The Cardiovascular Benefits of Empagliflozin, A Sodium Glucose Cotransporter Inhibitor: Is NHE1 a Viable Target?

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Background

- Poor glycaemic control is an important contributor to the development of heart failure (HF).
- Type 2 diabetes mellitus (T2DM) has almost doubled the risk of developing heart failure.
- Increased the levels of intracellular Na+ lead to cardiac hypertrophy.
- Empagliflozin (EMPA), a sodium-glucose cotransporter-2 (SGLT-2) inhibitor, regulates glucose levels and reduces myocardial intracellular Na+ levels.
- The Na+/H+ exchanger isoform 1 (NHE1) is located in the heart and functions by regulating the cardiomyocytes pH.
- Enhanced expression of NHE1 has been implicated in the progression of heart failure by promoting cardiac hypertrophy.
- Activation of NHE1 is directly coupled to the activation of sodium/calcium exchanger (NCX).
- Elevated levels of cytoplasmic Na+ leads to increased levels of cytoplasmic Ca2+ levels, which induces cardiomyopathy and injury to the heart.

Hypothesis/ Objectives

- **Hypothesis** EMPA mediates its cardiovascular benefits partially by inhibiting NHE1 activity.
- **The objectives of this study were** to evaluate whether ANG II induces cardiomyocyte hypertrophy and if so, whether EMPA reduces angiotensin-induced cardiomyocyte hypertrophy by regressing NHE1 protein expression

Methods

- **Study design** In-vitro experimental study
- **Cell culture** H9c2 cardiomyoblasts were plated at 35 mm dish and cultured in DMEM/F12 1:1 culture media containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37° C for 24 hours, were starved in FBS free DMEM medium for additional 24 hours and then treated with ANG II (100nM), EMPA (500nM), and ANG II+ EMPA for 18 hours.
- **Crystal violet staining** After treatment, cells were fixed and stained with crystal violet stain. Measurement of the cell surface area was done using AxioVision Imaging software. An average of 50-70 measures was taken from each treatment group and represented as one n value for each group.
- **Protein assay** Fresh Bovine serum albumin (BSA) stock solution and 6 dilutions of protein standards were prepared. BSA dilutions and samples were loaded into wells. After 15 minutes, the absorbance was read at 750 nm using Spectramax M2 spectrophotometer located in Dr. Ashraf lab (corridor C).
- **Western blot** Cells were lysed with RIPA and transferred into microcentrifuge tubes. Protein samples were loaded into gels. The gels were run and then transferred onto nitrocellulose membrane. The membrane was then blocked, primary antibodies were added, and incubated overnight. Secondary antibodies were incubated for 1.5 hours. Proteins were visualized using chemiluminescence and imaged using the Alpha Innotech FluorChem® Imager (R&D Systems).

Results

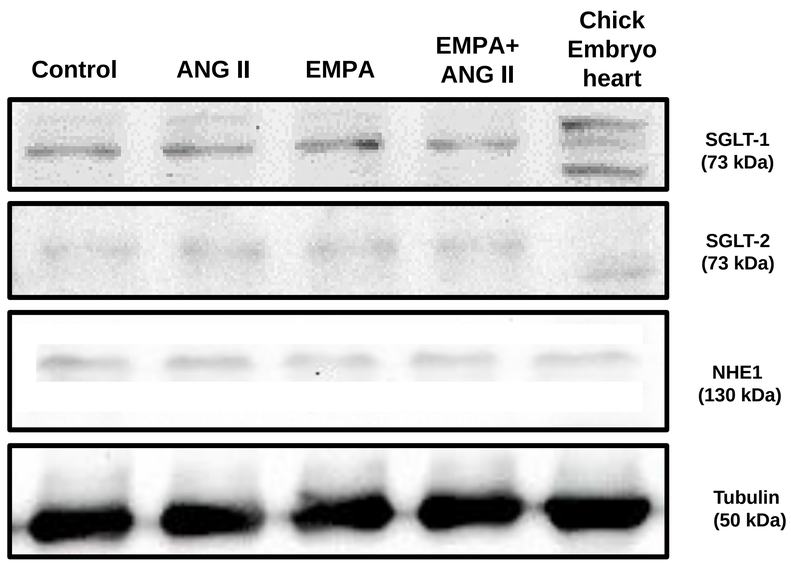

Figure 1: EMPA reduced SGLT-1 expression following stimulation with ANG II in H9c2 cardiomyoblasts
Representative western blot of SGLT-1 (73 kDa), SGLT-2 (73 kDa) and NHE1 (130 kDa) protein expression, normalization was against alpha-tubulin (50 kDa) in H9c2 cardiomyoblasts. Protein expression was tested in non-treated (control), 100nM ANG II, 500nM EMPA, and EMPA + ANG II treated cells for 18 hours in 3 sets.

Table 1: Protein concentrations of H9c2 cardiomyoblasts treated with ANG II in the presence and absence of EMPA

Set #	Samples	Conc. (mg/mL)
Set 4	Control	1.386089425
	Ang II	**1.591199432**
	EMPA	**1.375916726**
	EMPA+ANG	**1.304944405**
Set 5	Control	1.387863733
	Ang II	**1.407381121**
	EMPA	**1.310622191**
	EMPA+ANG	**1.400757038**

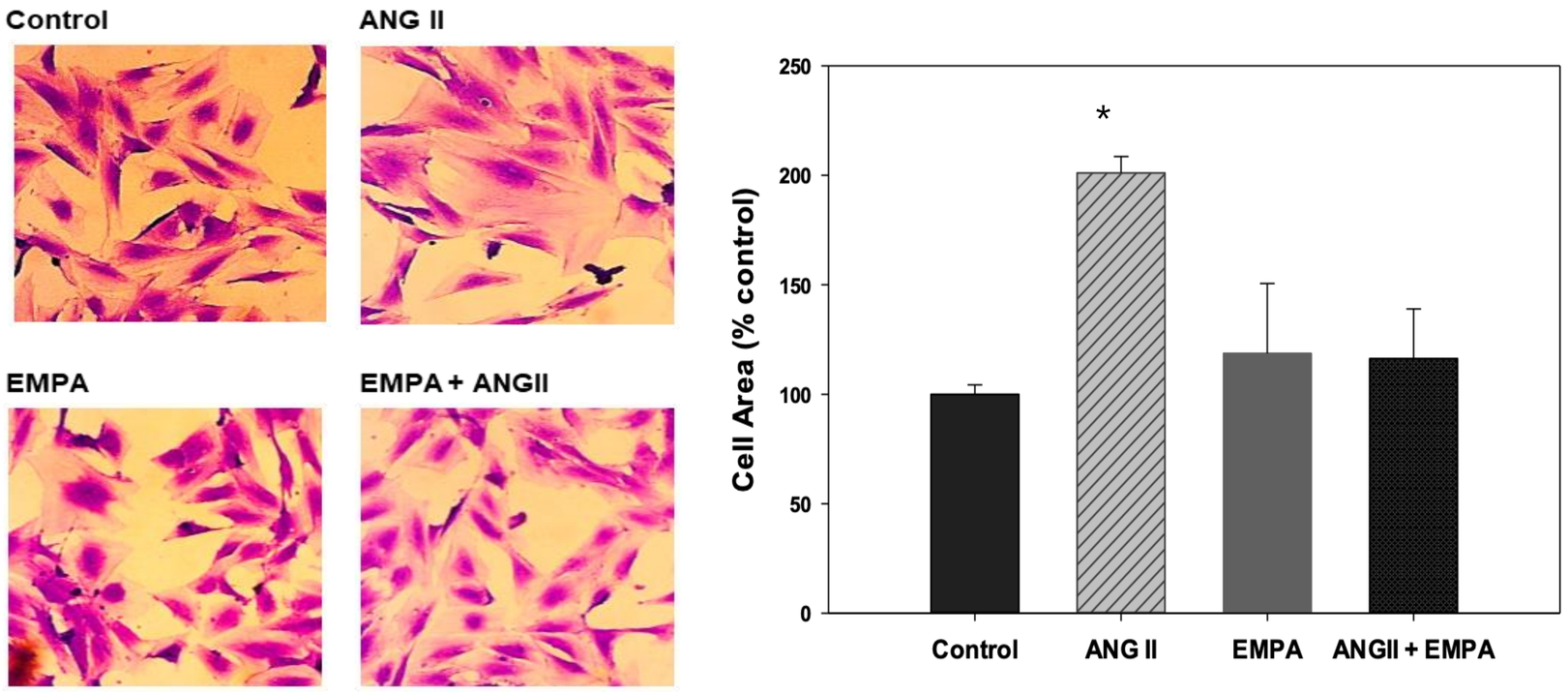

Figure 2: ANG II induced cardiomyocyte hypertrophy in H9c2 cardomyolabsts
Cell area: Left panel: Representative crystal violet stained images of H9c2 cardiomyoblasts treated with 100nM ANG II, with or without 500nM EMPA for 18 hours. **Right panel:** Cell area of at least 50-70 H9c2 cardiomyoblasts from 3 sets were measured. Statistical significant increase in the cell area was observed in the ANGII group when compared to the control group (*p=0.009).

Cell area measurements:

- H9c2 cardiomyoblasts treated with ANG II for 18 hours showed a statistically significant increase in cell area (p=0.0009) compared with control. This increase was reduced in the presence of EMPA. **(Figure 2).**
- An average of 3 sets cell area measurements had shown a 2 fold increase in cell size in cells treated with ANG II. EMPA resulted in an increase of cell size but to a lesser extent and an 82.5% reduction compared to ANG II group.
- A 84.9% reduction was seen with the combination group compared to the ANG II group. **(Figure 2).**
- EMPA has some hypertrophic effect on H9c2 cardiomyoblasts.

Protein assay:

- There were no big differences in the protein concentrations between the different treatment groups. **(Table 1).**

Protein expression (Western blot):

- EMPA reduces SGLT-1 expression following stimulation with ANG II with no increase observed in ANG II group **(Figure1)**
- Less SGLT-2 protein expression was detected in all treatment groups
- No change in NHE1 protein was detected.

Conclusions

- Despite that EMPA reduced ANG II-induced cardiomyocytes hypertrophy, we can not conclude whether EMPA significantly reduces the risk of hypertrophy.
- The study was not able to identify the effect of NHE1 protein expression in the cardiovascular benefits of EMPA.

Limitations

- Low protein expression in WB may have contributed to the difficulties in quantifying the bands.
- Low concentration of EMPA used compared to clinical dosage regimens (500nM vs. 10 or 25 mg) limited our abilities to generalize our results to humans.
- The cell line used is H9c2 cardiomyoblasts (immature cells) are not cardiomyocytes.

Future work

- Future study will use transgenic mice that overexpress NHE1 treated with the presence and absence of EMPA to identify the exact effect of EMPA on NHE1 expression.
- Future studies can measure the expression of NHE1 activator proteins like p90 Ribosomal S6 Kinase (p90RSK).

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