Effects of canagliflozin on human myocardial redox signalling: clinical implications

Hidekazu Kondo, Ioannis Akoumianakis, Ileana Badi, Nadia Akawi, Christos P. Kotanidis, Murray Polkinghorne, Ilaria Stadiotti, Elena Sommariva, Alexios S. Antonopoulos, Maria C. Carena, Evangelos K. Oikonomou, Elsa Mauricio Reus, Rana Sayeed, George Krasopoulos, Vivek Srivastava, Shakil Farid, Surawee Chuaiphichai, Cheerag Shirodaria, Keith M. Channon, Barbara Casadei, and Charalambos Antoniades

1Division of Cardiovascular Medicine, Radcliffe Department of Medicine, University of Oxford, L6 West Wing, John Radcliffe Hospital, Headley Way, Oxford OX3 9DU, UK; 2Department of Cardiology and Clinical Examination, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama, Yufu, Oita 879-5593, Japan; 3Department of Genetics and Genomics, College of Medicine and Health Sciences, United Arab Emirates University, Khalifa Ibn Zayed Street, Al Maqam, Al-Ain, P.O. Box 17666, United Arab Emirates; 4Unit of Vascular Biology and Regenerative Medicine, Centro Cardiologico Monzino IRCCS, via Carlo Parea 4, 20138, Milan, Italy; 5Acute Vascular Imaging Centre, University of Oxford, Headley Way, Oxford OX3 9DU, UK; 6Caristo Diagnostics, 1st Floor, New Barclay House, 234 Botley Rd, Oxford OX2 0HP, UK; and 7Acute Vascular Imaging Centre, University of Oxford, Headley Way, Oxford OX3 9DU, UK

Received 14 June 2020; revised 14 January 2021; editorial decision 15 June 2021; accepted 18 June 2021; online publish-ahead-of-print 19 July 2021

See page 4961 for the editorial comment for this article ‘Canagliflozin and myocardial oxidative stress: SGLT1 inhibition takes centre stage’, by G.S. Schiattarella and D. Bode, https://doi.org/10.1093/eurheartj/ehab519.

Aims
Recent clinical trials indicate that sodium-glucose cotransporter 2 (SGLT2) inhibitors improve cardiovascular outcomes in heart failure patients, but the underlying mechanisms remain unknown. We explored the direct effects of canagliflozin, an SGLT2 inhibitor with mild SGLT1 inhibitory effects, on myocardial redox signalling in humans.

Methods and results
Study 1 included 364 patients undergoing cardiac surgery. Right atrial appendage biopsies were harvested to quantify superoxide (O$_2^-$) sources and the expression of inflammation, fibrosis, and myocardial stretch genes. In Study 2, atrial tissue from 51 patients was used ex vivo to study the direct effects of canagliflozin on NADPH oxidase activity and nitric oxide synthase (NOS) uncoupling. Differentiated H9C2 and primary human cardiomyocytes (hCM) were used to further characterize the underlying mechanisms (Study 3). SGLT1 was abundantly expressed in human atrial tissue and hCM, contrary to SGLT2. Myocardial SGLT1 expression was positively associated with O$_2^-$ production and pro-fibrotic, pro-inflammatory, and wall stretch gene expression. Canagliflozin reduced NADPH oxidase activity via AMP kinase (AMPK)/Rac1 signalling and improved NOS coupling via increased tetrahydrobiopterin bioavailability ex vivo and in vitro. These were attenuated by knocking down SGLT1 in hCM. Canagliflozin had striking ex vivo transcriptomic effects on myocardial redox signalling, suppressing apoptotic and inflammatory pathways in hCM.

Conclusions
We demonstrate for the first time that canagliflozin suppresses myocardial NADPH oxidase activity and improves NOS coupling via SGLT1/AMPK/Rac1 signalling, leading to global anti-inflammatory and anti-apoptotic effects in the human myocardium. These findings reveal a novel mechanism contributing to the beneficial cardiac effects of canagliflozin.
Keywords

SGLT2 inhibitor • SGLT1 • Myocardial redox state • NADPH oxidase activity • NOS coupling • AMPK

Graphical Abstract

Proposed mechanism of canagliflozin-induced improvement of myocardial redox state. Canagliflozin increases intracellular AMP/ATP ratio through inhibition of SGLT1, which can activate AMPK/NOS signalling and increase NO that suppresses pro-inflammatory signalling. AMPK activation also inhibits activation of Rac1 and membrane translocation of Rac1 and p47phox, which decrease NADPH oxidase activity and superoxide (O$_2^-$) production, attenuates inflammatory and apoptotic pathways and increasing the bioavailability of tetrahydrobiopterin (BH4), a key factor for NOS coupling.

Translational perspective

Sodium-glucose cotransporter 2 (SGLT2) inhibitors are a novel class of anti-diabetic drugs that have recently emerged as cardioprotective agents in heart failure, even in the absence of diabetes. Despite their clinical benefit, the underlying effects on the human myocardium remain largely unexplored. In this work, we demonstrate a novel, SGLT1-mediated effect of canagliflozin, a clinically used SGLT2 inhibitor, on myocardial biology affecting its redox state, inflammation, fibrosis, and apoptosis in humans. Our work suggests novel cellular mechanisms underlying the observed clinical benefits of SGLT2 inhibitors; further characterization of the mechanistic effects of the SGLT2 inhibitor drug class on the human myocardium will broaden their use and indications in cardiovascular disease more efficiently.

Introduction

Sodium-glucose cotransporter 2 (SGLT2) inhibitors comprise an anti-hyperglycaemic drug class regulating plasma glucose by inhibiting glucose reabsorption through SGLT2 blockade in the proximal renal tubules. Clinically available SGLT2 inhibitors also display variable affinity to SGLT1. While SGLT2 is the main responsible for glucose reabsorption from the glomerular filtrate, SGLT1, which is essential for the fast uptake of glucose and galactose in the intestine, plays a minor role in renal reabsorption. These two sodium-glucose cotransporters have different affinity and capacity for glucose transport and diverse expression patterns in the renal proximal tubule as well as in other organs. The cardioprotective effects of SGLT2 inhibitors have been demonstrated in recent clinical trials (EMPA-REG OUTCOME, CANVAS, DECLARE-TIMI 58) in diabetic as well as non-diabetic patients with heart failure (DAPA-HF). Accordingly, the 2019 ESC guidelines on diabetes, pre-diabetes, and cardiovascular diseases recommend empagliflozin, canagliflozin, or dapagliflozin in patients with type 2 diabetes mellitus and cardiovascular disease, or at high/very high cardiovascular risk, to reduce cardiovascular events, as well as in diabetic patients to lower the risk of heart failure hospitalization. However, the mechanisms behind these effects remain unclear.

Limited evidence suggests that SGLT2 inhibitors have direct cardioprotective effects that extend beyond their systemic glucose-lowering function. Empagliflozin attenuates cardiac fibrosis in diabetic mice and preserves myocardial function in a mouse model of pressure overload-induced heart failure. Canagliflozin activates AMP kinase (AMPK), whose cardioprotective effects are well established, by inhibiting mitochondrial function and increasing cellular AMP levels in vitro. However, several aspects of the direct effects of SGLT2 inhibition on the human heart remain unclear.

Sustained oxidative stress is central to the pathogenesis of cardiac diseases, such as heart failure and atrial fibrillation. NADPH oxidases (Nox) are major sources of superoxide anions (O$_2^-$) in the human heart, and the activity of Nox1 and Nox2...
isoforms is dependent on activation and membrane translocation of the GTPase Rac1 and the presence of p47phox. On the other hand, nitric oxide synthases (NOSs) are redox-related enzymes responsible for nitric oxide (NO) synthesis in the human heart. In disease states such as long-standing persistent atrial fibrillation, myocardial NOS appears to be uncoupled, therefore producing O$_2$$^-$ rather than NO as a result of oxidative depletion of its co-factor tetrahydrobiopterin (BH4). Similar NOS uncoupling is also observed in various diseases like diabetes and hypertension. However, despite the importance of myocardial redox state regulation in cardiac biology, the putative antioxidant effects on the human heart of different SGLT2 inhibitors have not been well investigated so far.

In this study, we first explored the expression profiles of SGLT2 and SGLT1 in the human heart to determine the molecular target of direct SGLT1/2 inhibition in the human atrial myocardium. Secondly, we evaluated the direct effects of the SGLT2-specific inhibitor empagliflozin and the dual SGLT1/2 inhibitor canagliflozin on myocardial redox signalling in the human heart.

### Methods

#### Study population

The study population consisted of patients undergoing cardiac surgery, all of whom were under the Oxford Heart Vessels and Fat (ox-HVF) programme (www.oxhvf.com) at Oxford University Hospitals NHS Foundation Trust, UK. The patient’s demographics, indication for surgery, and medication are presented in Table 1.

Study 1 was used for cohort-wide associations and consisted of 364 patients. Myocardial biopsies were obtained intraoperatively from the right atrial appendage cannulation site, transferred in ice-cold buffer, and processed for O$_2$$^-$ quantification or gene expression studies as described. The exclusion criteria were any active inflammatory disease (e.g. autoimmune disease in active/relapse phase) or active infectious disease, advanced liver failure (known or suspected cirrhosis) or end-stage renal disease (estimated glomerular filtration rate <15 mL/min), active malignancy (untreated or under treatment) and systemic use of nonsteroidal anti-inflammatory drugs (i.e. cyclooxygenase-2 inhibitors) or antioxidant vitamins. As chronic obstructive pulmonary disease (COPD) has not been previously associated with changes in myocardial oxidative stress, COPD was not included as an exclusion criterion.

#### Table 1  Demographic characteristics of the study participants

|                          | Clinical study (Study 1) | Ex-vivo study (Study 2) |
|--------------------------|--------------------------|-------------------------|
| Patients, n              | 364                      | 51                      |
| Age (years)              | 69 [60–75]               | 66 [57.25–74.00]        |
| Male sex                 | 301 (82.70)              | 41 (80.40)              |
| Hypertension             | 265 (72.80)              | 36 (70.60)              |
| Hyperlipidaemia          | 282 (77.50)              | 34 (66.70)              |
| T2DM                     | 69 (19.00)               | 9 (17.65)               |
| Smoking                  |                          |                         |
| Active                   | 193 (53)                 | 24 (47.10)              |
| Past                     | 30 (8.2)                 | 3 (5.90)                |
| CrCl (mL/min/1.73 m$^2$) | 88.14 [25.11–108.99]     | 97.93 [71.96–116.70]    |
| BMI (kg/m$^2$)           | 27.59 [25.10–30.33]      | 29.26 [26.03–32.44]     |
| CABG                     | 279 (76.65)              | 43 (84.31)              |
| AVR due to AR/AS         | 58 (15.93)               | 6 (11.77)               |
| MVR due to MR/MS         | 27 (7.42)                | 2 (3.92)                |
| NYHA class               |                          |                         |
| I                        | 133 (36.54)              | 17 (33.34)              |
| II                       | 154 (42.31)              | 18 (35.29)              |
| III                      | 64 (17.58)               | 14 (27.45)              |
| IV                       | 13 (3.57)                | 2 (3.92)                |
| Medication               |                          |                         |
| Antplatelet              | 280 (76.90)              | 36 (70.59)              |
| ACEI/ARBs                | 210 (57.70)              | 28 (54.90)              |
| Statins                  | 298 (81.90)              | 34 (66.70)              |
| β-blocker                | 235 (64.60)              | 21 (41.20)              |
| CCB                      | 85 (23.40)               | 12 (23.50)              |
| Insulin                  | 24 (6.60)                | 6 (11.80)               |
| Oral anti-diabetics      | 47 (12.90)               | 4 (7.80)                |

Values are presented as n (%) or median [25th–75th percentile]. ACEI, angiotensin-converting enzyme inhibitor; AR, aortic regurgitation; ARB, angiotensin receptor blocker; AS, aortic stenosis; AVR, aortic valve replacement; BMI, body mass index; CABG, coronary artery bypass grafting; CCB, calcium channel blocker; CrCl, creatinine clearance; MR, mitral regurgitation; MS, mitral stenosis; MVR, mitral valve replacement; T2DM, type 2 diabetes mellitus.
patients were not excluded if they were not receiving systemic anti-inflammatory treatments.

Study 2 included patient samples used for mechanistic ex vivo experiments and consisted of 51 prospectively recruited patients undergoing cardiac surgery with the same exclusion criteria as above. Atrial myocardium specimens were collected during surgery, transferred to the lab, and used for ex vivo experiments as described.25

Hyperlipidaemia and hypertension were defined according to the current European Society Cardiology guidelines.23,24 Diabetes mellitus was defined according to the American Diabetes Association guidelines.25 Study protocols were in agreement with the Declaration of Helsinki and all participants had provided written informed consent. The collection of human ventricle biopsy was approved by the Istituto Europeo di Oncologia and Centro Cardiologico IRCCS—Ethics Committee (R1020/19-94 CCM1072). Participant demographic characteristics are reported in Table 1.

**Cell culture models**

The effects of SGLT1/2 on cardiomyocytes were first screened in the rat cardiac myocyte-derived cell line, H9c2, and the key findings were replicated in human cardiomyocytes (hCM, PromoCell, Heidelberg, Germany). H9c2 cells were differentiated to cardiac myocytes in Dulbecco’s Modified Eagle Medium (glucose 5.5 mM, Sigma-Aldrich, cat. number D6046) supplemented with 1% horse serum (Sigma-Aldrich). Primary hCM isolated from adult heart ventricles were purchased and differentiated to cardiac myocytes in dedicated Myocyte Growth Medium (glucose 5.5 mM) with SupplementMix (PromoCell) for 60 days after reaching 100% confluence. Troponin I expression and myotube formation were used to confirm efficient cardiomyocyte differentiation (Supplementary material online, Figure S1).

Both hCM and H9c2 cells were cultured in a high-glucose medium (25 mM) for 72 h before in vitro incubations to mimic the human ex vivo experimental environment and the in vivo diabetes environment, as 25 mM is the most prevalent concentration of glucose used in the literature to mimic diabetic hyperglycaemia in H9c2 cells.26 In selected experiments, glucose was replaced by mannitol 25 mM to study the role of the canagliflozin-induced glucose transfer. Cells were treated with canagliflozin 10 μmol/L (Cayman) ± Compound C (CC, an AMPK inhibitor, 10 μmol/L) or carrier (DMSO) for 60 min or 24 h and used for chemiluminescence experiments, gene expression studies and downstream signalling experiments. The specificity of CC as an AMPK inhibitor was confirmed by tracking downstream phosphorylation of its validated surrogate target, acetyl-CoA carboxylase (ACC).

**Human tissue harvesting**

Human myocardial biopsies were processed as described (see Supplementary material online, Data supplement).22

**Superoxide quantification**

O2 production was measured in human atrial myocardium homogenates and cell lysates using lucigenin (5 μmol/L)-enhanced chemiluminescence evaluating NADPH oxidase activity and NOS coupling status as described (see Supplementary material online, Data supplement).17,22,27

**RNA isolation and quantitative real time-polymerase chain reaction**

RNA was purified in a semi-automated way and processed for cDNA generation and gene expression studies as described (see Supplementary material online, Data supplement).28

**Western blotting**

Western immunoblotting was performed on myocardium homogenates and cell lysates (see Supplementary material online, Data supplement).

**Rac1 activation assay**

Rac1 activation was detected in myocardial homogenates and cell lysates using a commercial kit (Cell Signalling, see Supplementary material online, Data supplement).

**Evaluation of myocardial Rac1 and p47phox membrane translocation**

Membrane translocation of Rac1 and p47phox in human myocardial tissue was estimated by differential centrifugation of myocardial homogenates to isolate membrane proteins as described (see Supplementary material online, Data Supplement).22,29

**Bioppterin measurements**

BH4, dihydروبpterin (BH2), and bioppterin levels were determined using high-performance liquid chromatography as described (see Supplementary material online, Data Supplement).27

**Oxidative fluorescent microtopography**

In situ O2 production was determined in human atrial myocardium cryo-sections and hCMs with an oxidative fluorescent dye (see Supplementary material online, Data Supplement).

**TUNEL assay**

Apoptotic cells in human myocardial sample sections were detected with the in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) method using a commercially available kit (see Supplementary material online, Data Supplement).

**JC-10 mitochondrial membrane potential assay**

The mitochondrial function of samples derived from human atrial myocardium was analysed by means of a commercially available kit (see Supplementary material online, Data Supplement).

**Intracellular ADP and ATP measurement**

Adenosine triphosphate/Adenosine diphosphate (ATP/ADP) ratio was quantified in hCM by a commercial kit (see Supplementary material online, Data Supplement).

**SGLT1 siRNA transfection studies**

SGLT1 siRNA and negative control siRNA (Thermo Fisher Scientific) were used to knock down SGLT1 in hCMs and in differentiated H9c2 cells by lipofectamine RNAiMax and BlockiT Alexa Fluor Red Fluorescent control as a positive transfection control (see Supplementary material online, Data Supplement).

**Transcriptome profiling of canagliflozin-treated human cardiomyocytes**

**Treatments and RNA extraction**

Human cardiomyocytes were incubated 24 h with 10 μmol/L canagliflozin or DMSO. RNA was extracted with the RNeasy Micro/Mini kit (Qiagen), and samples were purified with an RNA purification kit (ReliaPrep RNA Clean-up and Concentration System, Promega), achieving 260/280 ratio > 2, 260/230 ratio > 1.8.
**Clariom™ S Assay-HT, Human Array Plate**

Genome-wide expression profiling was done using the GeneChip® WT PLUS assay kit and processed on the GeneTitan using Human Clariom S Assay-HT 16-Arry Plate at the High-Throughput Genomics Wellcome Trust Centre for Human Genetics (Oxford, UK). RNA quality was controlled using Agilent Tape Station. The latest version of Affymetrix Genechip Command Console software for GeneTitan was used for quality check and data analyses.

**Statistical analysis**

For Study 1, continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test. Non-normally distributed variables are presented as median [25th-75th percentile], whereas normally distributed variables are presented as mean ± SD. The correlation of two continuous variables was evaluated with Pearson’s r or Spearman’s rank correlation coefficients, as appropriate. Continuous variable comparisons were performed using Student’s t-test or Mann–Whitney U-test (for two groups, as appropriate) or by ANOVA (for >2 groups), followed by Bonferroni correction for multiple comparisons. Power calculations were based on myocardial NADPH oxidase activity and we estimated that with 363 patients we could detect a difference of 0.25 in log(NADPH-stimulated O2) between high vs. low myocardial SGLT1 expression with power 0.9 [assuming a standard deviation of 1.04 in log(NADPH-stimulated O2)].

To test whether the association of myocardial NADPH-stimulated O2 with myocardial SGLT1 expression was independent of other risk factors, we performed multivariable linear regression analysis where myocardial NADPH-stimulated O2 was used as a dependent variable and myocardial SGLT1 expression, age, sex, diabetes, hypertension, hypercholesterolemia, and NYHA class were used as independent variables. The standardized betas (Bstand) are presented for each covariate.

In order to assess the independent effect of myocardial SGLT1 expression on NADPH-stimulated and Vas2870-inhibitable O2 as well as myocardial TNFα, IL6, ANP, BNP, and COL1A1 expression, each of the aforementioned variables was used as a dependent variable in individual multivariable linear regression analyses, and myocardial SGLT1 expression was used as an independent variable. Age, sex, diabetes, hypertension, smoking (categorized as previous/active smoker vs. never-smoked) and NYHA class (class IV vs. lower classes) were used as covariates.

For Study 2, we estimated that with a minimum of five pairs of samples we would be able to identify a change of log(NADPH-stimulated O2) by 0.37 with α = 0.05, power 0.9 and SD for paired response difference of 0.30 upon canagliflozin treatments. Paired comparisons between two groups were performed using Wilcoxon signed-rank tests. When the changes between two paired analyses were compared that was performed using two-way ANOVA with interaction terms as stated in the respective figure legends. All tests were two-sided and alpha (α) was set at 0.05. Statistical analysis was performed using SPSS version 20.0 and R version 3.6.0.

**Results**

**SGLT1/2 expression profile in the human heart**

We first evaluated the gene expression levels of SGLT1 and SGLT2 in the human myocardium (Study 1). SGLT2 was undetectable in ~96% of tested human myocardial samples whilst SGLT1 was abundantly expressed (Figure 1A and Supplementary material online, Figure S2). SGLT1 expression in atrial myocardium was positively associated with NADPH-stimulated (Figure 1B) and Vas2870-inhibitable O2 (Figure 1C) in 244 biopsies. Importantly, myocardial SGLT1 expression was positively related to NADPH oxidase activity, independently of traditional cardiac risk factors (Table 2). In a multivariable linear regression analysis (log-transformed values), SGLT1 expression in the human atrial myocardium was positively associated with basal O2 and NADPH oxidase activity independently of left ventricular ejection fraction and NYHA class (markers of heart-pumping function), suggesting an independent redox association with SGLT1 expression (Supplementary material online, Table S1). A multivariable linear regression analysis also revealed that the association between atrial SGLT1 expression and NADPH-stimulated O2 (stand.β: 0.449, P < 0.001) was independent of medical treatment with hypoglycaemic agents (stand.β: 0.202, P = 0.031), insulin (stand.β: 0.025, P = 0.791), statins (stand.β: -0.007, P = 0.940), angiotensin-converting enzyme inhibitors/angiotensin receptor blockers (stand.β: 0.081, P = 0.388), calcium channel blockers (stand.β: -0.117, P = 0.2), diuretics (0.092, P = 0.301), beta-blockers (stand.β: -0.048, P = 0.603), nitrates (stand.β: -0.026, P = 0.780), and antiplatelet therapy (stand.β: 0.046, P = 0.618).

SGLT1 expression in atrial myocardium was also strongly related with markers of myocardial inflammation [tumour necrosis factor-α (TNFα), Figure 1D and interleukin-6 (IL6, Figure 1E)], wall stretch [atrial natriuretic peptide (ANP), Figure 1F and brain natriuretic peptide (BNP), Figure 1G], as well as fibrosis [collagen 1A1 (COL1A1) expression, Figure 1H]. We also ran multivariable regression analyses to explore whether SGLT1 gene expression was related to each one of these readouts independently of the baseline demographic characteristics. Indeed, SGLT1 expression was related to myocardial IL6 expression (stand.β: 0.110, P = 0.042), myocardial TNFα expression (stand.β: 0.133, P = 0.014), myocardial ANP expression (stand.β: 0.342, P < 0.001), myocardial BNP expression (stand.β: 0.161, P = 0.003), myocardial COL1A1 expression (stand.β: 0.290, P < 0.001), NADPH-stimulated O2 (stand.β: 0.196, P = 0.003), and Vas2870-inhibitable O2 (stand.β: 0.163, P = 0.014), independently of age, sex, smoking, diabetes, hypertension, and NYHA class.

**Direct effect of SGLT inhibitors on human myocardial redox state**

Given the previous associations of myocardial SGLT1 expression with oxidative stress, we explored the direct effects of canagliflozin, an SGLT2 inhibitor with significant affinity to SGLT1,30 on the redox state in the human heart. Incubation of human atrial myocardium with canagliflozin ex vivo reduced basal (Figure 2A), NADPH-stimulated (Figure 2B) and Vas2870-inhibitable (Figure 2C) O2 generation; importantly, all canagliflozin concentrations (3, 10, or 100 μM) had significant effects on these readouts of myocardial redox state. In addition, canagliflozin reduced O2 production from uncoupled NOS, as estimated by the L-NAME-induced reduction in O2 (Figure 2D).

Canagliflozin reduced oxidative stress in myocardial tissue from patients with or without diabetes mellitus, but it showed stronger effects on the myocardial tissue from diabetic patients (Supplementary material online, Figure S3). Canagliflozin reduced total and Vas2870-inhibitable dihydrouiodidium fluorescence in human atrial myocardium (Figure 2E–G), similar to the chemiluminescence experiments. In contrast, treatment with the SGLT2 inhibitor...
Figure 1 Sodium-glucose cotransporter (SGLT)1/2 expression in human atrial myocardium and relations with myocardial redox state and inflammation biomarkers. (A) SGLT1 was abundantly expressed in the human atrial myocardium, contrary to SGLT2. n = 357 [290 non-diabetic patients (no DM); 67 diabetic patients (DM)]. SGLT1 expression was positively correlated with NADPH-stimulated (B) and Vas2870-inhibitable (C) O₂⁻ as well as tumour necrosis factor-α (TNFα, D), interleukin-6 (IL6, E), atrial natriuretic peptide (ANP, F), brain natriuretic peptide (BNP, G), and collagen 1A1 (Col1A1, H) expression. P-values by Mann–Whitney U-test for no DM vs. DM (A) and highest quartile of SGLT1 expression vs. rest (B–H). Data are presented as mean ± SD (A) and median [25th–75th percentile] (B–H).
empagliflozin, which has little affinity to SGLT1, did not affect myocardial \text{O}_2^\text{•} \text{ generation, NADPH} oxidase activity, or NOS coupling (Figure 2H–K). This finding could be explained by the very low affinity of empagliflozin to SGLT1, which is the main SGLT isoform in the human atrial and ventricular myocardium, and implies that clinically used SGLT2 inhibitors may exert direct myocardial effects depending on the degree of SGLT1 affinity. Therefore, we focused our next experiments on evaluating the direct effects of canagliflozin on myocardial redox signalling in humans, due to its dual SGLT1/SGLT2 affinity.

We first investigated the mechanism by which canagliflozin affects NADPH oxidase activity and NOS coupling status. To understand how canagliflozin reduced NADPH oxidase activity, we explored which subunits of the enzyme are mostly affected. Canagliflozin prevented the GTP activation of Rac1 (an important Nox1/2 subunit, Figure 3A) and its membrane translocation (Figure 3B), which lead to Nox1/2 isoform activation, while preventing the membrane translocation of the p47\textsuperscript{phox} subunit of Nox2 (Figure 3C). To assess whether NADPH oxidase activity increased \text{O}_2^\text{•} \text{ generation, not only directly, but also indirectly, we evaluated the oxidation of the NOS co-factor BH4. Canagliflozin increased BH4 levels in the atrial myocardium without affecting total biopterin content (Figure 3D–F), suggesting that it decreased BH4 oxidation (e.g. as a result of NADPH oxidase inhibition) rather than increased the biopterin biosynthetic pathway. Indeed, we also observed a reduction in BH2, the direct oxidation product of BH4, while biopterin, the terminal oxidation product of BH2 oxidation, was borderline decreased (Supplementary material online, Figure S4A–F). This would lead to improved enzymatic coupling of NOS and to a reduction in NOS-derived \text{O}_2^\text{•}.

We next explored the upstream mechanism of the effects of canagliflozin on the myocardial redox state. Canagliflozin induced rapid phosphorylation of AMPK\textsubscript{\alpha}2 (the main isoform in the heart) at the activation site Thr172 (Figure 3G), resulting in increased AMPK\textsubscript{\alpha}2 activity documented by phosphorylation of its downstream target ACC at Ser79 (Figure 3H). Canagliflozin did not affect ERK phosphorylation at Thr202/Tyr204 or AKT at Ser473 (Figure 3I and J). Interestingly, canagliflozin also induced endothelial NOS phosphorylation at the activation site Ser1177 (Figure 3K), whereas it did not affect inducible NOS phosphorylation (Supplementary material online, Figure S4G and H). We next used CC, as an allosteric modulator of AMPK activity, to prove the concept that downstream SGLT1 effects are prevented by inhibiting AMPK activity. Compound C inhibited ACC phosphorylation (Figure 3G and H) as well as the ability of canagliflozin to inhibit Rac1 activation or increase BH4 bioavailability (Figure 3A–E). Compound C prevented the canagliflozin-induced reduction of myocardial \text{O}_2^\text{•} generation (Figure 3L–N) and of the increase in NOS coupling (Figure 3O), identifying AMPK\textsubscript{\alpha}2 as a link between canagliflozin, NADPH oxidase activity, and NOS coupling. Canagliflozin could also reduce apoptosis and mitochondrial dysfunction through AMPK\textsubscript{\alpha}2 (Supplementary material online, Figure S5).

Direct effects of canagliflozin on primary human cardiomyocytes

We next studied the effects of canagliflozin specifically on primary terminally differentiated hCM (Supplementary material online, Figure S1). To mimic the \textit{in vivo} human atrial myocardium experimental conditions as well as a clinically relevant diabetic environment, hCM were cultured in high-glucose medium. Osmolality changes associated with high glucose had no effect on myocardial redox state using mannitol as osmolality control (Supplementary material online, Figure S6). As observed in the human atrial myocardium, canagliflozin increased AMPK\textsubscript{\alpha}2 Thr172 phosphorylation (Figure 4A) leading to AMPK\textsubscript{\alpha}2 activation, as assessed by the phosphorylation status of ACC at Ser79 (Figure 4B). In addition, canagliflozin increased BH4 bioavailability, without altering total biopterins in hCM, in a CC-reversible way (Figure 4A–E). Similarly to the human atrial myocardium, exogenous canagliflozin reduced basal \text{O}_2^\text{•} \text{ generation (Figure 4F) and NADPH oxidase activity (Figure 4G and H) and improved NOS coupling (Figure 4I) in an AMPK\textsubscript{\alpha}2-dependent manner (since all those effects were reversed by CC) in hCM. Dihydroethidium staining confirmed the effect of canagliflozin on NADPH oxidase-derived \text{O}_2^\text{•} \text{ in hCM (Figure 4J–L). Canagliflozin also induced NOS phosphorylation at its activation site Ser1177 in hCM (Supplementary material online, Figure S7). These findings were replicated in H9c2 cells (Supplementary material online, Figures S8 and S9), where the results were more striking after culture in a high-glucose medium.}

The role of SGLT1 in myocardial redox regulation by canagliflozin

To explore how canagliflozin affects the upstream phosphorylation of AMPK\textsubscript{\alpha}2, we next evaluated the intracellular ADP-to-ATP ratio in hCM, which can allosterically regulate AMPK\textsubscript{\alpha}2 activity and auto-phosphorylation. Canagliflozin increased ADP/ATP in a dose-dependent manner (Figure 5A). Depleting glucose from the culture media resulted in increased ADP/ATP (Figure 5A) and activated AMPK\textsubscript{\alpha}2, evidenced by ACC phosphorylation. Adding canagliflozin to glucose-starved hCM did not lead to further change on either AMPK\textsubscript{\alpha}2 or ACC (Figure 5B and C). These indicate that the effect of canagliflozin on hCM is dependent on glucose uptake, which then affects intracellular ADP/ATP and subsequent AMPK\textsubscript{\alpha}2 activation.

Consistent with our findings in human cardiac samples, hCM SGLT2 expression was undetectable (Figure 5D–F). SGLT1 expression confirmed by qPCR and Western immunoblotting using HEK293 cells as positive control (Figure 5D and E). To further explore the

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**Table 2** Table Multivariable regression model of myocardial NADPH oxidase activity

| Covariate          | Standardized \( \beta \) | Adjusted \( P \)-value |
|--------------------|--------------------------|-------------------------|
| Myocardial SGLT1 expression | 0.339                    | 0.001                   |
| Age (years)       | 0.152                    | 0.021                   |
| NYHA class        | 0.137                    | 0.032                   |
| Smoking           | 0.067                    | 0.284                   |
| Diabetes          | 0.054                    | 0.378                   |
| Hypertension      | 0.022                    | 0.737                   |
| Sex               | -0.052                   | 0.401                   |
| Hypercholesterolaemia | -0.034                | 0.601                   |

SGLT1, sodium-glucose cotransporter 1. Statistically significant values are in bold.
Figure 2  Direct effects of canagliflozin and empagliflozin on human myocardial redox state. Ex vivo canagliflozin (3, 10, and 100 μM) treatment for 1 h reduced basal (A), NADPH-stimulated (B), and Vas2870 inhibitable O$_2^-$ (C) and increased L-NAME-(delta O$_2^-$) (D) dose-dependently in human atrial myocardium. Canagliflozin decreased the intensity of basal and Vas2870 inhibitable 2-hydroxyethidium (2-OH-ethidium) fluorescence (E–G) in human atrial biopsies stained with dihydroethidium (DHE). Empagliflozin had non-significant direct effect on either of these measures (H–K). n = 5–7 in panels A–L. Data are presented as mean ± SD. P-values are calculated by Wilcoxon signed-rank test (A–C, F–J) and paired t-test (D, K).
Effects of canagliflozin on human heart

Figure 3 Effects of canagliflozin on myocardial NADPH oxidase activity and nitric oxide synthase (NOS) coupling status in the human atrial myocardium. Canagliflozin (10 μM for 1 h) inhibited GTP activation (A) and membrane translocation (B) of Rac1, as well as the membrane translocation of p47phox (C). Canagliflozin increased myocardial BH4 but not total biopterin content (D–F). Canagliflozin induced AMPK Thr172 phosphorylation (G) and downstream acetyl-coA carboxylase (ACC) Ser79 phosphorylation (H). These were prevented by the AMPK inhibitor, compound C (CC) (G and H). Canagliflozin did not affect ERK or AKT phosphorylation (I and J). Canagliflozin induced NOS Ser1177 phosphorylation (K). Results are mean ± SD. $P$-values are calculated by Wilcoxon signed-rank test (A–N) and paired $t$-test (O).
Figure 4 Direct effects of canagliflozin on human cardiomyocytes (hCM). Canagliflozin (10 μM) induced phosphorylation of AMPK and acetyl-coA carboxylase (ACC) in high glucose (HG)-treated human cardiomyocytes (A and B, n = 8) and increased BH4 levels without affecting total biopterin levels (C–E, n = 8). Canagliflozin decreased basal, NADPH-stimulated and Vas2870-inhibitable O2•− and increased the value of L-NAME delta(O2•−) in human cardiomyocytes (F–I, n = 7). Dihydroethidium (DHE) staining combined with Vas2870 confirmed these (J–L, n = 8). Data are presented as mean ± SD. P-values are calculated by Wilcoxon signed-rank test (A–H, J, K) and paired t-test (I).
Figure 5 SGLT1 mediates the effects of canagliflozin on myocardial redox state. Canagliflozin increased ADP/ATP dose-dependently in human cardiomyocytes, while glucose deprivation from the culture medium (NG) induced similar changes in ADP/ATP ratio (A, n = 8). There were no differences in AMPK and ACC phosphorylation among the NG-incubated cells with or without canagliflozin (10μM), and high glucose (HG)-incubated cells with canagliflozin (panels B and C, n = 8). SGLT1 expression was detected, while SGLT2 was not detected in human cardiomyocytes (D–F, n = 8). SGLT1 was knocked down using siRNA (transfection low toxicity and efficiency was confirmed by transfecting BlockiT Alexa Fluor Red Fluorescent control, G), resulting into ~76% down-regulation of SGLT1 mRNA (panel H, n = 6), and ~85% protein down-regulation (panel I, n = 6). Canagliflozin-induced AMPK and ACC phosphorylation was attenuated in SGLT1 knocked down human cardiomyocytes compared with siRNA ctrl human cardiomyocytes (J and K, n = 8). SGLT1 deletion diminished canagliflozin-induced decrease of basal (L), NADPH-stimulated (M), and Vas2870-inhibitable O₂ production (N) and increased L-NAME delta O₂⁻ (O). n = 8 in L–O. Data are presented as mean ± SD. P-values are calculated by Wilcoxon signed-rank test (A–C, H–I). Comparisons in canagliflozin responses between siControl and siSGLT1 cells were performed with two-way ANOVA with treatment (canagliflozin) × cell type (siControl or siSGLT1) interaction (J–O).
Direct effects of canagliflozin on redox-sensitive pro-inflammatory signalling in the human myocardium

After establishing a role for canagliflozin as a regulator of the myocardial redox state, we explored its downstream effects on the transcriptomic profile of hCM. Canagliflozin (10 μM) treatment for 24 h altered the expression of 946 differentially expressed genes (DEGs, 466 up-regulated and 480 down-regulated) in hCM, with 127 DEGs demonstrating an effect size of >50% or >466 up-regulated and 480 down-regulated) in hCM also prevented the ability of canagliflozin to improve NOD coupling (Figure 5F). SGLT1 knock down did not lead to a compensatory increase in SGLT2 expression (Supplementary material online, Figure S10). Interestingly, knock down of SGLT1 did not fully mimic the effects observed with canagliflozin, and this could be explained either by the incomplete SGLT1 knock down technically achieved in these cells, or even by the presence of off-target effects of canagliflozin, independent of SGLT1/2 inhibition.

Discussion

In this work, we identify SGLT1 as the main SGLT isoform in the human myocardium, its expression being positively related with myocardial oxidative stress. As the main objective of this study was to evaluate the direct effects of SGLT1 vs. SGLT2 inhibition on the myocardium and SGLT2 is not expressed in human myocardium and cardiac myocytes, we focused on canagliflozin, which has significant affinity for SGLT1. We further demonstrate that a clinically relevant concentration of canagliflozin directly suppresses NADPH oxidase activity, increases BH4 bioavailability, and improves NOS coupling in isolated hCM and myocardial tissue via an SGLT1/AMPK/Rac1-dependent mechanism. These suppress redox-sensitive pro-inflammatory and pro-apoptotic signalling in the human cardiomyocyte, providing a novel mechanism contributing to the beneficial effects of canagliflozin on cardiac adverse events (Graphical abstract).

The role of SGLT isoforms in myocardial physiology is controversial. SGLT2 expression has been detected in hCM after exposure to high glucose; however, other studies have shown no SGLT2 expression in human hearts. In contrast, SGLT1 was found in hCM while functionally damaging variants in SGLT1 have been associated with reduced death/heart failure risk. We definitively demonstrate, using a large number of human myocardial biopsies and hCM, that SGLT1 is abundantly expressed in the human heart, whereas SGLT2 is barely detectable.

Clinically used SGLT2 inhibitors have variable SGLT1 affinities. Canagliflozin displays higher SGLT1 affinity than other SGLT2 inhibitors, whilst empagliflozin is the most selective SGLT2 inhibitor. Evidence has suggested differing cellular responses to canagliflozin vs. empagliflozin, e.g. canagliflozin is a stronger inducer of AMPK phosphorylation in HEK293 cells or human vascular endothelial cells. Importantly, we demonstrate for the first time that canagliflozin (in concentrations comparable to in vivo pharmacological levels) directly inhibits NADPH oxidase-derived oxidative stress in human myocardial redox signalling via SGLT1/AMPK/Rac1 signalling, which may be more potent than empagliflozin. Furthermore, canagliflozin could reverse the decrease in ADP/ATP ratio due to a hyperglycaemic environment by inhibiting glucose uptake in hCM.

Myocardial oxidative stress facilitates apoptosis and inflammation, leading to cardiac fibrosis and promoting cardiac dysfunction. Myocardial inflammation is influenced by redox-sensitive transcriptional factors such as NFκB. Our transcriptome analysis demonstrated that canagliflozin suppresses several redox-sensitive pro-inflammatory and pro-apoptotic pathways in hCM, such as those of TNF-α and IL-1 as well as, crucially, on NFκB activity. The link of canagliflozin with myocardial redox regulation was further validated in ~400 human atrial biopsies, by demonstrating a clear relationship between myocardial NADPH oxidase-derived O₂⁻ and the expression of key genes regulated by canagliflozin.

Certain aspects of the interplay between SGLT2 inhibitors and myocardial disease are not explored in our work. SGLT2 inhibitors with little SGLT1 selectivity (e.g. empagliflozin) also demonstrate cardioprotective effects despite lacking SGLT1 affinity. A very recent study from Koliijn et al. described an antioxidant effect of empagliflozin, in humans with heart failure with preserved ejection fraction as well as in ZDF obese rats, evaluated by lipid peroxidation levels, which was accompanied by improved NO-sGC-PKG signalling. Although in our study empagliflozin did not affect NADPH oxidase-derived superoxide production in human atrial myocardium, these two pieces of work may describe distinct mechanisms, which could be complementary and the discrepancies in results may be due to different participant characteristics, use of different (indirect) readings of redox state (we specifically targeted NADPH oxidase-derived superoxide, whilst Koliijn et al. targeted lipid peroxidation, which is influenced by reactive oxygen species levels, antioxidant capacity, and cell metabolism) and tissue type differences (atrial vs. ventricular tissue). Furthermore, this suggests that systemic SGLT2 inhibition has also indirect effects on the human heart, most likely by affecting kidney function as well as the secretome of other tissues with indirect, endocrine effects on cardiac function, which is not explored here. However, some SGLT2 inhibitors (like canagliflozin) may well have...
Figure 6 Canagliflozin has a global anti-inflammatory and anti-apoptotic effect on human cardiomyocytes. In this experiment, human cardiomyocytes were cultured in high-glucose medium (25 mM) for 72 h and then treated with canagliflozin (10 μM) or DMSO for 24 h. Heat map of 127 down- or up-regulated genes by canagliflozin (fold change >1.5 or /C18 -1.5, P < 0.05) in canagliflozin-treated human cardiomyocytes from n = 7 patients (A). NFkB, Wnt, IL1, IL3, TNFα, chemokine, MAPK pathways, and apoptotic pathways (highlighted by red font) were down-regulated by canagliflozin (more than 50% of pathway genes down-regulated by canagliflozin, B). TNFRSF11, TRAF5, FZD7, CASP7, and BAD were the most down-regulated individual genes upon canagliflozin treatment, implicated in NFkB, TNFα, and apoptosis pathways. The mRNA expression of these genes was positively correlated with myocardial NADPH oxidase activity (i.e. high Vas2870-inhibitable signal) in 240 atrial biopsies from Study 1 (C). Data are presented as median [25th–75th percentile]. P-values are calculated by Mann–Whitney U-test for high (above median) vs. low (below median).
chronic direct effects on the heart via cardiac SGLT1 inhibition, and this direct effect on the human myocardium was the objective of our study. This concept warrants further validation in appropriate in vivo animal models, such as Sglt1 loss- and gain-of-function mouse models.

Of note is the fact that the ex vivo experiments were performed on myocardial tissue of atrial origin, whereas our mechanistic experiments were carried out in cardiomyocytes derived from ventricles. The fact that our proposed mechanism is shown in a similar way in all models (human atrial myocardium, human ventricular cardiomyocytes, and terminally differentiated H9c2 cells) further strengthens the validity of the proposed mechanisms by which canagliflozin affects myocardial biology. In this study, we evaluate the membrane translocation of NADPH oxidase subunits by measuring their levels on isolated membranes vs. the cytosolic phase using an ultra-centrifugation protocol, rather than electron microscopy, and this is a methodological limitation that needs to be acknowledged. Finally, in translational studies like this one, surgical human tissue availability varies, and this has led to variability in sample sizes across the various experiments in this study.

In conclusion, we demonstrate for the first time that canagliflozin inhibits NADPH oxidase activity and improves NOS coupling via SGLT1/AMPK signalling, whilst suppressing several pro-inflammatory and pro-apoptotic pathways in the human myocardium. Our work describes an important SGLT1-mediated mechanism that could contribute to the cardioprotective effects of the SGLT2 drug class.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

**Acknowledgements**

H.K. conceived and performed experiments, performed data analysis, and drafted the manuscript; I.A. conceived and performed experiments, contributed to participant recruitment, performed data analysis, and contributed to the writing of the manuscript; I.B. performed experiments and data analysis and reviewed the manuscript; N.A. performed data analysis and reviewed the manuscript; C.P.K. contributed to participant recruitment and human sample collection, conceived and performed experiments, performed data analysis, and contributed to the writing of the manuscript; M.P. performed experiments and data analysis; I.S. performed experiments and data analysis; E.S. contributed to biopsy collection and performed data analysis; A.S.A. performed experiments, contributed to participant recruitment, and performed data analysis; M.C.C. conceived and performed experiments; E.K.O. contributed to the project design; E.M.R. contributed to participant recruitment; R.S., G.K., V.S., and S.F. contributed to surgical specimen collection; S.C. performed experiments; C.S. and K.M.C. contributed to the project design and reviewed the manuscript; B.C. conceived the project, secured funding, oversaw the implementation of individual experiments, performed data analysis, and corrected the manuscript.

**Funding**

This study was supported by the British Heart Foundation (FS/16/15/32047, and RGI/F/21/110040 to C.A., CH/16/1/32013 to K.C., and Centre of Research Excellence award RG/13/1/20181), the National Institute for Health Research Oxford Biomedical Research Centre. H.K. acknowledges support by the Japanese Heart Rhythm Society-European Heart Rhythm Association fellowship grant sponsored by Biotronik. The authors thank the High-Throughput Genomics Group at The Wellcome Trust Centre for Human Genetics (funded by Wellcome Trust grant reference 090532/Z/09/Z) for the generation of the microarray data.

**Conflict of interest:** C.A. declared past consultancy agreement with Mitsubishi Tanabe, past grants from Sanofi and Novo Nordisk. C.A. and K.M.C. are founders, shareholders, and directors of Caristo Diagnostics, and C.S. is an employee and shareholder of Caristo Diagnostics. C.A. is the chair of British Atherosclerosis Society. B.C. is the past president of the European Society of Cardiology. The remaining authors have nothing to disclose.

**Data availability**

The data underlying this article will be shared on reasonable request to the corresponding author.

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