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SGLT2 inhibitors and the cardiac Na⁺/H⁺ exchanger-1: the plot thickens

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With the ever-mounting evidence for a profound and direct effect of SGLT2 inhibitors (SGLT2i's) on the heart, understanding their mechanism of action becomes increasingly important. So, we are pleased that our paper¹ published in this edition of *Cardiovascular Research*, has generated a lively debate.^{2,3} In our work, we use a variety of methods to show that, at least in our hands, empagliflozin (EMPA) from two independent suppliers, as well as two other chemically-related SGLT2i's, are not potent inhibitors of the cardiac Na⁺/H⁺ exchanger-1 (NHE1) and, related to this, have no effect on intracellular Na⁺ concentration ([Na⁺]_i) in the healthy heart. This is contrary to several previous reports (see references 1-4 in Zuurbier *et al.*²).

Our findings are in contrast with Zuurbier and colleagues in Amsterdam, who have responded to our work with a short letter in this issue of *Cardiovascular Research*.² Their letter contains some misunderstandings and errors that warrant a response from us. However, before briefly responding to this letter, it is important to say that our labs in London and Oxford, and those of Zuurbier and colleagues in Amsterdam, have been in useful and regular correspondence over the last 6 months to try to understand the reasons for our contrasting results. We also add that we have the highest regard for the Amsterdam group, and the quality of their science, as well as a long-standing personal friendship between our groups. So, in the spirit of constructively trying to understand what underlies these apparently contrasting findings, we make the following observations:

- 1. EMPA and intracellular Na concentration:** In their letter, Zuurbier *et al* claim that, when 'calibrated', our SBFI data support the notion that EMPA lowers the intracellular Na concentration in isolated cells. They arrive at this conclusion by transcribing and re-analysing our SBFI ratios, read from our original figure (Figure 3A in Chung *et al*¹). While a post-hoc application of an arbitrary calibration curve is unlikely to be reliable, the Amsterdam group were kind enough to share their spreadsheet and analysis with us. Unfortunately, our data has been mis-transcribed and includes some outliers that were not present in our original data set as well as other transcription errors. Never the less, using our real observed values and the calibration equation provided in Zuurbier *et al*, a retrospective calibration of our data does not alter our original assertion that EMPA (1 or 10 μM) has no effect on intracellular Na⁺ (see Figure 1 - inset table).

50 2. **Type I vs Type II errors:** The Zuurbier *et al* letter claims that they have published extensively
51 showing evidence for SGLT2i's inhibiting NHE1 activity in rabbits and mice. However, these
52 studies, taken individually, are based on a relatively small number of observations: the
53 primary observation of NHE1 inhibition in Baartscheer *et al* (2017) for example, is made in
54 5-6 cells from 4 rabbits (Figure 2c), in Uthman *et al* (2018) in 8 cells from 4 mice (Figure 1)
55 and Uthman *et al* (2019) 8 cells from 5 mice (Figure 5) (see reference 1-3 in Zuurbier *et al*²).
56 In the recent letter by Zuurbier *et al*, the primary observation (Figure 1A) is based on 3 cells
57 from 3 rabbits.² The [Na⁺]-lowering effect is based on similarly small sample sizes.

58
59 As David Eisner points out in a separate recent review, the use of the number of cells as the
60 statistical sample size is valid only when comparing 'before' and 'after' drug interventions,
61 as is the case for our cellular [Na⁺] responses (Figure 3 in Chung *et al*¹).⁴ The use of
62 hierarchical statistical analysis can also limit bias due to inter-animal variability. However,
63 the likelihood of a Type I error (i.e. false positive) increases as sample size declines, when
64 data are clustered, or when not implementing repeated measures or hierarchical analyses.⁴

65
66 Our inferences, on the other hand, may be prone to a Type II error (false negative). We have
67 therefore reanalysed the intracellular [Na⁺] data using hierarchical (nested) analysis (to avoid
68 clustering bias) as well as paired t-tests (more likely to detect a systematic small difference).
69 However, these analyses also fail to detect any statistically significant reduction of
70 intracellular Na by EMPA in healthy myocytes (Figure 1). Our single-cell Na studies are based
71 on 24-33 cells in each experimental group and are 'paired'. These observations are supported
72 by 'unpaired' intracellular Na measurements made using ²³Na NMR spectroscopy in isolated
73 rat, mouse (n=6/group) and guinea pig hearts beating at physiological rates where again no
74 changes in Na are observed. Our measurements of NHE1 activity are 'unpaired' as they were
75 made in separate cells as myocytes do not usually tolerate two consecutive NH₄ prepulses.
76 However, hierarchical cluster analysis based on 24-39 cells from at least 3 rats (8 cells from
77 2 rats for cariporide) using the summary variable of NHE1 flux at pH 6.9, shows no effect of
78 EMPA (and a significant effect of cariporide) (Figure 1).

79
80 3. **Specificity and sensitivity of the NHE1 assay:** The Zuurbier *et al* letter suggests that our
81 inability to detect an inhibition of NHE1 activity using our set-up is compromised by the
82 "non-specificity" and "low-sensitivity" of our NHE1 assay. We respond to these
83 unsubstantiated claims by arguing that the method used in Amsterdam is, in fact, more
84 prone to be affected by non-specificity and low-sensitivity.

85
86 An NHE1-specific assay that is based on measurements of intracellular pH (pH_i) must ensure
87 that the only transporter responsible for producing a H⁺-equivalent flux is NHE1. We do this
88 by eliminating any contribution from HCO₃⁻-dependent transporters (by buffering our
89 solution with HEPES only). In contrast, the 'Amsterdam' protocol adds bicarbonate to their
90 solutions, which inadvertently activates transporters in addition to NHE1. Thus, non-
91 specificity is a greater concern with the Amsterdam approach. Zuurbier *et al* point out that
92 our recordings show a partial recovery of pH_i in the presence of the NHE1 inhibitor
93 cariporide, and conclude that our system thus has a non-NHE1 component. This reasoning
94 is, however, flawed because it ignores the fact that the dose of drug used - 10 μM - is not
95 a concentration at which cariporide is a full inhibitor. Previously, it was determined by
96 Ch'en *et al* (2008) that 30 μM is required to block NHE1 in rat myocytes.⁵ Nonetheless, we
97 observe a 90% inhibition of flux in the presence of 10 μM cariporide. The pH_i recovery is
98 not a sign of non-NHE1 components, but rather the product of residual NHE1 activity. In

99 other studies, we have consistently used 30 μ M cariporide to block NHE1, but in this
100 instance, we opted for a concentration to match that of EMPA. In our NHE1 assay using
101 HCT116 cells (Chung *et al* Supplement Figure 4S), we show a 97% inhibition of NHE1 flux
102 with 30 μ M cariporide.¹

103

104 With regard to sensitivity, an NHE1 assay must (i) ensure that the transporter's activity
105 under control conditions is sufficiently large to detect even a small inhibitory effect of
106 candidate-drugs, (ii) that the actions of drugs are expressed in terms of flux, i.e the most
107 accurate functional measure of NHE1 activity, and (iii) that fluxes are compared at matching
108 levels of transport substrate (i.e. pH_i). With respect to the first point, we were perplexed
109 to read that our assay was deemed to be 'not sensitive enough' because our NHE1 activity
110 is too high. Our measurements peaked at 20 mM/min at low pH_i ; as expected for rats and
111 consistent with the literature.^{6,7} Yamamoto *et al* (2005) have previously showed that NHE1
112 flux in rabbit myocytes is over four times slower than in rat myocytes.⁸ Rabbit myocytes
113 are thus a less sensitive system to study NHE1 inhibitors. The sensitivity of NHE1
114 measurements in rabbits (and those in mice) by the Amsterdam protocol was further
115 compromised by performing recordings at the unphysiological extracellular pH of 7.2-7.3,
116 an inhibitory influence. As shown by Vaughan-Jones and Wu (1990), the relationship
117 between extracellular pH and NHE activity is particularly steep between pH 7.0 and 7.5,
118 thus the use of mildly acidotic conditions will further reduce NHE activity and hence
119 compromise its ability to resolve inhibition.⁹ At the lower NHE1 activity in rabbits, it is not
120 surprising that even a low dose of cariporide results in an apparent block of transport; in
121 reality, there is a small residual activity that is simply not big enough to resolve. We argue
122 that to measure the inhibitory effect of a drug, transmembrane H^+ -equivalent flux should
123 be calculated correctly, i.e. from the product of pH_i change and buffering capacity and
124 plotted against the corresponding pH_i at which it was calculated to generate a pH -flux
125 curve, as has been the standard established in our lab for over 2 decades. Additional
126 transformations such as normalizations performed in the Zuurbier letter and comparing
127 these slopes without taking into account the level of substrate (i.e. pH_i) are problematic.
128 NHE1 is steeply sensitive to pH_i , therefore the effects of drugs must be compared at
129 precisely matching levels of pH_i .

130

131 4. **Isolated heart studies:** Both groups appear to be in agreement that EMPA has no effect on
132 contractility in isolated hearts. The lack of a negative inotropic effect of SGLT2i's has been
133 widely reported - not only by our respective groups but also in many other studies in a wide
134 range of models. The Uthman *et al* and Baartcheer *et al* studies report a fall in Na of 20-
135 25%. Given the steep relationship between $[Na^+]_i$ and contractility (for example see
136 Eisner),¹⁰ a reduction in $[Na^+]_i$ of this magnitude would be expected to elicit a negative
137 inotropic response which is not observed. The lack of changes in inotropy reported in these
138 studies is therefore surprising and suggests there are some, as yet undefined, confounding
139 factors. Alternatively, the lack of a negative inotropic effect with this degree of $[Na^+]_i$
140 reduction supports our contention that intracellular $[Na^+]$ does not fall acutely in intact
141 healthy hearts in response to SGLT2i's.

142

143 5. **SGLT2i's in pathology:** While Na may not fall in healthy hearts, we agree that the beneficial
144 effects of SGLT2i's may be particularly apparent under pathological conditions. Indeed,
145 with regard to Na fluxes, a recent study by Philippaert *et al* has reported that EMPA blocks
146 the slowly inactivating Na channel in failing myocytes (but not in healthy myocytes).¹¹

147

148 In this regard it is also interesting that Zuurbier *et al* cite the excellent study of Cappetta *et*
 149 *al* (see reference 5 in Zuurbier *et al.*²). In this study, Cappetta and colleagues report that
 150 dapagliflozin inhibits NHE in HUVECs. Studies originating from the Amsterdam group have
 151 also reported that SGLT2i's can inhibit ROS production and improve NO bioavailability in
 152 HUVECs.¹² So, while SGLT2i's may inhibit NHE in endothelial cells, it is far from certain that
 153 this is a direct effect - particularly as high concentrations of NO have been shown to inhibit
 154 NHE1.⁷ Perhaps of more relevance to the present debate is Cappetta *et al*'s observation
 155 that in cardiomyocytes, dapagliflozin had no acute effect either on systolic or diastolic Ca
 156 or on diastolic intracellular Na. They concluded that "These observations suggest that the
 157 beneficial effects on Ca and Na homeostasis that we observed after 6 weeks of dapagliflozin
 158 treatment in vivo were not caused by a direct acute modification of [Na] and Ca ion fluxes
 159 and concentrations by the drug. Therefore, in our experimental setting, dapagliflozin did
 160 not directly target cardiomyocyte ion transporters or channels that would otherwise
 161 determine instantaneous changes in intracellular Ca and Na." This therefore seems an odd
 162 paper to cite in support of their argument.

163

164 6. **Conclusions:** At present, we remain puzzled as to why we can find no evidence for SGLT2's
 165 inhibiting NHE1 or lowering $[Na^+]_i$ in the healthy myocardium, as reported by the
 166 Amsterdam group and by Trum *et al* (see reference 4 in Zuurbier *et al.*²) Zuurbier *et al* in
 167 their recent letter have explored some differences, and we have discussed others.
 168 However, while there are clear protocol differences between our studies, we do not believe
 169 that any of them are likely to be sufficient to explain such profoundly different results.
 170 Indeed, this is the conclusion also reached by Zuurbier *et al*. The mechanisms by which
 171 SGLT2i's elicit their important beneficial effects in the heart remain unresolved and
 172 therefore fertile ground for further research. We would therefore welcome suggestions
 173 from the wider community and, when we hopefully emerge from this COVID pandemic, our
 174 labs will get together in Oxford, London and Amsterdam to try to unravel this conundrum.

175

176 **Conflict of Interest:** None Declared.

177

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 179 original data, experimental contributions and funding were as detailed in Chung *et al.*¹

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182 References

183

- 184 1. Chung YJ, Park KC, Tokar S, Eykyn TR, Fuller W, Pavlovic D, Swietach P, Shattock MJ. Off-
 185 target effects of SGLT2 blockers: empagliflozin does not inhibit Na⁺/H⁺ exchanger-1 or
 186 lower $[Na^+]_i$ in the heart. *Cardiovasc Res* 2021
- 187 2. Zuurbier CJ, Baartscheer A, Schumacher CA, Fiolet JWY, Coronel R. SGLT2 inhibitor
 188 Empagliflozin inhibits the cardiac Na⁺/H⁺ exchanger 1: persistent inhibition under
 189 various experimental conditions. *CardiovascRes* 2021
- 190 3. Murphy E, Eisner DA. Does the cardioprotective effect of Empagliflozin involve inhibition
 191 of the sodium-proton exchanger? *CardiovascRes* 2021
- 192 4. Eisner DA. Pseudoreplication in physiology: More means less. *J Gen Physiol* 2021;**153**
- 193 5. Ch'en FF, Villafuerte FC, Swietach P, Cobden PM, Vaughan-Jones RD. S0859, an N-
 194 cyanosulphonamide inhibitor of sodium-bicarbonate cotransport in the heart. *Br J*
 195 *Pharmacol* 2008;**153**:972-982

- 196 6. Kandilci HB, Richards MA, Fournier M, Simsek G, Chung YJ, Lakhal-Littleton S, Swietach
 197 P. Cardiomyocyte Na(+)/H(+) Exchanger-1 Activity Is Reduced in Hypoxia. *Front*
 198 *Cardiovasc Med* 2020;**7**:617038
- 199 7. Richards MA, Simon JN, Ma R, Loonat AA, Crabtree MJ, Paterson DJ, Fahlman RP, Casadei
 200 B, Fliegel L, Swietach P. Nitric oxide modulates cardiomyocyte pH control through a
 201 biphasic effect on sodium/hydrogen exchanger-1. *Cardiovasc Res* 2020;**116**:1958-1971
- 202 8. Yamamoto T, Swietach P, Rossini A, Loh SH, Vaughan-Jones RD, Spitzer KW. Functional
 203 diversity of electrogenic Na⁺-HCO₃⁻ cotransport in ventricular myocytes from rat, rabbit
 204 and guinea pig. *J Physiol* 2005;**562**:455-475
- 205 9. Vaughan-Jones RD, Wu ML. Extracellular H⁺ inactivation of Na(+)-H⁺ exchange in the
 206 sheep cardiac Purkinje fibre. *J Physiol* 1990;**428**:441-466
- 207 10. Eisner DA, Lederer WJ, Vaughan-Jones RD. The quantitative relationship between twitch
 208 tension and intracellular sodium activity in sheep cardiac Purkinje fibres. *J Physiol*
 209 1984;**355**:251-266
- 210 11. Philippaert K, Kalyanamoorthy S, Fatehi M, Long W, Soni S, Byrne NJ, Barr A, Singh J,
 211 Wong J, Palechuk T, Schneider C, Darwesh AM, Maayah ZH, Seubert JM, Barakat K, Dyck
 212 JRB, Light PE. The Cardiac Late Sodium Channel Current is a Molecular Target for the
 213 Sodium-Glucose Co-Transporter 2 Inhibitor Empagliflozin. *Circulation* 2021
- 214 12. Uthman L, Homayr A, Juni RP, Spin EL, Kerindongo R, Boomsma M, Hollmann MW,
 215 Preckel B, Koolwijk P, van Hinsbergh VWM, Zuurbier CJ, Albrecht M, Weber NC.
 216 Empagliflozin and dapagliflozin reduce ROS generation and restore NO bioavailability in
 217 tumor necrosis factor alpha-stimulated human coronary arterial endothelial cells. *Cell*
 218 *Physiol Biochem* 2019;**53**:865-886

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223 Figure Legend:

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226 **Figure 1: Intracellular Na measurements and NHE1 flux at pH 6.9 estimated from our original**
 227 **data (Chung et al). Left Panel:** Using the calibration described by Zuurbier *et al* the SBFI
 228 ratiometric values were converted into intracellular Na ([Na⁺]_i). **Right Panel:** NHE1 flux was
 229 measured at pH 6.9. In both panels, each data point represents a single observation and these
 230 are colour-coded to identify individual cell isolations. The mean values are shown in the inset
 231 tables. Using hierarchical analysis, the intraclass correlation coefficient of the [Na⁺]_i and NHE1
 232 flux data were 32% and 3.2% respectively - demonstrating the need to use hierarchical
 233 statistical test on these type of data. When tested with hierarchical (nested) t-tests, EMPA has
 234 no effect on [Na⁺]_i or NHE1 flux as previously reported. Cardiporide very significantly reduced
 235 NHE1 flux whether tested by unpaired t-test or by hierarchical analysis. A paired t-test (but not
 236 nested hiererchical analysis) detects a small but significant (likely erroneous) reduction in [Na⁺]_i
 237 by cariporide. ns = not significant. Note: we have undertaken the retrospective Na calibration
 to match that of Zuurbier *et al*, however, we recognise that this is unlikely to be reliable.