A Novel Role for Epidermal Growth Factor Receptor Tyrosine Kinase and Its Downstream Endoplasmic Reticulum Stress in Cardiac Damage and Microvascular Dysfunction in Type 1 Diabetes Mellitus

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Abstract—Epidermal growth factor receptor tyrosine kinase (EGFRtk) and endoplasmic reticulum (ER) stress are important factors in cardiovascular complications. Understanding whether enhanced EGFRtk activity and ER stress induction are involved in cardiac damage, and microvascular dysfunction in type 1 diabetes mellitus is an important question that has remained unanswered. Cardiac fibrosis and microvascular function were determined in C57BL/6J mice injected with streptozotocin only or in combination with EGFRtk inhibitor (AG1478), ER stress inhibitor (Tudca), or insulin for 2 weeks. In diabetic mice, we observed an increase in EGFRtk phosphorylation and ER stress marker expression (CHOP, ATF4, ATF6, and phosphorylated-eIF2α) in heart and mesenteric resistance arteries, which were reduced with AG1478, Tudca, and insulin. Cardiac fibrosis, enhanced collagen type I, and plasminogen activator inhibitor 1 were decreased with AG1478, Tudca, and insulin treatments. The impaired endothelium-dependent relaxation and -independent relaxation responses were also restored after treatments. The inhibition of NO synthesis reduced endothelium-dependent relaxation in control and treated streptozotocin mice, whereas the inhibition of NADPH oxidase improved endothelium-dependent relaxation only in streptozotocin mice. Moreover, in mesenteric resistance arteries, the mRNA levels of Nox2 and Nox4 and the NADPH oxidase activity were augmented in streptozotocin mice and reduced with treatments. This study unveiled novel roles for enhanced EGFRtk phosphorylation and its downstream ER stress in cardiac fibrosis and microvascular endothelial dysfunction in type 1 diabetes mellitus. (Hypertension. 2012;60:71-80.) ● Online Data Supplement

Key Words: EGFRtk ■ ER stress ■ type 1 diabetes ■ Tudca ■ cardiac fibrosis ■ resistance arteries ■ endothelial function

Diabetes mellitus is a major cause of morbidity and mortality worldwide and is a threat to human health.1,2 Increasing evidence from experimental and clinical studies indicates a higher prevalence of cardiac damage and microvascular complications in diabetic patients.3–7 Epidermal growth factor receptor (EGFR) is a glycoprotein containing a single transmembrane domain with intracellular portion harboring the tyrosine kinase domain. The EGFRtk is regulated by glucose through EGFR-N-glycosylation,8 and although there is a plethora of information on the growth-promoting effects of EGFR, its role in cardiovascular complications in type 1 diabetes mellitus remains unknown. We and others have demonstrated that increased EGFRtk phosphorylation contributes to resistance artery dysfunction in type 2 diabetes mellitus.9,10 In addition, it has been reported that the inhibition of EGFR activity promotes vasodilatation and reduces elevated arterial blood pressure in hypertensive animal models with or without insulin resistance.11,12

Recently, several studies in different cancer cell lines and human tissues have reported a relationship between aberrant...
EGFRtk expression-activation and endoplasmic reticulum (ER) stress-related proteins. ER stress plays a critical role in the pathogenesis of diabetes mellitus and associated cardiovascular complications. Various cellular stresses, including ischemia, hypoxia, gene mutation, oxidative stress, and protein synthesis overload, lead to impairment of ER function and create a state termed “ER stress” that leads to the activation of a complex signaling network called the unfolded protein response. The unfolded protein response is regulated in the cell by 3 ER membrane-associated proteins that act as sensors of ER homeostasis. The 3 membrane-bound protein are protein kinase-like ER eukaryotic initiation factor 2α kinase, inositol requiring ER-to-nucleus signaling protein-1a, and activating transcription factor 6 (ATF6). The involvement of ER stress in the development of diseases, such as obesity, stroke, myocardial ischemia, and type 2 diabetes mellitus, has been widely demonstrated and is considered a key element in pancreatic β-cell dysfunction and peripheral insulin resistance.

The significance and role of exacerbated EGFRtk and ER stress in cardiac damage and microvascular dysfunction in type 1 diabetes mellitus are important questions that have remained unanswered. Thus, the aim of this study was to determine the role of increased EGFRtk activity and its downstream ER stress as important factors in cardiac damage and microvascular dysfunction in the type 1 diabetic mouse model.

Materials and Methods
See the online-only Data Supplement.

Results
Effect of EGFRtk Inhibition on Glucose Level, Body Weight, Insulin, Cardiac Fibrosis, and ER Stress Markers in the Heart
The induction of type 1 diabetes mellitus with streptozotocin (STZ) injection increased blood glucose levels to 408% compared with control mice (100%; Figure 1A), decreased the body weight to 40% (Figure 1B), and induced cardiac fibrosis associated with increased plasminogen activator inhibitor (PAI) 1 (3.5-fold) and collagen type 1 expression (3-fold; Figure 1C through 1E). In STZ mice, the inhibition of EGFRtk reduced blood glucose level to 337% and increased the body weight to 84% (Figure 1A and 1B). Fibrosis was reduced by 50%, and collagen type 1 expression was reduced to 2-fold compared with STZ mice, whereas PAI-1 was blunted (Figure 1C through 1E). Interestingly, the mRNA levels and phosphorylation of EGFRtk were augmented by 2.5- and 2.0-fold, respectively, in cardiac tissue from STZ mice and were significantly reduced after EGFRtk inhibition (Figure 1F and 1G). ER stress marker expression assessed by real-time RT-PCR revealed an increase by 2.0- and 2.5-fold for CHOP, ATF4, and ATF6, respectively, in cardiac tissue from STZ mice and were reduced after EGFRtk inhibition, with the exception of ATF6 (Figure 1H, 1J, and 1K). The protein expression of CHOP was increased by 4-fold in STZ mice and blunted after EGFRtk inhibition (Figure 1I).

Effect of ER Stress Inhibition on Glucose Level, Body Weight, Insulin, Cardiac Fibrosis, and ER Stress Markers in the Heart
The injection of STZ significantly increased blood glucose concentration to 436% compared with control mice (100%) and was significantly reduced to 326% after ER stress inhibition and normalized with insulin injection (94%; Figure 2A). The ER stress inhibition increased body weight from 58% in STZ mice to 72%, whereas the treatment with insulin completely restored the body weight to control mice value (Figure 2B). Insulin level was not detectable in STZ mice treated with or without Tudca (0.036±0.0 and 0.035±0.0 ng/L, respectively) but was restored with insulin treatment (0.99±0.2 ng/L; Figure 2C).

We observed cardiac fibrosis induction in STZ mice evidenced by an increase in collagen type I deposition and an increase by 2.5- and 2.0-fold in PAI-1 and collagen type 1 expression, respectively, which were blunted with Tudca and insulin treatments (Figure 2D through 2F). The cardiac fibrosis in STZ mice was associated with ER stress evidenced by augmented mRNA levels of ATF4 (2.5-fold), CHOP (3.0-fold), and ATF6 (2.5-fold), which were reduced with Tudca and insulin treatment to control levels (Figure 2G, 2I, and 2J). The expression of CHOP was increased by 3.5-fold in STZ mice and was blunted after insulin treatment and ER stress inhibition (Figure 2H). In addition, Western blot analysis in heart showed no changes in EGFRtk phosphorylation in the STZ group compared with the STZ+Tudca group (Figure S1A).

Effect of EGFRtk Inhibition on Vascular Relaxation and ER Stress Markers in Mesenteric Resistance Arteries
The EGFRtk expression in mesenteric resistance arteries (MRAs) assessed by Western blot and real-time RT-PCR revealed no changes in all of the groups of mice (Figure 3A and 3B), whereas phosphorylated EGFRtk was 2-fold increased in MRA from STZ mice compared with control and diabetic mice treated with the EGFRtk inhibitor (Figure 3B).

To determine the role of EGFRtk in microvascular dysfunction in type 1 diabetes mellitus, we examined the endothelium-dependent relaxation (EDR) response in MRA from STZ mice infused with and without EGFRtk inhibitor. EDR was significantly decreased in STZ mice (33.6%) compared with control (84%; Figure 3C) and was associated with reduced endothelial NO synthase (eNOS) expression and phosphorylation and cGMP levels (Figure 3E and 3F). Endothelium-independent relaxation was shifted to the right in STZ mice (EC50=6.3±0.07) compared with control mice (EC50=7.29±0.03; Figure 3D). Importantly, the inhibition of EGFRtk in STZ mice improved EDR (76.6%), restored endothelial independent sensitivity (EC50=7.47±0.04), increased by 2-fold eNOS phosphorylation, and restored cGMP levels to control in MRAs (Figure 3C through 3F).

The phosphorylation of eIF2α and mRNA levels of CHOP, ATF4, and ATF6 were increased by 3.0-, 2.5-, 4.0-, and 7.0-fold, respectively, in MRAs from STZ mice compared with control mice and were reduced after EGFRtk inhibition by 2.5-, 1.6-, and 2.8-fold, respectively, with the exception of the
Figure 1. Effect of epidermal growth factor receptor (EGFR) tyrosine kinase (EGFRtk) inhibition on plasma glucose, body weight, fibrosis, EGFRtk, and endoplasmic reticulum (ER) stress markers in heart from streptozotocin (STZ) mice. A and B, Glucose levels (mg/dL) and body weight (BW) were determined in control, STZ, and STZ/AG1478 groups; n=7. The vertical dashed lines denote the start of infusion of AG1478. , control; , STZ/AG1478; , STZ. C, Representative histological sections from the heart stained with Sirius-red; bars indicate the quantitative data, n=5. D and E, Representative Western blot analysis and quantitative data for plasminogen activator inhibitor (PAI) 1 and collagen I in heart in all groups, n=4 to 5. F and G, EGFR mRNA levels, n=5 and representative Western blot analysis and quantitative data for phosphorylated EGFR and total EGFR in all groups; n=5. H and I, CHOP mRNA levels, n=5, and CHOP representative Western blot and quantitative data in all groups, n=3. J and K, ATF-4 and ATF-6 mRNA levels, normalized to 18S rRNA, in all of the groups, n=5. *P<0.05 for STZ vs control; #P<0.05 for STZ vs STZ/AG1478; $P<0.05 for STZ/AG1478 vs control.
Figure 2. Effect of endoplasmic reticulum (ER) stress inhibition on plasma glucose, body weight, plasma insulin, cardiac fibrosis, and ER stress markers expression in heart from streptozotocin (STZ) mice. A and B, Glucose levels (mg/dL) and body weight (BW) were determined in control, STZ, STZ+insulin, and STZ+Tudca groups; n=10. The vertical dashed lines denote the start of the injection of insulin or Tudca. Control; STZ; STZ+insulin; STZ+Tudca. C, Plasma insulin levels (ng/dL) in all groups, n=10. D, Representative histological sections from the heart stained with Sirius-red, bars indicate the quantitative data, n=5. E and F, Representative Western blot analysis and quantitative data for plasminogen activator inhibitor (PAI) 1 and collagen I in heart from all of the groups, n=4 to 5. G and H, CHOP mRNA levels, n=5, and CHOP representative Western blot and quantitative data in all groups, n=3. I and J, ATF-4 and ATF-6 mRNA levels, normalized to 18S rRNA, in all groups, n=5 to 6. *P<0.05 for STZ vs control or STZ+insulin, #P<0.05 for STZ vs STZ+Tudca, $P<0.05 for STZ+Tudca vs control.
Figure 3. Effect of epidermal growth factor receptor (EGFR) kinase inhibition on EGFR expression, endothelial function, and endoplasmic reticulum (ER) stress markers in mesenteric resistance arteries (MRAs) from streptozotocin (STZ) mice. A, EGFR mRNA levels in all groups, n=5. B, Representative Western blot analysis and quantitative data for phosphorylated EGFR and total EGFR in all groups, n=5. C, Endothelium-dependent relaxation in response to acetylcholine (ACh) in MRA from control, STZ + AG1478, and STZ groups, n=5. D, Endothelium-independent relaxation in response to single nucleotide polymorphism in MRAs from all groups, n=5. E, control; ×, STZ + AG1478; □, STZ. E, Representative Western blot analysis and quantitative data showing the phosphorylated endothelial NO synthase eNOS (P-eNOS) and total eNOS (T-eNOS), normalized to β-actin, in MRAs from all groups, n=4. F, Cyclic GMP levels in all the groups, n=4. G through J, Representative Western blot analysis showing the expression of phosphorylated eIF2-α, normalized to β-actin; total eIF2-α, n=4; and ATF-4, CHOP, and ATF-6 mRNA levels, normalized to 18S rRNA, in all groups, n=5. *P<0.05 for STZ vs control, #P<0.05 for STZ vs STZ + AG1478, and $P<0.05 for STZ + AG1478 vs control.
Figure 4. Effect of endoplasmic reticulum (ER) stress inhibition on endothelial function and ER stress markers expression in mesenteric resistance arteries (MRAs) from streptozotocin (STZ) mice. A and B, Endothelium-dependent and independent relaxation in response to acetylcholine (ACh) and single nucleotide polymorphism (SNP), respectively, in MRAs from control, STZ, STZ+insulin, and STZ+Tudca groups, n=5. •, control; □, STZ; ○, STZ+insulin; △, STZ+Tudca. C, Representative Western blot analysis and quantitative data
mRNA level of ATF6 (Figure 3F through 3I). The total eIF2α protein expression was similar in all of the groups of mice.

**Effect of ER Stress Inhibition on Microvascular Function in MRAs**

To delineate the role of ER stress in microvascular dysfunction in type 1 diabetes mellitus, we first examined EDR response in MRAs from STZ mice with or without Tudca or insulin. EDR was significantly improved in STZ mice treated with Tudca or insulin (61.3% and 84.3%, respectively; Figure 4A). Endothelium-independent relaxation was shifted to the right in STZ mice (EC50 = 6.27 ± 0.05) and was normalized after ER stress inhibition (EC50 = 6.94 ± 0.05) or insulin injection (EC50 = 7.43 ± 0.06; Figure 4B). These results were supported with the measurements of eNOS phosphorylation and expression and cGMP level, which were decreased by 4.0-, 3.0-, and 2.5-fold, respectively, in STZ mice and were normalized with Tudca and insulin treatments (Figure 4C and 4D). Microvascular endothelial dysfunction in STZ mice was associated with ER stress induction, as evidenced by enhanced phosphorylated eIF2α (4-fold) and the increase in the mRNA levels of ATF4 (4.5-fold), CHOP (3.0-fold), and ATF6 (9.0-fold; Figure 4E through 4H). Interestingly, insulin and ER stress inhibition (Tudca) were able to significantly reduce ER stress marker expression in MRAs from STZ mice (Figure 4E through 4H). The total eIF2α protein expression was similar in all of the groups of mice. In addition, EGFRtk phosphorylation in MRAs was similar in the STZ and STZ+Tudca groups (Figure S1B). The inhibition of NO–synthesis (Nω-nitro-L-arginine methyl ester; 100 μmol/L) reduced EDR by 17.3% in STZ mice, whereas a great reduction was observed in control and STZ mice treated with AG1478 (40%), Tudca (40%), or insulin (64%; Figure 5A through 5E).

To determine the link between ER stress and reactive oxygen species in microvascular endothelial dysfunction, we incubated MRAs with apocynin (100 μmol/L). The results revealed that apocynin significantly improved EDR in STZ mice (25%), whereas no effect was observed in control or STZ mice treated with AG1478, Tudca, or insulin (Figure 5A through 5E). The enhanced mRNA levels of Nox2 and Nox4 isoforms by 4- and 6-fold, respectively, in MRA from STZ mice supported these findings, which were blunted after inhibition of EGFRtk, ER stress, or insulin injection (Figure 5F through 5I). Moreover, the NADPH oxidase activity, determined in heart and MRA lysates, was increased in STZ mice by 3- and 4-fold, respectively, compared with control mice. The inhibition of EGFRtk and ER stress significantly decreased this activity (Figure S1C and S1D).

**Effect of ER Stress Induction by Tunicamycin on Phosphorylated EGFR Expression in Heart and MRAs**

The EGFRtk phosphorylation in heart and MRAs was similar in control and mice injected with Tunicamycin with and without Tudca (Figure S2A and S2B). In addition, the phosphorylated EGFRtk expression, in MRAs, remained unchanged in all of the groups (Figure S2C).

**Discussion**

In the present study, we found that EGFRtk phosphorylation and expression were upregulated in heart and microvessels of diabetic mice and were associated with ER stress induction, cardiac fibrosis, and microvascular endothelial dysfunction. Our results are supported by previous studies reporting that EGFRtk inhibition improved microvascular function in type 2 diabetes mellitus.9, 10 Interestingly, the inhibition of EGFRtk improved glucose levels, body weight, and microvascular function and reduced cardiac fibrosis and ER stress markers, with the exception of ATF6. These results suggest that exacerbated EGFRtk phosphorylation regulates cardiovascular dysfunction and metabolic alteration in type 1 diabetes mellitus, likely through phosphorylated protein kinase–like ER eukaryotic initiation factor 2α kinase-ATF4-derived ER stress branch but independent of the ATF6 branch.

Emerging evidence from experimental and clinical studies indicate that ER stress plays an important role in cardiovascular diseases17 and diabetes mellitus, as evidenced by peripheral insulin resistance and pancreatic β-cell dysfunction51, 23 related to ER stress.15, 19 In addition, ER stress has been demonstrated to be involved in the development of diabetes mellitus affecting different organs like liver, kidney, and skeletal muscle in several models of diabetic animals15, 24, 25; however, the role and mechanisms of ER stress in cardiac fibrosis and microvascular endothelial dysfunction in type 1 diabetes mellitus remain unclear. Previous studies have shown that ER stress is associated with heart failure and cardiomyopathy in nondiabetic and diabetic animals supporting the potential role of ER stress in cardiac damage.26–28 In the present work, we found that ER stress induction and cardiac fibrosis were associated with enhanced collagen type I and PAI-1 expression in diabetic mice. The inhibition of EGFRtk and ER stress reduced ER stress markers, suggesting that EGFRtk is upstream to ER stress activation. These data are supported by a recent publication showing that overexpression of aberrant EGFRtk in several cancers induces the expression of CHOP.14 In addition, chemical inhibition of EGFRtk and ER stress reduced cardiac fibrosis, collagen type 1, and PAI-1. Although the reduction on myocardial fibrosis appears pronounced in these animals, the effect of AG1487 and Tudca on blood glucose levels is modest, indicating that these drugs may act by mechanisms independent of their hypoglycemic effects. These results suggest that cardiac fibrosis in type 1 diabetes mellitus is regulated by an ER stress-dependent mechanism. However, it is unclear how ER stress controls collagen type 1 turnover, and additional studies are needed to delineate the mechanism.

Figure 4 (Continued). showing the phosphorylated eNOS (P-eNOS) and total eNOS (T-eNOS), normalized to β-actin, in all groups, n = 4. D, Cyclic GMP levels in all the groups, n = 4. E through H, Representative Western blot and quantitative data showing the expression of phosphorylated eIF2-α, normalized to β-actin, and total eIF2-α protein in all groups, n = 4, and CHOP, ATF-4, and ATF-6 mRNA levels, normalized to 18S rRNA, in all groups, n = 5 to 6. *P < 0.05 for STZ vs control, STZ+insulin; $P < 0.05 for STZ+Tudca vs STZ+insulin or control; #P < 0.05 for STZ vs STZ+Tudca.
It is well established that diabetes mellitus impairs microvascular function.\textsuperscript{29} It is known that hyperglycemia causes an enhancement in advanced glycation end products, oxidative stress levels, and increases EGFR tyrosine kinase activity leading to microvascular endothelial dysfunction, in part through the loss of NO bioavailability.\textsuperscript{9,30–32} The impaired EDR was also associated with a decrease in eNOS phosphorylation and expression in diabetic mice. These results are supported by previous studies showing a decrease in eNOS mRNA levels in KKAy mice\textsuperscript{33} and reduced eNOS phosphorylation in diabetic mice.\textsuperscript{9} Importantly, inhibition of EGFRtk and ER stress restored eNOS phosphorylation and expression in MRAs of diabetic mice indicating that eNOS expression and activity are regulated by EGFRtk and ER stress-dependent mechanisms. Although ER stress inhibition restored eNOS phosphorylation and expression to the control levels, EDR was partially improved, suggesting that other factors contribute to EDR impairment in MRAs.

Previous reports provided evidence that ER stress increases oxidative stress levels, which represent another mechanism regulating eNOS activity and NO bioavailability.\textsuperscript{34,35} NADPH oxidase seems to be the main source of oxidative stress in animal models of diabetes mellitus.\textsuperscript{36} Our data demonstrated that microvascular EDR was improved after the
inhibition of NADPH oxidase activity in diabetic mice, whereas no effect was observed in control and diabetic mice treated with EGFRtk and ER stress inhibitors. These data indicate that EGFRtk and ER stress-dependent mechanisms regulate NADPH oxidase activity. These results are supported by the reduction in NADPH oxidase activity and Nox-2 and Nox-4 expression in diabetic mice after EGFRtk and ER stress inhibition. In addition, Nox2 and Nox4 have been shown to be predominantly located in the perinuclear and/or ER membranes, suggesting a relationship between reactive oxygen species generation and ER stress in diabetes mellitus.37

In conclusion, we demonstrated that, in type 1 diabetes mellitus, the exacerbation in EGFRtk signaling contributes to ER stress induction as a mechanism in part responsible for cardiac fibrosis and microvascular dysfunction. Thus, EGFRtk and ER stress could be potential targets for novel therapeutic strategies to improve cardiovascular function in diabetes mellitus.

Perspectives
Diabetes mellitus is a metabolic disease associated with cardiovascular complications, including cardiac damage and impaired microvascular EDR. Most of clinical studies indicate that diabetic patients are at high risk for cardiovascular diseases. Despite the fact that treatments have progressed, the development of novel effective treatments for diabetic patients with vascular complications remains a major research goal. Therefore, there is a significant medical need to develop novel therapies to restore microvascular endothelial function in these patients. Our results indicate that exacerbated EGFRtk activity and ER stress play key roles in heart damage and vascular dysfunction in type 1 diabetic mice. Interestingly, inhibition of EGFRtk activity decreases ER stress markers, suggesting that ER stress is downstream of the EGFRtk pathway. The inhibition of EGFRtk and ER stress reduces cardiac fibrosis and improves microvascular function associated with enhance in eNOS phosphorylation, cGMP levels, and reduction in NADPH oxidase activity. Therefore, EGFRtk and ER stress could be potential targets for novel therapeutic strategies to improve cardiovascular function in diabetes mellitus.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is New?**

- This is the first study to demonstrate that the exacerbation in EGFRtk signaling contributes to ER stress induction as a mechanism responsible for cardiac fibrosis and microvascular dysfunction in type 1 diabetes mellitus.

**What Is Relevant?**

- This study unveils novel roles for enhanced EGFRtk phosphorylation and its downstream ER stress in cardiac fibrosis and microvascular endothelial dysfunction in type 1 diabetes mellitus.

**Summary**

EGFRtk phosphorylation and expression were upregulated in heart and microvessels of diabetic type 1 mice and were associated with ER stress induction, cardiac fibrosis, and microvascular endothelial dysfunction. Interestingly, the inhibition of EGFRtk and ER stress improved body parameters, cardiac fibrosis, and microvascular function.
A Novel Role for Epidermal Growth Factor Receptor Tyrosine Kinase and Its Downstream Endoplasmic Reticulum Stress in Cardiac Damage and Microvascular Dysfunction in Type 1 Diabetes Mellitus

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A novel role for EGFR tyrosine kinase and its downstream endoplasmic reticulum stress in cardiac damage and microvascular dysfunction in type 1 diabetes

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Running title: EGFR/ER stress and cardiovascular complication in diabetes

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MATERIALS AND METHODS

General protocol in mice

All experiments were performed according to the American Guidelines for the Ethical Care of Animals and were approved by Tulane University Animal Care and Use Committee. Mice (C57BL/6J, 8 weeks-old males) were purchased from Jackson Laboratories (Bar Harbor, ME) housed in groups of five and maintained at a temperature of 23°C with 12 hours light/dark cycle. Mice were fed on a solid standard diet (Na⁺ content 0.4%) and water.

Diabetes was induced by a single intra-peritoneal injection of streptozotocin (STZ, 200 mg/kg, dissolved freshly in citrate buffer, pH 4.5) to fasted mice for 12 hours. Hyperglycemia was confirmed by measuring tail vein blood glucose levels with glucometer (Accu-Chek, Roche Diagnostic, Germany). Mice with blood glucose levels ≥ 300 mg/dl were considered as type 1 diabetic.

One week after the induction of diabetes, mice were divided into five groups: 1) Diabetic group (STZ); 2) Diabetic group treated with AG1478 (10 mg/Kg/day) (STZ + AG1478); 3) Diabetic group treated with Tudca (150 mg/kg/day) (STZ + Tudca); 4) Diabetic group treated with Insulin (0.1 U/day) (STZ + Insulin) using insulin-implants placed underneath of the skin (Linshin, Canada); 5) Control group (Control). Mice were treated for 2 weeks.

Body weight and blood glucose levels were measured during the treatment period. At the end of treatment period, mice were sacrificed and blood samples were collected to determine the plasma concentration of insulin by the ELISA kit (Mercodia Ultrasensitive Mouse Insulin ELISA, USA). Heart and MRA were harvested immediately, placed in PSS solution (composition in mmol/L: NaCl 118; KCl 4.7; CaCl₂ 2.5; KH₂PO₄ 1.2; MgSO₄·7H₂O 1.2; NaHCO₃ 25 and glucose 11), pH=7.4 and processed appropriately for further studies.

In another set of experiments, we used 8 weeks-old C57BL/6J male mice divided into three groups: 1) Control group; 2) control group that received intra-peritoneal injection of Tunicamycin (Tunica, 1 mg/kg, 2 injections/week for 2 weeks, Control + Tunica); 3) control group that received Tunicamycin and Tudca (150 mg/kg/day) for 2 weeks (Control + Tunica + Tudca). At the end of treatment, mice were anaesthetized with isoflurane and then heart and mesenteric resistance arteries were immediately harvested and processed for further experiments.
**Cardiac fibrosis**

A transverse section of heart was fixed in 4% of formalin, embedded in paraffin and cut into 4 µm thick sections. Slices were stained with the collagen-specific stain Sirius-red (Sigma-Aldrich, USA). At least eight areas from each heart were captured using a high-resolution digital camera (Olympus DP50, Japan). The collagen was quantified using Adobe Photoshop CS2 (Microsoft). For each image, the percentage of interstitial fibrosis was determined as the ratio of collagen surface area to myocardial surface area.

**Mesenteric Resistance Arteries Reactivity**

Microvascular responses to acetylcholine (ACh) and sodium nitroprusside (SNP) were performed as previously described.\textsuperscript{40} To determine the role of NADPH oxidase in the impaired endothelium-dependent relaxation in diabetic mice, MRA were incubated with apocynin (100 µmol/L) for 30 minutes and then EDR responses were performed after pre-contraction with phenylephrine.

**Western blot analysis**

Mice were sacrificed, heart and MRA were immediately harvested and frozen in liquid nitrogen and then stored at -80°C. Western blot analysis for eNOS, PAI-1, eIF2-α, CHOP and EGFRtk (1:1000 dilution, Cell Signaling Technology, Inc, USA), collagen-1 and β-actin (1:500 dilution, Santa Cruz Biotechnology, Inc) was performed using specific antibodies as previously described.\textsuperscript{4}

**RT-PCR real-time assay**

EGFRtk, Nox, CHOP, ATF4 and ATF6 mRNA levels were determined in MRA and heart samples from all groups as previously described.\textsuperscript{4} Assays-on-Demand (Applied Biosystems) of TaqMan fluorescent real time PCR primers and probes were used for Egfr (Mm00433023_m1), Chop (Mm00492097_m1), Atf4 (Mm00515324_m1), Atf6 (Mm01295317_m1), Nox-2 (Mm01287743_m1), Nox-4 (Mm00479246_m1) and 18S rRNA (Hs99999901_s1), which was used as endogenous control to normalize results. Quantitative RT-PCR was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the following conditions: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at
95°C and 1 min at 60°C. Relative mRNA levels were determined using the $2^{-\Delta \Delta Ct}$ method. Results are expressed as the relative expression of mRNA in treated mice compared with untreated mice.

**Colorimetric Determination of cGMP**

The cGMP levels were measured in MRA lysates in all groups of mice. Measurements were performed using a sandwich enzyme-linked immunosorbent assay (ELISA; Cayman Chemical, Ann Arbor, MI) according to the manufacturer instructions.

**Immunohistochemistry**

Hearts and MRA were fixed in 4% of paraformaldehyde followed by zinc-saturated formalin and paraffin-embedded for immunoperoxidase staining using the Vectastain ABC Kit (USA). The sections were incubated overnight with anti-p-EGFR antibody (1:200, sc-101668, Santa Cruz Biotechnology, USA). At least eight sections from each heart and MRA were captured using a high-resolution digital camera (Olympus DP50, Japan).

**NADPH oxidase activity assay**

Superoxide anion levels generated by NADPH oxidase activity were measured in lysates of heart and MRA using lucigenin chemiluminescence. Briefly, lysates were prepared in a sucrose buffer containing KH$_2$PO$_4$ 50 mM, EGTA 1 mM, sucrose 150 mM; pH=7.0 and protease inhibitor cocktail (complete mini, Roche Diagnostics, IN, USA) in a Tissue Dounce homogenizer on ice, and aliquots of the homogenates were used immediately. To start the assay, a volume of 100 µL of each lysate was used in a total volume of 1 mL PBS buffer preheated at 37°C, containing lucigenin (5 µM) and NADPH (100 µM). Blank samples were prepared using 100 µL of sucrose buffer. Lucigenin activity was measured every 30 seconds during about 10 min in a luminometer (Turner biosystem 20/20, single tube luminometer) till enzymatic activity is reaching the plateau. Data are expressed as area under the curve of relative light units (RLU) normalized to protein content (µg protein).
Drugs

Phenylephrine hydrochloride, acetylcholine, sodium nitroprusside, U46619, apocynin, NADPH and L-NAME were obtained from Sigma-Aldrich (USA). Sustained release insulin implants were obtained from Linshin (Canada). Streptozotocin was obtained from Alexis Zonko (USA), Tudca from Calbiochem (USA and Canada) and AG1478 (T-7310 Tyrphostin AG1478) was purchased from LC Laboratories (USA and Canada). Stock solutions of drugs were prepared in ultrapure water, stored at -20 ºC and appropriate dilutions were made on the day of the experiments.

Statistical analysis

Data are expressed as mean ± SEM. Dose-response curves were analyzed using GraphPad Prism 4.0 software (GraphPad, USA). Statistical analysis for significant differences was performed using Student’s t test, one-way or two-way ANOVA as appropriate. Significance was accepted at p< 0.05.

REFERENCES


EGFRtk phosphorylation and NADPH oxidase activity in heart and MRA from control, STZ, STZ+AG1478 and STZ+Tudca mice groups. A-B: Western blot analysis and quantitative data for phosphorylated EGFR (P-EGFR) in heart and MRA, in all groups, n=3; C-D: NADPH oxidase activity in heart and MRA lysates quantified in all groups, n=5. *P<0.05 for STZ vs. control, #P<0.05 for STZ+Tudca vs. control.
Heart and MRA EGFR phosphorylation (P-EGFR) in control and mice injected with tunicamycin with and without Tudca. A-B: representative heart and MRA sections for P-EGFR, n=4. C: Western blot analysis and quantitative data for P-EGFR in MRA in all groups, n=3.