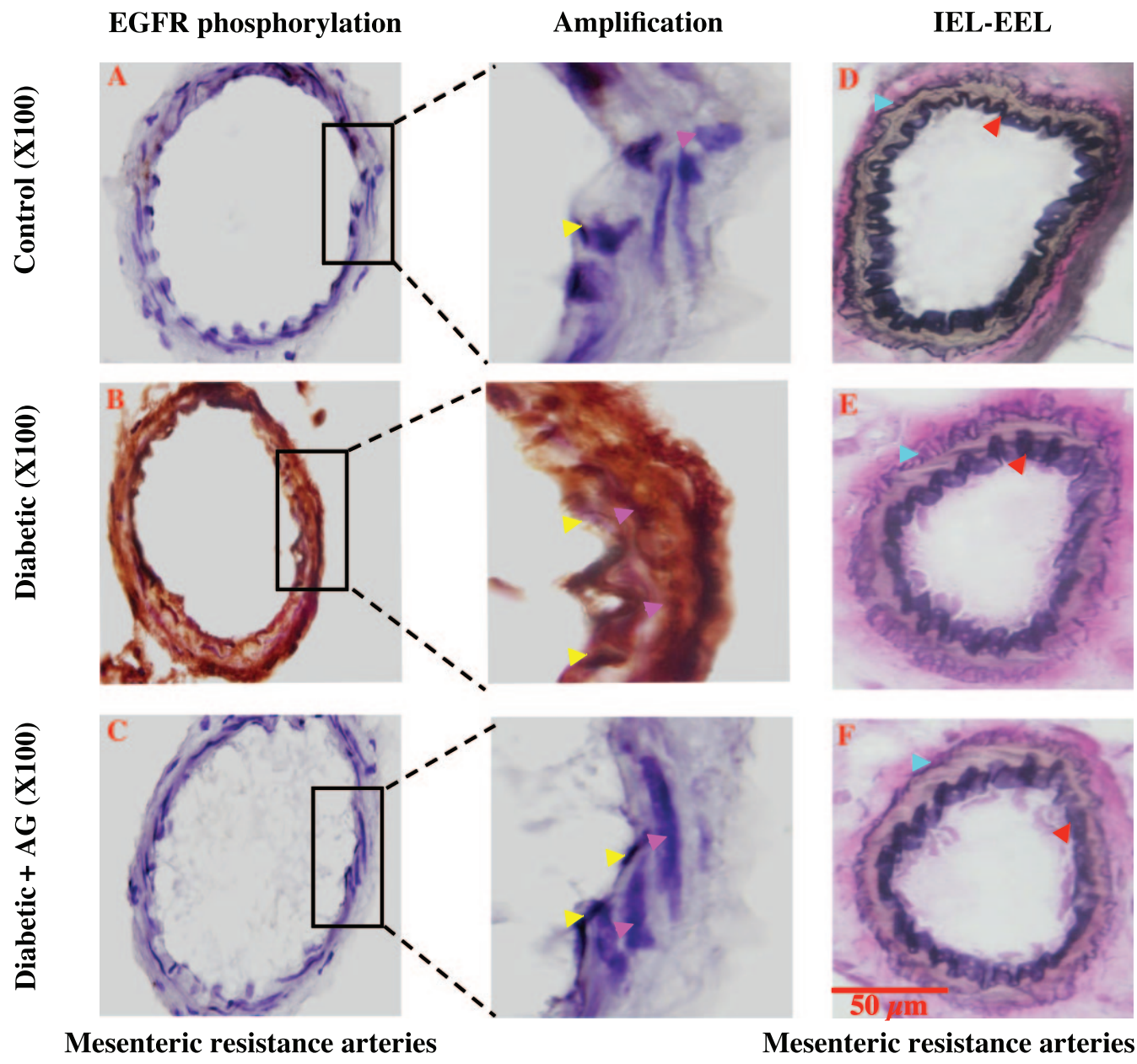

FIG. 5. Immunhistochemical staining showing the in vivo EGFR phosphorylation of mesenteric resistance arteries (MRA) from control mice (*A*), diabetic mice (*B*), and diabetic mice treated with EGFR inhibitor (AG, AG1478) (*C*). The brown staining indicated by yellow and magenta arrows shows the phosphorylated EGFR on endothelial and SMC of MRA, respectively. *D–F*: The IEL (red arrow) and the EEL (turquoise arrow) of different MRA from control mice, diabetic mice, and diabetic mice treated with AG1478, respectively. Each figure is representative of five experiments.

ing that elevated EGFR phosphorylation in type 2 diabetic mice is a consequence of the onset of type 2 diabetes. These data are in agreement with previous studies showing an increase of plasma glucose and serum insulin from db^-/db^- mice (20).

Resistance arteries play a crucial role in the control of local blood flow and, subsequently, tissue perfusion. These resistance arteries develop tone, which is mainly regulated by mechanical factors (pressure and shear stress) and hormonal factors (24,25).

It has been reported that type 2 diabetes is associated with small artery complications (26). Only limited studies have been conducted to establish the relationship between diabetes and altered vascular responsiveness in the microvasculature from diabetic models, with conflicting results. Lagaud et al. (5) demonstrate an increased myogenic tone in mesenteric resistance arteries from 12- and 16-week diabetic mice compared with the control that was independent of endothelium removal. In contrast, Bagi et al. (27) showed no significant increase in myogenic tone in coronary arterioles from 12-week-old db^-/db^- mice. Small arteries (65–230 μm) from patients with type 2 diabetes demonstrated decreased myogenic responsiveness (7). The reasons for these discrepancies are unclear but could be related to differences in vascular beds and species.

Emerging evidence suggests the existence of a new mode of EGFR signaling pathway in which activated receptor undergoes nuclear translocalization and subsequently regulates gene expression and potentially mediates other cellular processes. This signaling route is distinct from the better-characterized EGFR pathway that involves transduction of mitogenic signals through activa-

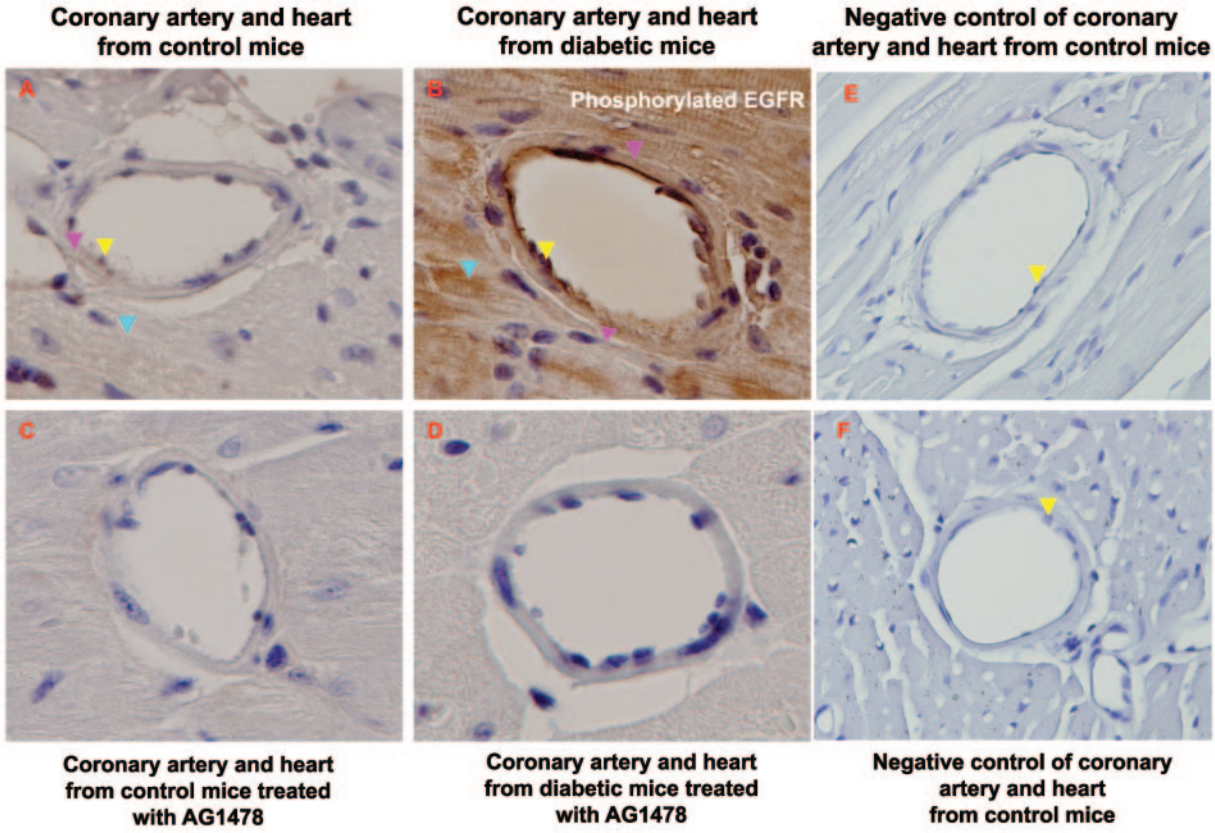

FIG. 6. Immunhistochemical staining showing the in vivo EGFR phosphorylation of coronary artery from control mice (*A*), diabetic mice (*B*), control + AG1478 mice (*C*), and diabetic mice treated with EGFR inhibitor (AG1478) (*D*). The brown staining indicated by yellow, magenta, and ice arrows shows the phosphorylated EGFR on endothelial cells, SMCs of coronary artery, and cardiomyocytes, respectively. *E* and *F*: Negative control sections of coronary artery from control mice, diabetic mice, and diabetic mice treated with AG1478. Each figure is representative of five experiments.

tion of multiple signaling cascades (28,29). Our data show increased myogenic tone in MRA and coronary artery from diabetic mice compared with their control mice, which is in accordance with a study conducted by Lagaud et al. (5). Interestingly, treatment with EGFR tyrosine kinase inhibitor reduces the myogenic tone potentiation of MRA and coronary artery from diabetic mice, indicating that elevated EGFR phosphorylation is a key element in the enhanced myogenic tone in type 2 diabetic mice. Contraction and endothelium-independent relaxation of MRA and coronary artery in response to G-protein–coupled receptor (phenylephrine) and nitric oxide donor were similar in all groups. In agreement with our study, Malik et al. (30) have shown that vasoconstriction to phenylephrine and angiotensin II was similar in small arteries from patients with or without type 2 diabetes. This study strengthens our data indicating that whether MRA and coronary artery develop hypersensitivity to vasoconstrictors G-protein–coupled receptor is not dependent on type 2 diabetes status. The mechanism leading to increased EGFR tyrosine kinase phosphorylation in type 2 diabetes is unknown. In a previous study, we showed that myogenic tone development is dependent on EGFR tyrosine kinase activation through metalloproteinase (MMP) 2 and MMP9 activity. We suggest that MMPs 2/9 and hyperglycemia signaling such oxidative stress and AGEs could be involved in the increased EGFR tyrosine kinase phosphorylation in type 2 diabetes. Further studies are needed to determine and emphasize the role of MMPs 2/9, AGEs, and oxidative stress in the increased EGFR phosphorylation in type 2 diabetes and microvessel dysfunction.

Endothelial dysfunction has been demonstrated to occur in small arteries from patients with type 2 diabetes (31–33). Our data revealed that endothelium-dependent relaxation assessed with flow changes was significantly altered in MRA from type 2 diabetic compared with control mice. Our data are concordant with other studies showing a dysfunction of endothelial cells in diabetes (27,34). To strengthen our conclusion, we used acetylcholine dose-response–induced relaxation, which was significantly decreased in MRA and coronary artery from type 2 diabetic mice compared with that in control mice. Interestingly, the treatment of type 2 diabetic mice with EGFR tyrosine kinase inhibitor significantly improved relaxation to shear stress and acetylcholine stimulation, and no effect was observed in control mice. The endothelium dysfunction was associated with a decrease in eNOS activator site (Ser1177) phosphorylation and expression in type 2 diabetic mice; the inhibitory eNOS site phosphorylation Thr495 was not affected. Ohashi et al. (35) showed a decrease of eNOS at the mRNA level in KKAy mice, which develop a maturity-onset obesity, type 2 diabetes, and hypertension. Similarly, a reduction in eNOS phosphorylation and expression in kidney from diabetic rats has been reported (36). Together, these data provide evidence of a link between endothelium dysfunction of resistance arteries and alteration of eNOS phosphorylation and expression in type 2 diabetic mice. Interestingly, the treatment of type 2 diabetic mice with EGFR tyrosine kinase inhibitor improved resistance artery relaxation in response to flow and acetylcholine and restored eNOS phosphorylation and expression. These data strongly sup-

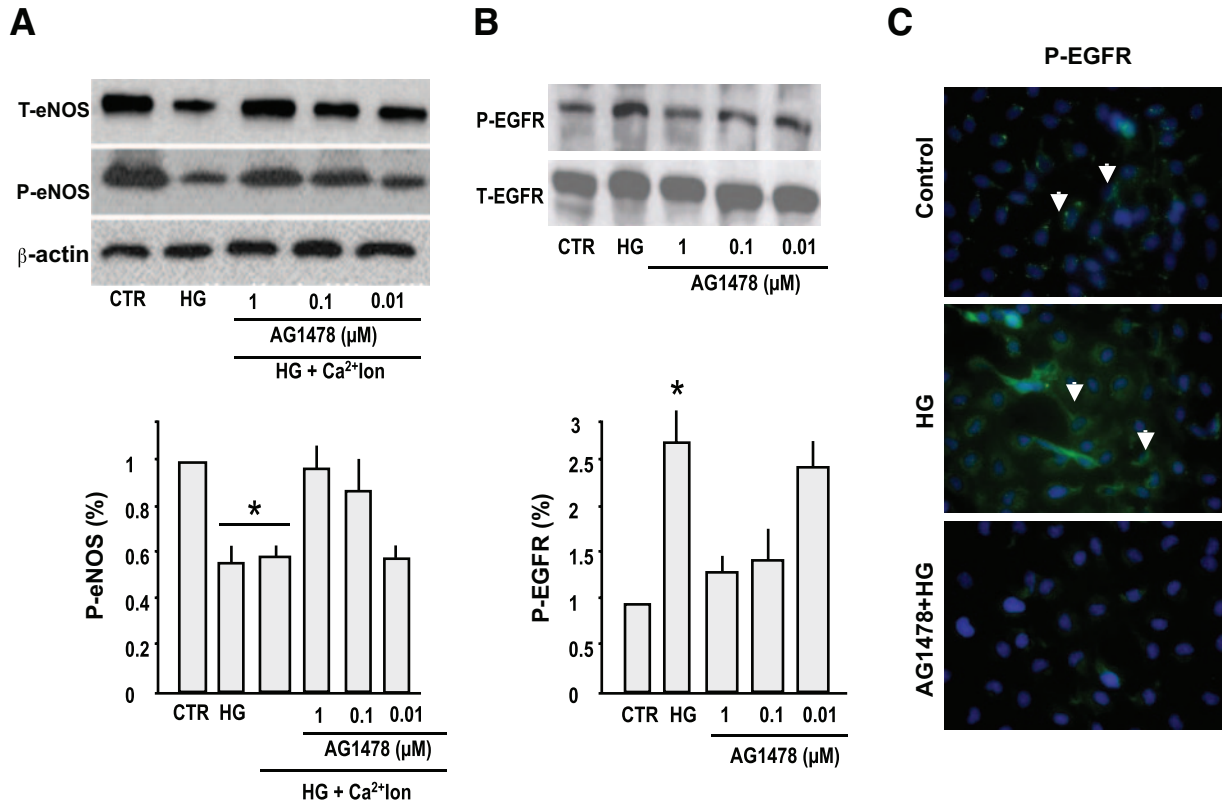

FIG. 7. *A*: Western blot analysis and cumulative data showing the effect of high glucose (HG), with or without AG1478 dose response (1, 0.1, and 0.01 μmol/l), on total eNOS and phosphorylated eNOS in response to calcium ionophore of primary cultured endothelial cells; $n = 8$. *$P < 0.001$ CTR vs. high glucose; high glucose vs. high glucose + AG1478. *B*: Western blot analysis and cumulative data showing total and phosphorylated EGFR tyrosine kinase of primary cultured endothelial cells in response to high glucose, with or without AG1478 dose response (1, 0.1, and 0.01 μmol/l); $n = 8$. *$P < 0.001$ CTR vs. high glucose; high glucose vs. high glucose + AG1478 (1 μmol/l). *C*: Immunostaining showing EGFR tyrosine kinase phosphorylation on primary cultured endothelial cells membrane under control, high glucose, and AG1478 + high glucose conditions. The green staining indicated by arrows shows the phosphorylated EGFR on cultured endothelial cells. Each figure is representative of five experiments.

port the link between EGFR tyrosine kinase and resistance artery relaxation, in part, through eNOS pathway. It is important to mention that endothelium of resistance artery is a source of different vasoactive compounds such as nitric oxide, prostacyclin I2, and endothelium-derived hyperpolarizing factor involved in SMC relaxation. Thus, the treatment of type 2 diabetic mice with AG1478 partially improved the relaxation while totally restoring the phosphorylation and expression of eNOS, indicating that type 2 diabetes could also affect prostacyclin and/or endothelium-derived hyperpolarizing factor (EDHF) pathways. Further studies are needed to explore the molecular mechanisms linking EGFR and vasoactive compounds in small arteries from type 2 diabetic mice.

The mechanism responsible for the elevated EGFR tyrosine kinase phosphorylation and its link to eNOS in resistance arteries endothelial cells from type 2 diabetic mice is unknown. It is likely that EGFR tyrosine kinase signaling such oxidative stress could be involved in eNOS pathway alteration in type 2 diabetes. Additionally, it has been reported that AGEs could have a potential role because it can also increase oxidative stress generation through their receptors. Future studies are needed to determine the mechanism leading to increased EGFR tyrosine kinase and its link to eNOS in type 2 diabetes.

To corroborate our functional studies, we performed immunostaining and found an increase of EGFR tyrosine kinase phosphorylation in endothelial cells and SMCs of MRA and coronary artery from type 2 diabetic mice compared with control mice, which was reduced with the EGFR tyrosine kinase inhibitor treatment. Together, these data strengthen a link between the increased EGFR tyrosine kinase phosphorylation and alteration of resistance artery function in type 2 diabetic mice.

To determine the contribution of hyperglycemia in endothelium dysfunction of resistance arteries from type 2 diabetic mice, we used primary cultured endothelial cells prepared from resistance artery and treated with high glucose for 48 h. Cell lysates subjected to Western blot analysis showed an increase of EGFR tyrosine kinase phosphorylation associated with alterations in eNOS expression and phosphorylation, which strengthens our in vivo observation, indicating that hyperglycemia participates in the dysfunction of small artery endothelial cells. An EGFR phosphorylation increase in response to hyperglycemia was supported by immunostaining. The pretreatment of endothelial cells with EGFR tyrosine kinase phosphorylation inhibitor prevented the effect of high glucose. This indicates that in type 2 diabetic mice, hyperglycemia-induced EGFR tyrosine kinase phosphorylation increase is, to some extent, responsible for endothelial cell dysfunction. It is well established that type 2 diabetes is characterized with hyperglycemia and insulin resistance, and it is likely that insulin resistance might have an impact on resistance artery endothelial and SMC dysfunction. Further studies are needed to investigate the insulin resistance in the absence of hyperglycemia on resistance artery reactivity.

Our data suggest that in type 2 diabetic mice, elevated EGFR tyrosine kinase phosphorylation is involved in resistance artery enhanced myogenic tone and alteration of endothelial cells through the nitric oxide synthesis pathway. The treatment of diabetic mice with AG1478 improved the MRA and coronary artery functions. We strongly believe that additional factors are implicated in the alteration of resistance arteries in type 2 diabetes. In addition, we believe that myriad signaling pathways are involved. The discovery of EGFR tyrosine kinase as regulator of endothelial and SMC dysfunction of resistance arteries might be relevant not only for type 2 diabetes small artery complications but also for many other pathological conditions in which EGFR has been suggested to play a role.

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