JACBTS 327 Online Appendix

SUPPLEMENTARY MATERIALS

Cardiac Microvascular Endothelial Enhancement of Cardiomyocyte Function is Impaired by Inflammation and Restored by Empagliflozin

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Brief title: Empagliflozin on endothelial-cardiomyocyte axis

Subject codes: Heart failure, Contraction and relaxation, Endothelial cell-derived nitric oxide, Oxidative stress, Empagliflozin

Supplementary methods

Adult rat ventricular cardiomyocyte isolation and culture

Rat cardiomyocytes were isolated using Liberase digestion of hearts as described previously (1,2). Adult wild-type male Wistar rats weighing (200-250 g) were sacrificed by isoflurane (Pharmachemie, 45.112.110, Harlem, The Netherlands) inhalation. The heart was injected with, quickly removed, and rinsed in cold isolation Tyrode solution pH 7.35 (130 mM NaCl, 4 mM KCl, 10 mM Hepes, 1.2 mM $MgSO_4$*$7H_20$, 1.2 mM NaH_2PO4*H_2O, 11 mM D-glucose) containing 0. 2 mM EGTA (Tyrode–EGTA, Titriplex VI, Merck, 67-42-5, Amsterdam, The Netherlands). The heart was then cannulated via the aorta to the Langendorff apparatus and perfused for 5 minutes with Tyrode–EGTA at 37°C. Thereafter, the heart was perfused with enzyme Liberase-Tyrode solution consisting of Tyrode solution and 75 mg/ml Liberase (Roche, 113211, Bazel, Zwitserland) for a period of 25 minutes. The heart was detached from the cannula, and the right ventricle and atria were removed. The left ventricle was cut into small pieces and triturated with a plastic Pasteur pipette for 5 minutes in Tyrode solution at 37°C. The supernatant was discarded and this step was repeated one more time. The left ventricle pieces were triturated in Tyrode solution supplemented with 1 M $CaCl_2$ (Sigma, 21115-100mL, St. Louis, Missouri) and 10 kU DNase 1 type 2 (Sigma, D4527-40KU) and filtered through a 300 µm nylon mesh filter into a 50 ml Falcon tube (Grenier bio-one, 227261, Monroe, North Carolina). Subsequently, the cell suspension was re-suspended in $CaCl_2$ buffers of increasing Ca^{2+} concentrations to reach a final concentration of 1 mM. Isolated adult cardiomyocytes were finally re-suspended in plating medium containing Medium 199 (Lonza Europe, BE12-117F, Breda, The Netherlands), 1% penicillin/streptomycin (Lonza, DE17-602DE) and 5% fetal bovine serum (PAA Cell Cutrure Company, A15-101, Cambridge, England), and seeded on 1% laminin (L2020-1MG, Sigma) coated plates (24-well format Costar culture plate, Corning, 3524, New York, US). One hour after plating, cells that were not attached were removed by replacing the plating medium with culture medium (EGM2-MV, Lonza, CC-3203). Subsequently, the cells were co-cultured with CMEC pre-seeded on inserts at 37°C in humidified air with 5% CO_2.

References

1. Kaestner L, Scholz A, Hammer K, Vecerdea A, Ruppenthal S, Lipp P. Isolation and genetic manipulation of adult cardiac myocytes for confocal imaging. J Vis Exp 2009;31:1-5.
2. van Deel ED, Najafi A, Fontoura D et al. In vitro model to study the effects of matrix stiffening on Ca(2+) handling and myofilament function in isolated adult rat cardiomyocytes. J Physiol 2017;595:4597-4610.

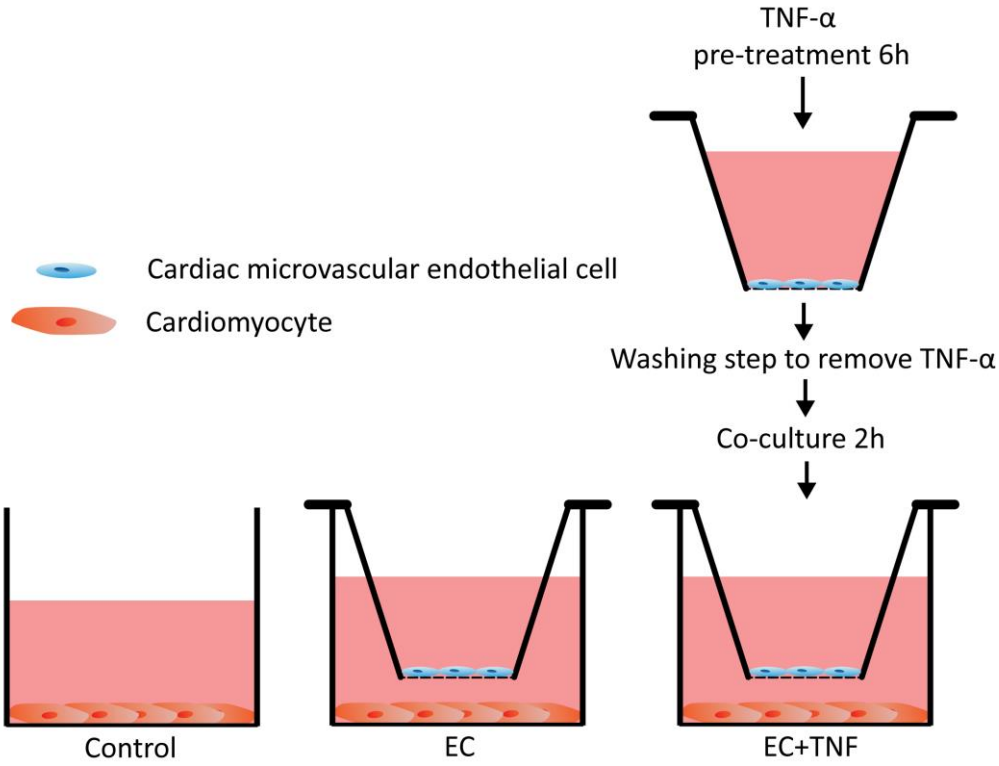
Supplementary Table

Supplementary Table 1. Primer list

Gene	Forward	Reverse
β2 microglobulin (B2M)	TTCATCCATCCGACATTGAA	CCTCCATGATGCTGCTTACA
Nitric oxide synthase 3 (NOS3)	TGGCTTTCCCTTCCAGTTC	AGAGGCGTTTTGCTCCTTC
NADPH oxidase 4 (NOX4)	ATGTTGGGGCTAGGATTGTG	CTCCTGCTTGGAACCTTCTG
Superoxide dismutase 1 (SOD1)	AGGCATGTTGGAGACTTGGG	CCACAAGCCAAACGACTTCC
Superoxide dismutase 2 (SOD2)	AGGATCCACTGCAAGGAACA	CATAAAGAGCTTAACATACTCAGCA
VCAM1	ACAAAGGCAGAGTACGCAAACA	GGCTGACCAAGACGGTTGTATC
E-selectin	TGTGCAAGTTCGCCTGTCC	CATGTCCGAGCTGCAGAGC

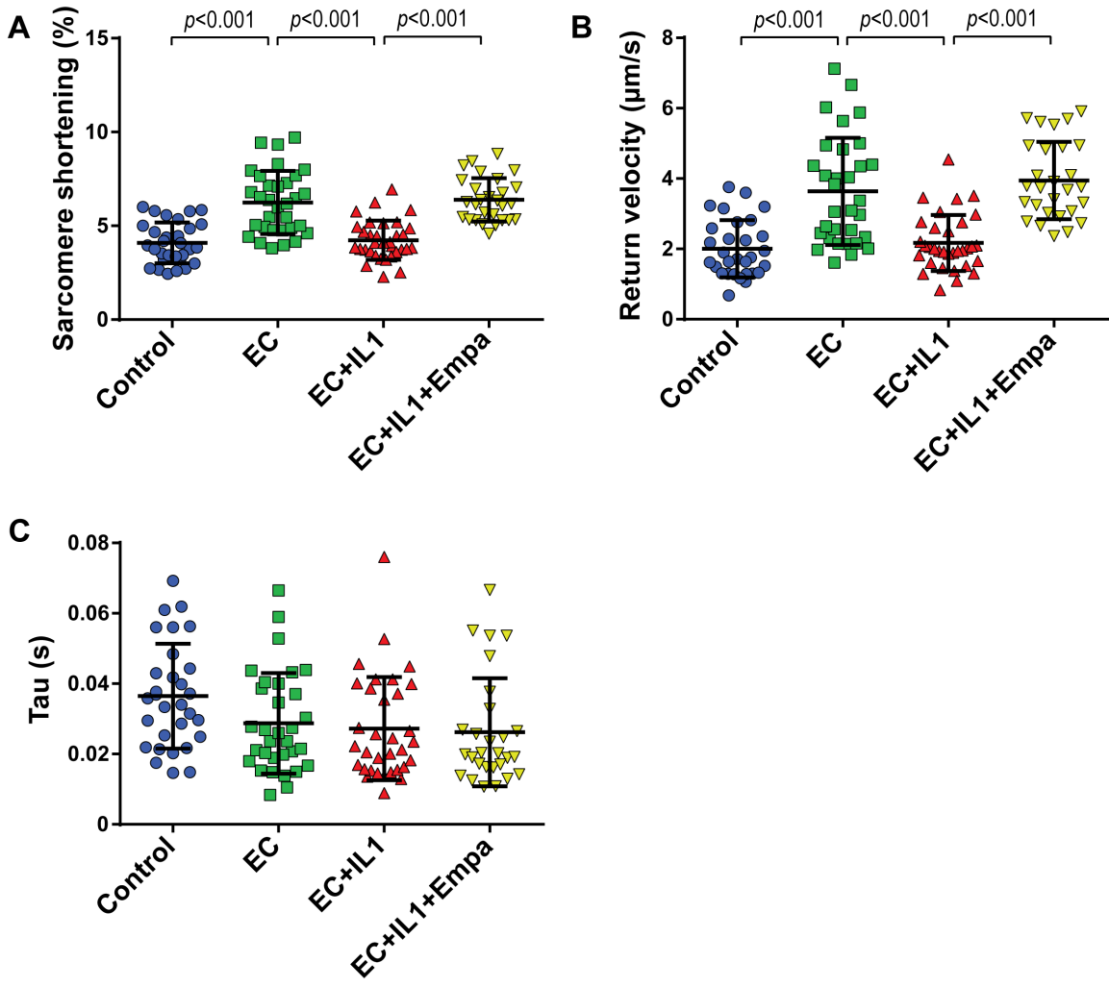
Supplementary figures

Supplementary Figure 1.



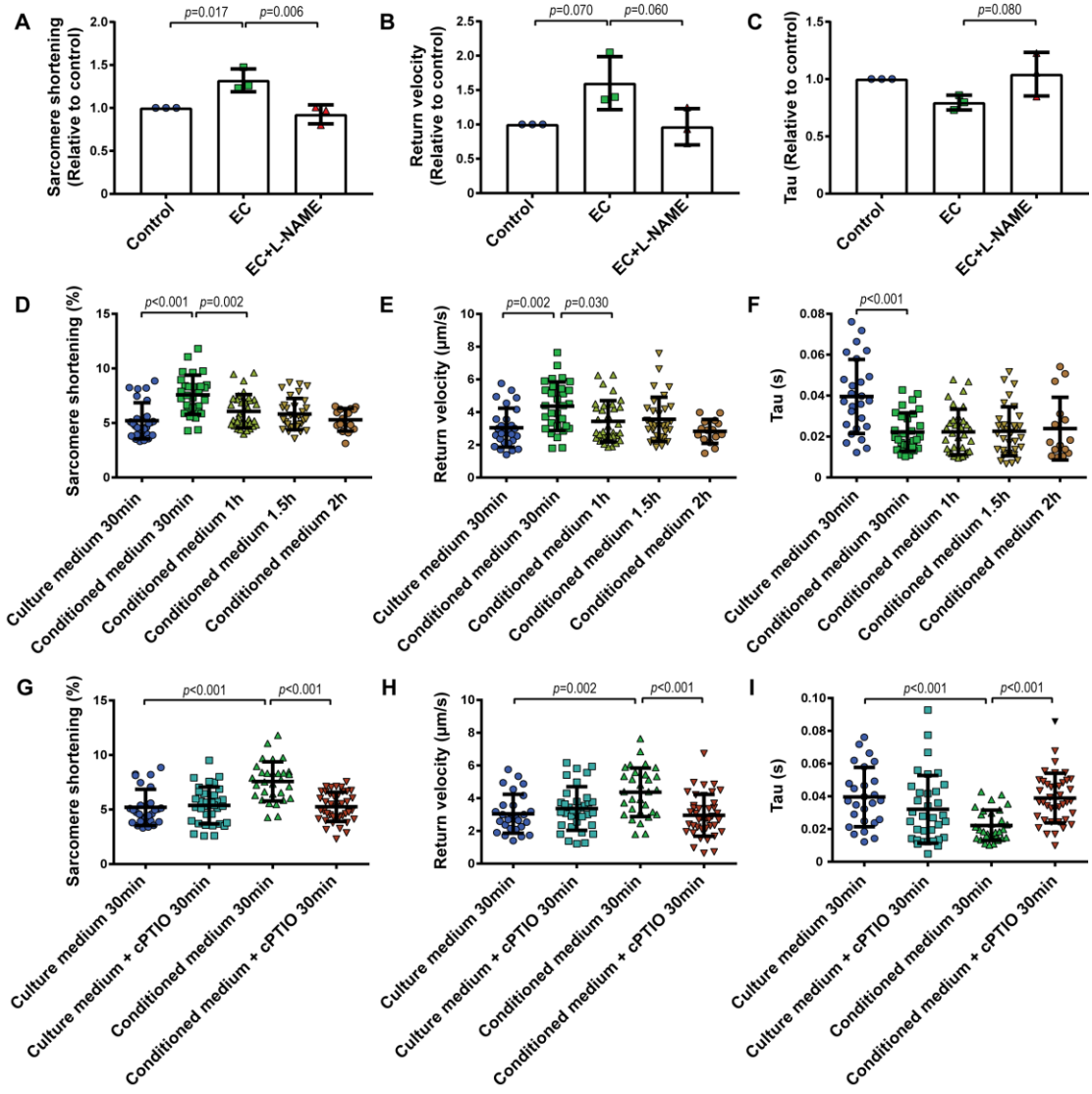
Supplementary figure 1. CMEC-CM co-culture experiment set-up. Experiment set-up with non-co-incubated CMs (control), co-culture with CMEC (EC), and co-culture with CMEC treated with TNF-α prior to co-culture procedure (EC+TNF), respectively.

Supplementary Figure 2.



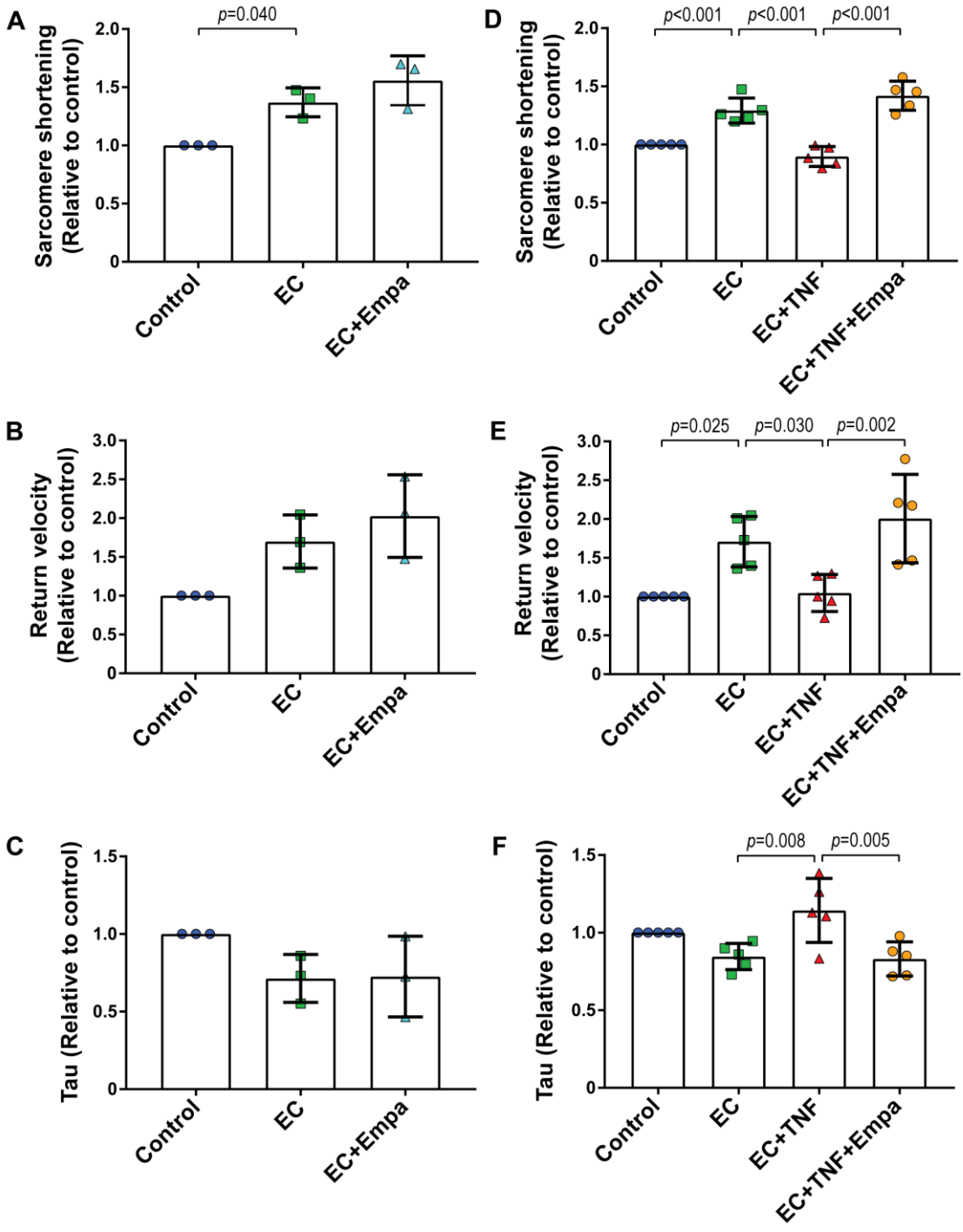
Supplementary figure 2. The beneficial effect of empagliflozin on CMEC-enhancing effect of CM function after IL-1β stimulation on CMEC. Co-treatment of IL1β-stimulated CMEC with empagliflozin prevented reduction of CM contraction in comparison to IL1β stimulation alone (A). Co-treatment with empagliflozin also maintained CMEC beneficial effect on CM relaxation velocity (B) and tau (C). (A, B, C are graphs representing single CMs isolated from one individual rat, distributed into the four corresponding experimental conditions; 40-45 CMs were measured per condition; data are represented as mean ± SD).

Supplementary Figure 3.



Supplementary figure 3. The effect of inhibition or scavenging of CMEC-derived NO on CM contraction and relaxation. Inhibition of CMEC NO generation with L-NAME abrogated the effect of CMEC on CM sarcomere shortening (A), return velocity (B), and tau (C). (A, B, C are graphs of combined average values obtained from 3 independent experiments corresponding to 3 individual rats; data are represented as mean ± SD). Incubation of CMs with CMEC-conditioned medium improved CM contractility performance in a time-dependent manner (D, E, F). (D, E, F are graphs representing single CMs isolated from one individual rat, distributed into the five corresponding experimental conditions; 40-50 CMs were measured per condition; data are represented as mean ± SD) . Incubation of CMs with cPTIO-treated CMEC-conditioned medium abolished the beneficial effect of the conditioned medium on CM function. Treatment of CMs with cPTIO-treated control culture medium did not affect CM contractility (G, H, I) (G, H, I are graphs representing single CMs isolated from one individual rat, distributed into the four corresponding experimental conditions; 40-50 CMs were measured per condition; data are represented as mean ± SD).

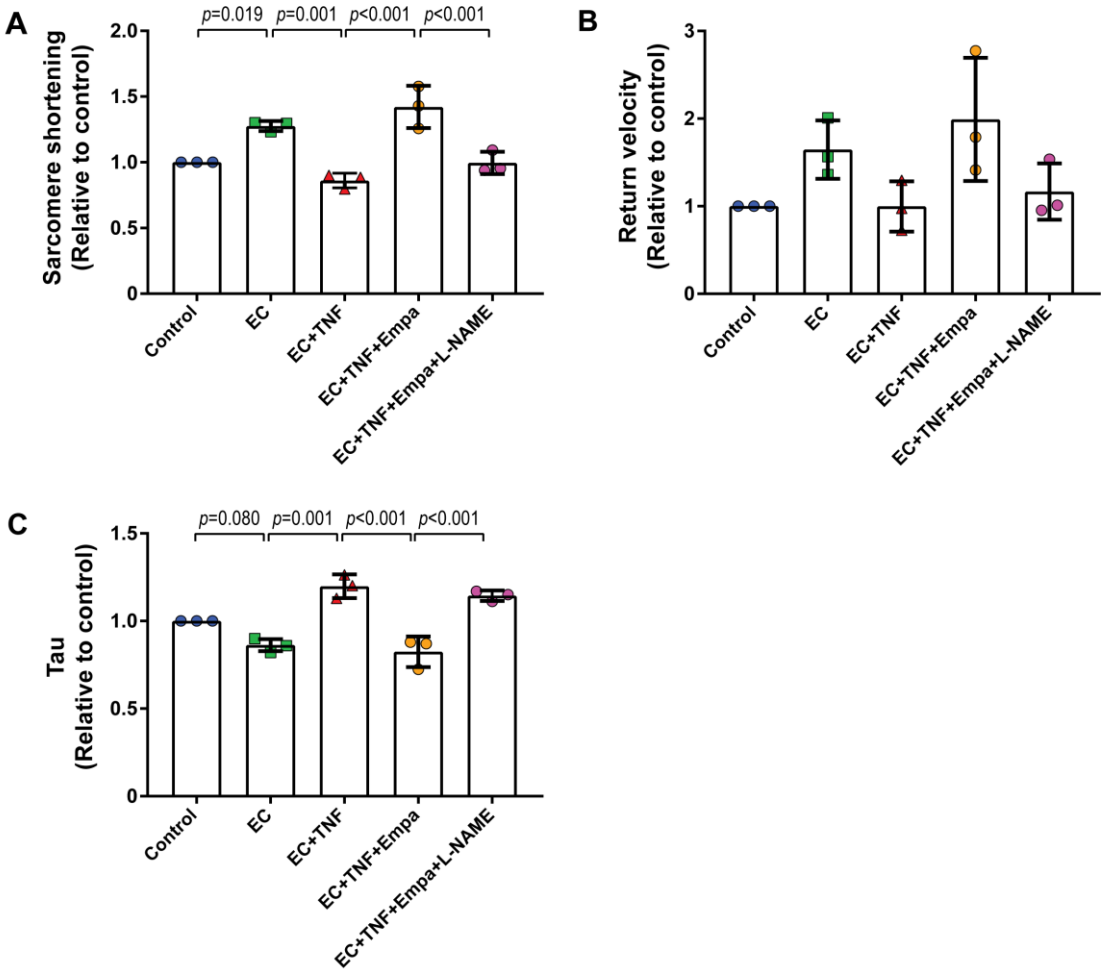
Supplementary Figure 4.



Supplementary figure 4. The effect of empagliflozin on CMEC-mediated CM function. Co-culture of CMEC with CMs increased CM shortening (A) and speed of relaxation (B), and shortened tau (C). Pre-treatment of CMEC with empagliflozin (EC+Empa) resulted in a slight increase of CM contraction compared to CMEC alone (EC) (A). Empagliflozin pre-treatment significantly increased CM return velocity (B), but did not affect relaxation time, in comparison to CMEC alone. Co-treatment of TNF-

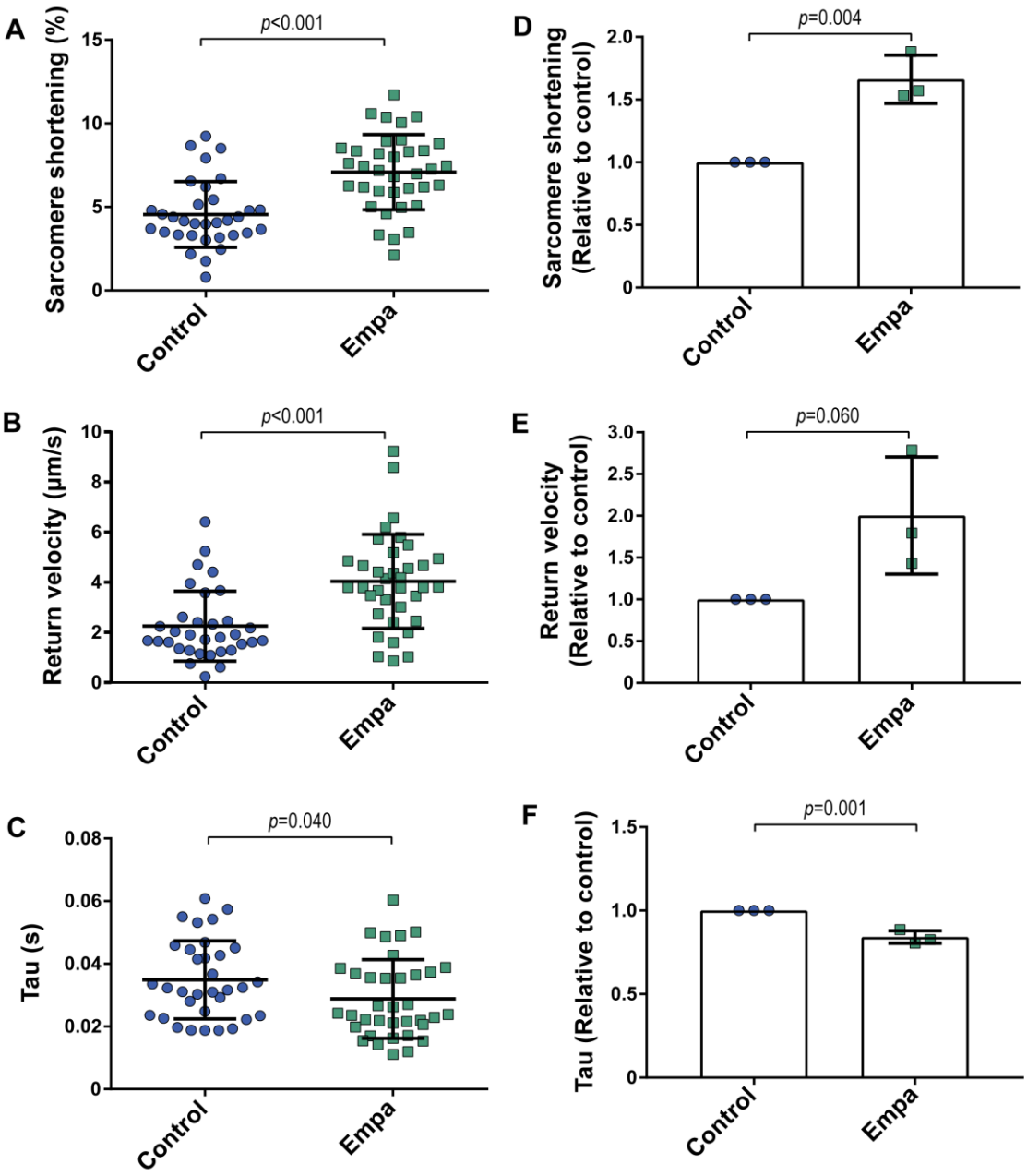
α-stimulated CMEC with empagliflozin prevented reduction of CM contraction in comparison to TNF-α stimulation alone (D). Co-treatment with empagliflozin also maintained CMEC effect on CM relaxation velocity (E) and tau (F). (A, B, C are graphs of combined average values obtained from 3 independent experiments corresponding to 3 individual rats; data are represented as mean ± SD; D, E, F are graphs of combined average values obtained from 5 independent experiments corresponding to 5 individual rats; data are represented as mean ± SD).

Supplementary Figure 5.



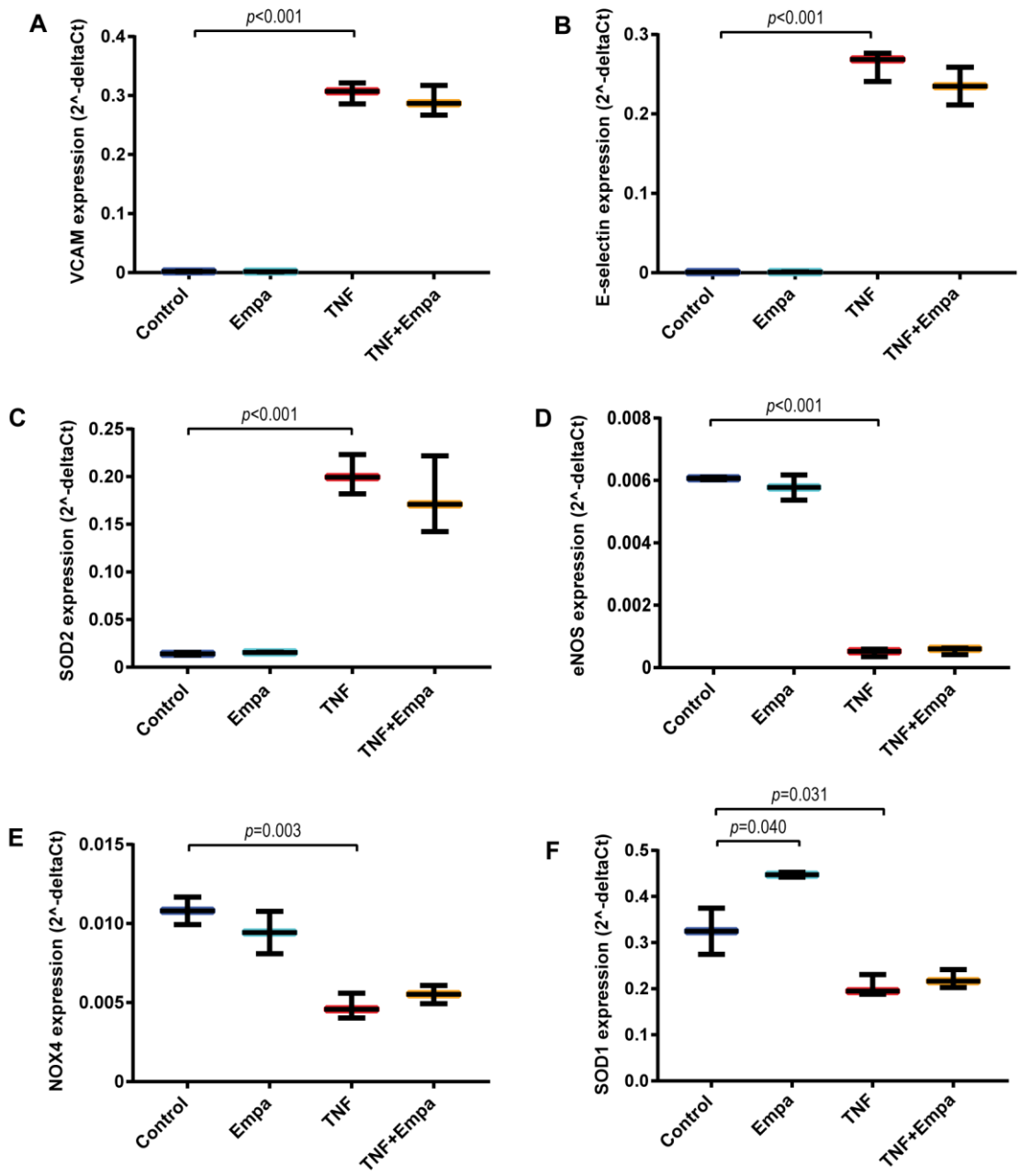
Supplementary figure 5. Abrogation of the beneficial effect of empagliflozin after co-treatment with L-NAME. Empagliflozin co-treatment eliminated the detrimental effect of TNF-α on CM contraction (A) and relaxation (B, C). The beneficial effect of empagliflozin was abolished when CMEC were co-treated with L-NAME (A-C). (A, B, C are graphs of combined average values obtained from 3 independent experiments corresponding to 3 individual rats; data are represented as mean ± SD).

Supplementary Figure 6.



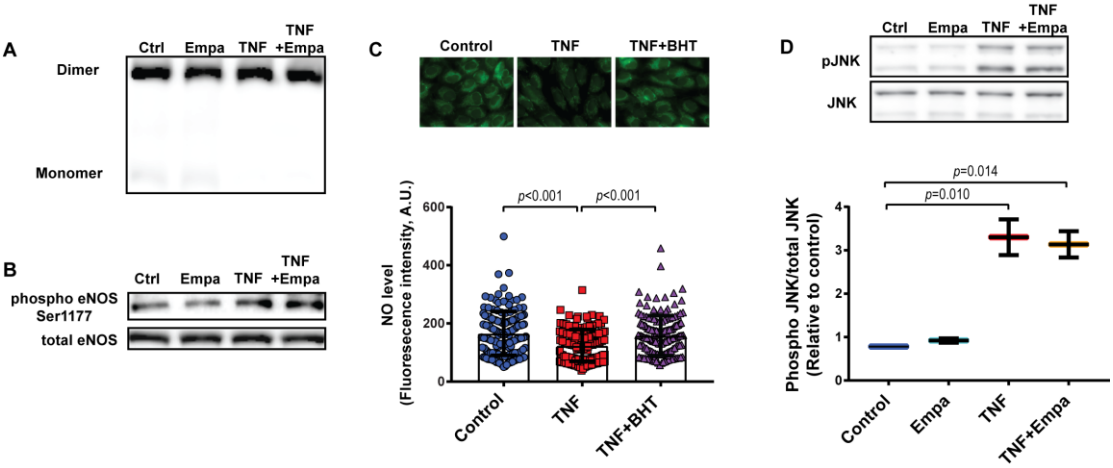
Supplementary figure 6. The direct effect of empagliflozin on CM contraction and relaxation performance. Treatment of CM with empagliflozin (Empa) increased CM contraction (A, C) and improved CM relaxation as shown by increased return velocity (B, D) and shortened tau (C, E). (A, B, C are graphs representing single CMs isolated from one individual rat, distributed into the two corresponding experimental conditions; 40-45 CMs were measured per condition; data are representative of 3 independent experiments as shown in D, E, F; D, E, F are graphs of combined average values obtained from 3 independent experiments corresponding to 3 individual rats; all data are represented as mean ± SD).

Supplementary Figure 7.



Supplementary figure 7. mRNA expression of VCAM, E-selectin, SOD1, SOD2, eNOS and NOX4, after TNF-α and empagliflozin treatment. TNF-α stimulation led to increased VCAM, E-selectin, and SOD2 mRNA expression in CMEC and co-treatment with empagliflozin did not change the expression (A, B, C). TNF-α reduced eNOS, NOX4, and SOD1 mRNA expression with no effect of empagliflozin observed (D, E, F). (n=3, refers to number of independent experiments; data represented as mean ± SD).

Supplementary Figure 8.



Supplementary figure 8. eNOS dimer/monomer status and phosphorylation, NO level after BHT treatment, and phosphorylation status of JNK after TNF-α and empagliflozin treatment. eNOS dimerization in CMEC was unchanged after 6h TNF-α exposure, while the small amount eNOS monomer decreased (A). Empagliflozin did not affect eNOS dimer/monomer status (A) (n=2, refers to number of independent experiments; data represented as mean ± SD). Empagliflozin did not change total eNOS protein content or the phosphorylation of eNOS at Ser1177 in control or TNF-α-treated CMEC (B) (n=2, refers to number of independent experiments; data represented as mean ± SD). BHT restored NO level after TNF-α stimulation in CMEC (C) (n=150-160, refers to number of cells per group from 3 independent experiments; data represented as mean ± SD). TNF-α stimulation induced phosphorylation of JNK while total protein expression was not changed. Co-treatment with empagliflozin did not change phosphorylation nor the total protein expression of JNK. (n=2, refers to number of independent experiments; data represented as mean ± SD).