Supplemental Materials

# 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 23ºC 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.[1] 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)[2] (STZ + AG1478); 3) Diabetic group treated with Tudca (150 mg/kg/day)[3] (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; $CaCl_2$ 2.5; $KH_2PO_4$ 1.2; $MgSO_4x7H_2O$ 1.2; $NaHCO_3$ 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.[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.[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.[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 $KH_2PO_4$ 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 37ºC, 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**


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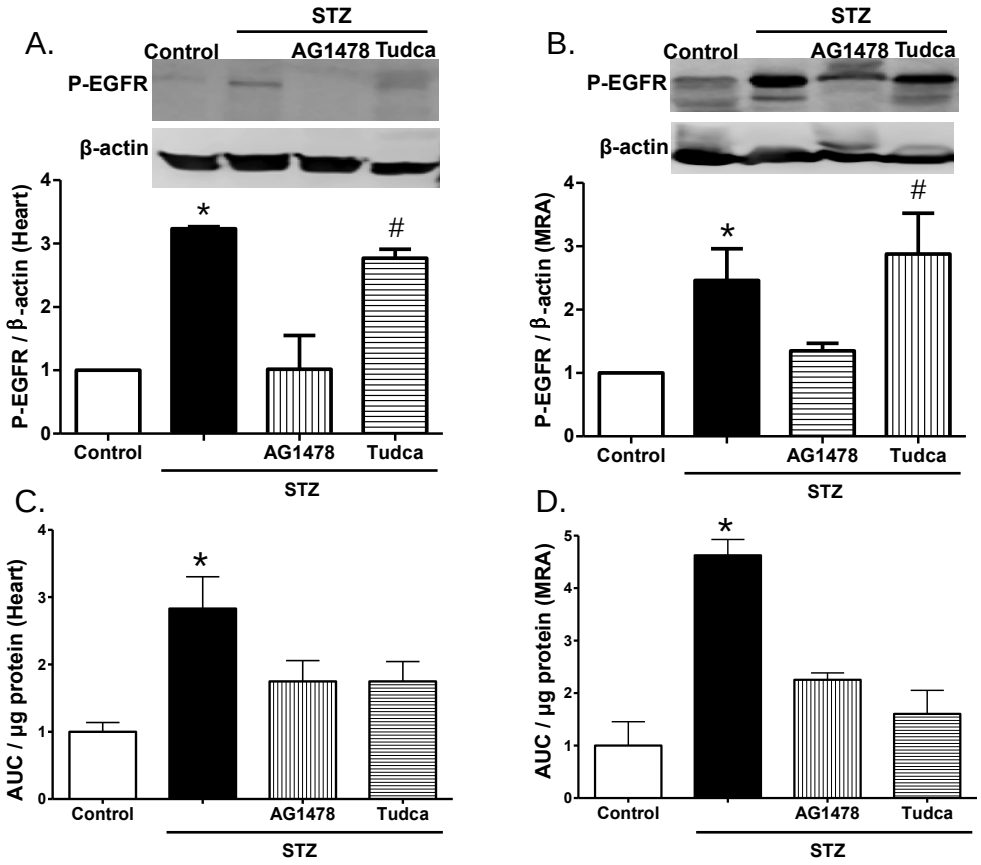


Figure S1

**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.

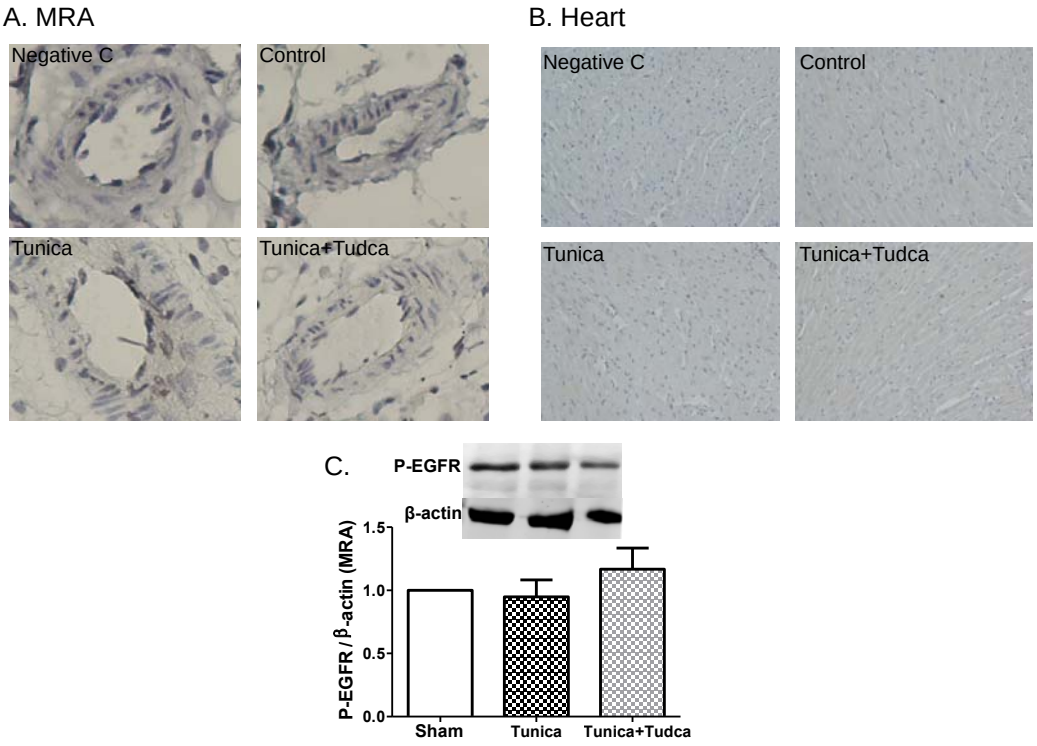


Figure S2

**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.