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Pioglitazone alters Ace/Ace 2 balance to favour Ace2 independently of glycaemia levels in diabetic rat heart

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Received May 12, 2023, accepted June 16, 2023

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Pharmazie 78: 122-127 (2023) *doi: 10.1691/ph.2023.3570*

The activation of the renin-angiotensin system (RAS) contributes to the pathogenesis of cardiac damage during diabetes. In the present study, we investigated the role of pioglitazone, dapagliflozin and their combination on RAS components in streptozotocin-induced diabetic cardiomyopathy in Wistar rats. Blood glucose, serum lipids, and ACE (angiotensin-converting enzyme), ACE2 levels were determined. mRNA levels of Myh6 (myosins heavy chain), Myh7, Ace, Ace2, Nppa, Nppb (natriuretic peptide A, B) and Ppars (peroxisome proliferator activating receptors) genes in the heart were determined by real-time PCR (polymerase chain reaction). Protein expression of ACE and ACE2 was assessed by western blotting. After six weeks pioglitazone suppressed Ace mRNA and protein levels ($p<0.05$) and modified the Ace/Ace2 ratio ($p<0.05$) in the cardiac tissue of diabetic rats. Pioglitazone significantly decreased serum lipids ($p<0.05$) but did not significantly influence blood glucose and ACE serum levels of diabetic animals. Dapagliflozin had a significant glucose-lowering action ($p<0.05$) however, it had no impact on the Ace/Ace2 ratio. The combination of both compounds markedly improved blood glucose ($p<0.05$) as well as the Myh6/Myh7 ratio ($p<0.05$) but had no further impact on the Ace to Ace2 balance in cardiac tissue compared to pioglitazone monotherapy. We found that pioglitazone improves the cardiac Ace/Ace2 ratio in diabetic rats suggesting a potential cardioprotective effect. This effect is independent of its antidiabetic and metabolic effects.

1. Introduction

The high prevalence of cardiovascular disease (CVD) among patients with type 2 diabetes (T2DM) has been widely recognized and extensively discussed over the past several decades. Yet, the risk of CVD is also greatly increased in patients with type 1 diabetes (T1DM) and it is a major cause of mortality in such patients because of an earlier age of onset (Vergés 2020).

The diabetes-associated changes in myocardial function have been called diabetic cardiomyopathy (Bugger and Dale Abel 2014). The pathogenesis of diabetic cardiomyopathy also involves the enhanced activation of PPAR (peroxisome proliferator-activated receptor) transcription factors, including PPARα, and to a lesser degree PPARβ and PPARγ (He and Quintana 2015). PPARγ agonist, pioglitazone, enhances the sensitivity of liver, muscle and adipose tissue to insulin and facilitates glucose uptake into the cells (Kavitha et al. 2016). Pioglitazone is authorised for oral therapy of T2DM patients (Rőszer and Ricote 2010) and furthermore, some studies suggested it potentially positive influence on the treatment and prevention of T1DM as well, since pioglitazone reduced pancreatic β cells destruction and therefore partially helped to preserve insulin secretion (Tafuri 2013; Bhat 2007). Experimental and human data suggest vasculoprotective effects of pioglitazone, including delayed atherosclerosis progression and reduced cardiovascular events (Nesti et al 2021). Concordantly with human observations (Dorkhan 2009; Hughes 2013) pioglitazone exhibited some cardioprotective effects as well in animal models (Rodriguez et al. 2006; Kim et al. 2003).

The disrupted renin-angiotensin-system (RAS) has a role in the pathogenesis of diabetic cardiomyopathy in T1DM, too (Bugger and Dale Abel 2014; Miller 1999). Hyperglycaemia increases the expression and concentration of RAS components in the pancreatic islets (Lupi et al. 2006), rat kidneys (Siragy and Huang 2008) and rat heart (Dostal and Baker 1999). ACE catalyzes the conversion of angiotensin I (Ang I) into angiotensin II (Ang II) (Costela-Ruiz et al. 2020). Ang II induces vasoconstriction, oxidative stress and hypertension (Stelzig et al. 2020; Fiorino and Evangelista 2014). ACE activity is counterbalanced by ACE2 converting Ang II into Ang (1-7) which is a vasodilator (Varagic et al. 2014). In diabetic patients, ACE hyperactivity shifts the balance between Ang (1-7) and Ang II towards enhanced Ang II. This imbalance results in cardiovascular complications (Rabelo et al. 2011). The gene expression of RAS components is modulated by PPARs. PPARγ agonists like pioglitazone strongly reduce Ang II levels, Ace and AT1r (angiotensin 1receptor) gene expression and enhance Ace2 gene expression in vascular tissues and thus may diminish cardiovascular complications associated with RAS activation (Kvandova et al. 2016).

Dapagliflozin, a highly selective sodium-glucose co-transporter 2 (SGLT2) inhibitor, was shown to improve glycaemic control by decreasing renal glucose reabsorption in the kidneys (Heerspink et al. 2016). Dapagliflozin treatment also showed a statistically significant reduction in serious cardiovascular events both in diabetic patients with heart failure and in patients without diabetes mellitus (McMurray et al. 2019). Some experimental (Shin et al. 2016) and clinical studies (Kopecky et al. 2019) showed that SGLT2 inhibitors suppress activity (renin, aldosterone) and expression of RAS components (AT1r, Ace, Ace2) and increase production of RAS component Ang1-7 (angiotensin 1-7). These changes can improve the balance of both types of systemic and intrarenal RAS components in both types of DM and thus further support current glucose-lowering, cardiovascular- and renoprotective treatment.

Thus, in the present study, we examined the effect of the PPARγ agonist pioglitazone and SGLT 2 inhibitor dapagliflozin and their combination on ACE as a component of RAS in diabetic rats. We aimed to investigate the alterations in Ace vs Ace2 balance in the heart of diabetic rats treated with pioglitazone, dapagliflozin

and their combination potentially resulting in a cardioprotective effect. We further studied the association between changes in ACE balance and the antidiabetic effects of the drugs.

2. Investigations and results

2.1. Biochemical and biometric parameters of the experimental groups

Following STZ injection, the rats displayed elevated blood glucose, water, and food intake and also urine output at the time of stratification at week 6, when compared to controls. The results in Table 1 show that both monotherapies, as well as the combined therapy, attenuated STZ-induced manifestations of diabetes ($p<0.05$ for all). Diabetes-related dyslipidemia, manifesting as significantly elevated levels of TAG and total cholesterol was decreased only in the STZ+DapaPio group when compared to the other groups ($p<0.05$). As shown in Table 1, monotherapy and combined therapy resulted in increased body weight as well as absolute (but not relative) heart weight when compared to the STZ-only treated animals ($p<0.05$).

2.2. Serum concentrations of ACE and ACE2 enzymes determined by ELISA

Serum concentrations of ACE and ACE2 enzymes are summarized in Table 2. The application of STZ caused significantly increased ($p<0.05$) levels of ACE and ACE2 enzymes in blood serum when compared to controls. We observed a significant decrease in ACE levels in the STZ+ DapaPio group only in comparison to the STZ group and STZ + Dapa group ($p<0.05$). The levels of ACE2 remained unchanged in all treated groups when compared to the untreated STZ group.

Table 1: Characteristics of experimental groups

	Control	STZ	STZ+Pio	STZ+Dapa	STZ+DapaPio
n	10	10	10	8	9
Food intake (g/24h)	17 ± 3	37 ± 2*	23 ± 4#	30 ± 3*	25 ± 6#
Water intake (ml/24h)	20 ± 2	105 ± 10*	68 ± 15*#	76 ± 7*#	85 ± 5*#$£
Urine output (ml/24h)	6 ± 1	72 ± 6*	27 ± 13*#	31 ± 2*#	51 ± 5*#$£
Fasting glucose (mol/ml)	7.18 ± 0.15	14.62 ±0.78*	12.21 ± 1.01*	10.02 ± 0.32*#	10.11 ±0.21*#£
Random glucose (mol/ml)	7.90 ± 0.22	20.23 ± 0.65*	18.10 ± 0.43*	16.24 ± 0.62*	14.98 ± 0.81*# £
Total cholesterol (mol/ml)	1.87 ± 0.07	2.33 ± 0.37*	2.09 ± 0.01*#	2.30 ± 0.04*	2.18 ± 0.09*#$
TAG (mol/ml)	1.05 ± 0.12	1.85 ± 0.24*	1.62 ± 0.19*#	1.79 ± 0.27*	1.53 ± 0.08*#$
ACE (ng/ml)	24.26±1.05	46.66±3.07*	39.89±2.72*	47.30±3.77*	31.89 ± 3.73*#$
ACE2 (ng/ml)	28.23±1.72	54.98±5.10*	64.18±3.99*	55.41±5.27*	55.05±3.40*
Body Weight (g)	368 ± 14	267 ± 12*	334 ± 13*#	345 ± 9*#	315 ± 9*#$£
Heart weight (g)	1.13 ± 0,04	0.82 ± 0.04*	1.02 ± 0.05#	1.04 ±0.03#	1.03 ±0.03#
Heart weight/body weight	3.10±0.10	3.09 ±0.10	3.05 ±0.09	3.02±0.05	3.18±0.06

Groups labelling: STZ – streptozotocin administered diabetic rats; STZ+ Pio – pioglitazone-treated STZ rats; STZ+Dapa – dapagliflozin-treated STZ rats; STZ+DapaPio – dapagliflozin- and pioglitazone-treated STZ rats. Data are presented as mean±SEM; *p < 0.05 vs Control . # p < 0.05 vs STZ, $ vs STZ+Dapa, £ vs STZ+Pio.Abbreviations: TAG – triglycerides, Ace – angiotensin-converting enzyme

Table 2: Expression of ACE and ACE2 at the protein level in the heart

Protein	Control	STZ	STZ+Pio	STZ+Dapa	STZ+DapaPio
ACE	100 ± 4	110 ± 9	79 ± 5#	84 ± 6	94 ± 8
ACE2	100 ± 7	84± 6*	109± 9	101 ± 7	118 ± 7
ACE/ACE2 ratio	1.00 ±0.17	1.10 ± 0.13	0.77 ± 0.08 #	0.86 ± 0.09	0.78 ± 0.08 #

For the labelling of the groups, see Table 1. Data are presented as mean±SEM; *p < 0.05 vs Control #p < 0.05 vs STZ

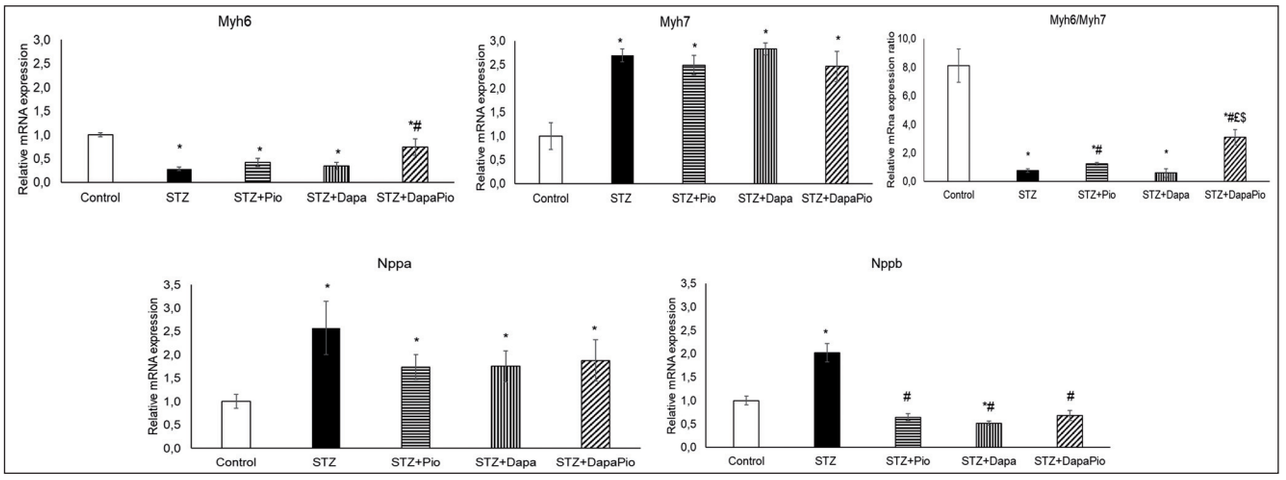

Fig. 1: Relative cardiac mRNA expressions of Myh6 myosin heavy chain 6 (alpha-myosin heavy chain), Myh7 myosin heavy chain 7 (beta-myosin heavy chain) proteins, their ratio, Nppa and Nppb enzymes in the heart. For labelling of the groups, see Table 2. Data are presented as mean±SEM; p < 0.05 * vs Control, p < 0.05 # vs STZ, $ vs STZ+Dapa, £ vs STZ+Pio.

2.3. Pioglitazone in monotherapy and combination therapy increased Myh6/Myh7 mRNA ratio levels and decreased mRNA of the BNP in the heart

The presence of cardiac damage was suggested by a significant ($p < 0.05$) myosin-isoform shift and elevated ANP and BNP mRNA levels in the STZ group (Fig. 1). We observed the (Nppa) of (Nppb) exclusively in the group with the treatment combination ($p<0.05$ *vs.* untreated STZ). This was supposedly influenced by Pio, as only groups with its administration exhibited an elevated Myh6 to Myh7 ratio when compared to untreated STZ rats ($p<0.05$). In mRNA expression, the experiment reported that the levels of ANP were not changed in treatment groups compared to the STZ group. In contrast, after 42 days, treatment with Pio, Dapa and DapaPio had significantly ($p < 0.05$) down-regulated BNP mRNA expression as compared to STZ rats.

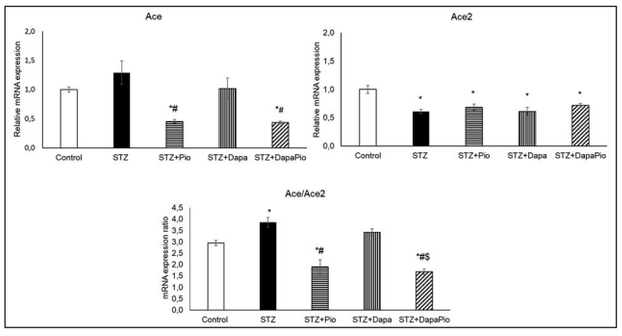

Fig. 2: Relative cardiac mRNA expressions of the Ace and Ace2 enzymes in heart and their ratio. For labelling of the groups, see Table 2. Data are presented as mean±SEM; $p < 0.05$ * vs Control, $p < 0.05$ # vs STZ, $ vs Dapa

2.4. Pioglitazone monotherapy and combination with dapagliflozin decreased the mRNA and protein expression of Ace but not Ace2 in the diabetic heart

Due to impact on myocardial remodelling, as evidenced in the myosin's heavy chain alteration, we assumed the possible involvement of the tissue RAS system (Fig. 2). STZ significantly decreased ($p<0.05$) Ace2 gene expression and significantly increased ($p<0.05$) Ace/Ace2 ratio as compared to control. In comparison to the untreated STZ rats, cardiac Ace mRNA levels were significantly decreased by administration of Pio and DapaPio ($p<0.05$ for both). On the other hand, cardiac Ace2 mRNA and protein levels remained stable across the groups (Fig. 3, Table 2). Consequently, Pio and DapaPio treatment altered the equilibrium between angiotensin-converting enzymes, as evidenced by the significantly decreased Ace/Ace2 mRNA and level protein ratio ($p<0.05$ *vs.* both STZ and STZ+Dapa, resp.). Additionally, we also found that the cardiac expression of AT1r remained stable in all treated groups when compared to the untreated STZ group (STZ=1.04±0.03; STZ+Pio=1.08±0.06; STZ+Dapa=1.06±0.06; STZ+DapaPio=0.93±0.04, *NS*). The mRNA levels of At2r and Mas were below the detection limit of the method used.

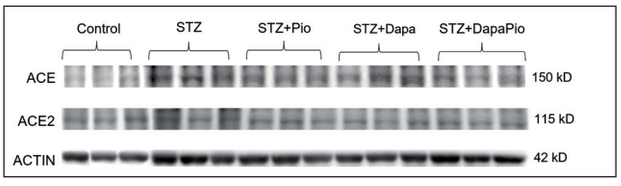

Fig. 3: Protein expression of ACE and ACE2 in the heart. Actin was used as a loading control. Representative autoluminograms. For labelling of the groups, see Table 1.

Table 3 : Primer sequences for quantitative RT-qPCR

Gene	GenBank PCR accession number	Primer sequence (5´-3´)	PCR product length (bp)
Hprt1	NM_012583.2	Forward: CAGCTTCCTCCTCAGACCGCTTT	82
		Reverse: TCACTAATCACGACGCTGGGACTG	
B2m	NM_012512.1	Forward: ATGGAGCTCTGAATCATCTGG	105
		Reverse: AGAAGATGGTGTGCTCATTGC	
Myh6	NM_017239.2	Forward: GCCCTTTGACATCCGCACAGAGT	152
		Reverse: TCTGCTGCATCACCTGGTCCTCC	
Myh7	NM_017240.1	Forward: GCGGACATTGCCGAGTCCCAG	133
		Reverse: GCTCCAGGTCTCAGGGCTTCACA	
Ace	NM_012544.1	Forward: TCCTGCTAGACATGGAGACGA	142
		Reverse: CAGCTCTTCCACACCCAAAG	
Ace2	NM_001012006.1	Forward: CGCTGTCACAGACAAGAA	129
		Reverse: CGTCCAATCCTGGTTCAAG	
Ppara	NM_013196.1	Forward: AATCCACGAAGCCTACCTGA	132
(Pparα)		Reverse: GTCTTCTCAGCCATGCACAA	
Ppard	NM_013141.1	Forward: GCAGCCTCAACATGGAGTG	167
(Pparβ/δ)		Reverse: GTTGCGGTTCTTCTTCTGGA	
Pparg	NM_001145366.1	Forward: GCCCTTTGGTGACTTTATGG	166
(Pparγ)		Reverse: CAGGTTGTCTTGGATGTCCTC	
Nppa (ANP)	NM_012612.2	Forward: GGGGGTAGGATTGACAGGAT	104
		Reverse: GGATCTTTTGCGATCTGCTC	
Nppb (BNP)	NM_031545.1	Forward: GACCGGATCGGCGCAGTCAGT	78
		Reverse: GGAGTCTGCAGCCAGGAGGTCT	

Abbreviations: HPRT1: hypoxanthine phosphoribosyltransferase 1; B2m: β2-Microglobulin; Myh6: myosin heavy chain 6 (α-myosin heavy chain); Myh7: myosin heavy chain 7 (β-myosin heavy chain); Ace: angiotensin-converting enzyme; Ace2: angiotensin-converting enzyme 2; Ppara: peroxisome proliferator-activated receptor alpha; Ppard: peroxisome proliferator-activated receptor beta/delta; Pparg: peroxisome proliferator-activated receptor gamma

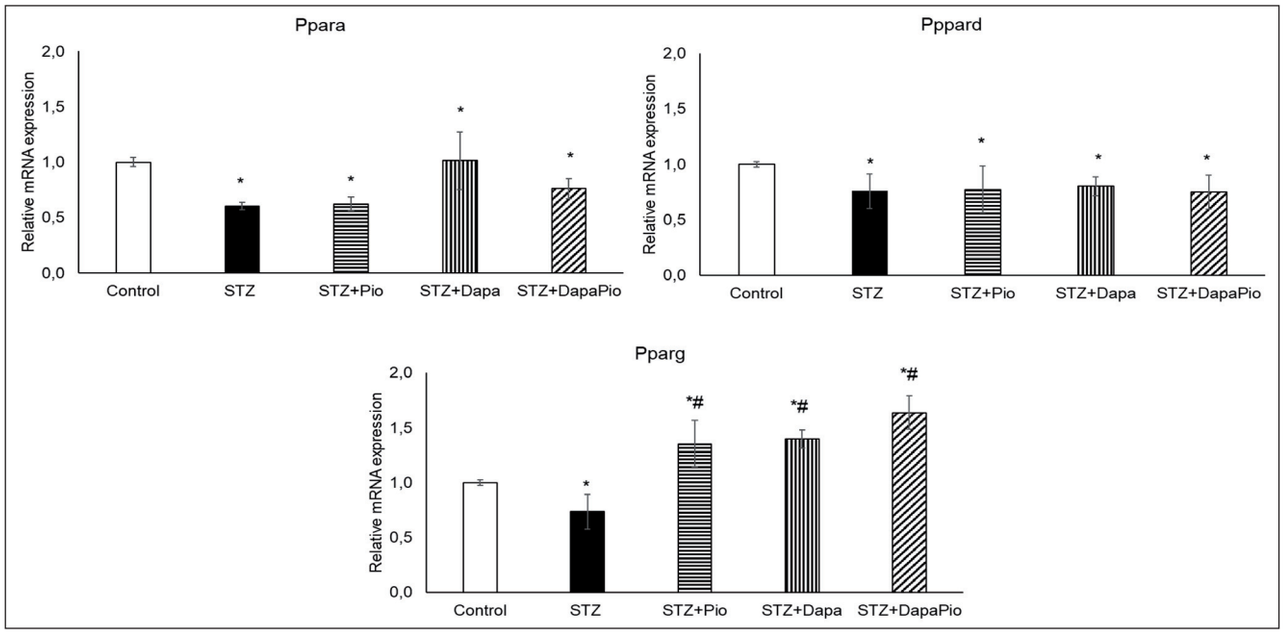

Fig. 4: Relative cardiac mRNA expressions of peroxisome proliferator-activated receptors (Ppara – Ppar alpha; Ppard – Ppar beta/delta and Pparg – Ppar gamma). For labelling of the groups, see Table 2. Data are presented as mean±SEM; $p < 0.05$ * vs Control, $p < 0.05$ # vs STZ.

2.5. Glucose-lowering treatment increased the mRNA expression of Pparγ in the heart

We also evaluated the involvement of PPARs in the damaged hearts after STZ administration (Fig. 4). In the STZ model, decreased mRNA levels of all three isoforms were observed. The Pparα and Ppar β/δ mRNA levels remained unchanged compared to the STZ group but Pparγ significantly increased in all the treated groups (Fig. 4, $p<0.05$ *vs.* untreated STZ).

3. Discussion

In the present study, we have shown that pioglitazone affects the cardiac RAS system by decreasing the protein and mRNA level of the Ace/Ace2 ratio in rats with STZ-induced T1DM. This effect was observed when pioglitazone was administrated alone or in combination with dapagliflozin and was independent of the antidiabetic action of the drug. Many pathological conditions, including those of the cardiovascular system, are associated with an increase in the Ace/Ace2 ratio within the organs and systems. This Ace/Ace2 imbalance is very often due to a downregulation of ACE2 levels and is accompanied by a disturbance in RAS homeostasis (Pagliaro and Penna 2020). Increased Ace/Ace2 ratio may induce angiotensin II over-activation and accelerate cardiac remodelling in patients with heart failure (Wang et al 2015). Prolonged exposure to high glucose is also associated with the upregulation of Ace and the downregulation of Ace2 (Lavrentyev and Malik 2009). Therefore, the cardiac Ace/Ace2 ratio decreasing effect of pioglitazone may result in the balancing of RAS and potential cardioprotection. It is well known that PPARγ agonists can attenuate the adverse effects of angiotensin II by reducing the angiotensin II concentration and ACE and AT1r gene expression in the body (Zhang et al. 2014). In addition, pioglitazone can decrease Ace and increase Ace2 expression in vascular and cardiac tissue in rats with T1DM (Efrati et al. 2007; Qiao et al. 2015). Similarly, in this study, we report a downregulation of cardiac ACE and a decrease in ACE serum levels after treatment with pioglitazone as well as the combined treatment of pioglitazone and dapagliflozin. Dapagliflozin alone did not affect ACE and ACE2 expression (both on protein and mRNA levels), as well as serum levels, compared to the STZ-only, treated group. Although SGLT2 inhibitors treatment showed beneficial effects on renal RAS (McMurray et al. 2019, Shin et al. 2016) their effect on myocardial RAS is still not clear. The mechanism explaining the influence of dapagliflozin on ACE and ACE2 levels in serum and myocardial tissue is currently unknown and warrants further studies. Our results suggest that pioglitazone and not dapagliflozin contribute to decreased levels of cardiac and serum ACE in diabetic rats.

One could hypothesize that the improvement in the Ace/Ace2 ratio in our experiment might be due to the antidiabetic actions of the drugs. Multiple diabetic rodent models have demonstrated clinical improvement of hyperglycaemia induced by STZ when pre-treated with TZDs (thiazolidinediones) (Tafuri et al. 2013; Takamura et al. 1999). Additionally, SGLT2 inhibitors were reported to effectively reduce hyperglycaemia in both T1DM and T2DM animal models of diabetes (Kojima et al 2015; Han et al. 2008). However, in our model, pioglitazone did not lower fasting and random glucose. In general, pioglitazone needs insulin for its effect and animals after STZ application had very low insulin levels (data not published) that's why pioglitazone did not change them compared to the STZ group. On the other hand, dapagliflozin reduced fasting glucose in STZ-induced diabetes and the drug combination significantly reduce fasting and random glucose. The improvement of the Ace/Ace2 ratio and no reduction in blood glucose after pioglitazone treatment and *vice versa* after treatment with dapagliflozin suggests no connection between the antidiabetic effect of the drugs and the action of the drugs on the cardiac RAS system in diabetic animals. However, both pioglitazone and dapagliflozin and their combination significantly alleviated the symptoms of STZ-induced diabetes as loss of weight, polydipsia, polyuria, and polyphagia (Hakim et al. 1997). Interesting is the effect of dapagliflozin on urine output in our experiment. We measured a decrease in urine output after dapagliflozin administration. This could be caused by compensatory changes in medullary transport proteins which tended to conserve solute and water even with persistent glycosuria (Chen et al. 2016). Emerging evidence points toward the role of dyslipidemia as a crucial attendant risk factor in the pathogenesis of diabetic complications. Moreover, ACE, a key component of the RAS pathway is associated with dyslipidemia in a hyperglycemic milieu by promoting the development of insulin resistance, and interfering with insulin signaling (Mahwish et al. 2020). Pioglitazone and its combination with dapagliflozin but not dapagliflozin in monotherapy significantly decreased TAG, total cholesterol and ACE protein and Ace gene expression as well compared to STZ-treated rats. This effect of pioglitazone agrees with other studies reporting the effect of pioglitazone on plasma lipids in diabetic patients (Brackenridge et al. 2009) and experimental

animals (Refaat et al. 2016; Tenenbaum et al. 2005). Patients with T2DM treated with dapagliflozin experienced minor changes in lipid levels (Bays et al. 2017), as we also confirmed in our model experimental T1DM. These data confirmed the metabolic effects of both drugs described in other studies.

The pathophysiology of diabetic cardiomyopathy is frequently associated with cardiac tissue remodelling resulting in altered cardiac function (Nemoto et al. 2006). Although the absolute heart weight remained unchanged following therapy, pioglitazone elevated the Myh6-to-Myh7 ratio what could indicated an improvement in cardiac function. Cardiac contractility depends on the expression of two myosins' heavy chains (MHC) genes, Myh6 (encodes alpha-MHC) and Myh7 (for beta-MHC) (Doka et al. 2017). Alpha-MHC is crucial for normal myocardial function and is down-regulated in myocardial hypertrophy, cardiomyopathy and failing hearts (Carniel et al. 2005). The amount of myocardial Myh7 mRNA is minimal in the normal adult rat heart, but it increases during the development of ventricular dysfunction (Depre et al. 2000). In our experiments, we observed decreased mRNA expression of Myh6 and increased mRNA expression of Myh7 after STZ application. It has been published, that Pio increased alpha-MHC expression levels and also increased the alpha-MHC/beta-MHC ratio in diabetic rats (Pelzer et al. 2005) and also empagliflozin, another SGLT2 inhibitor, decreased diabetes-induced expression of beta-MHC in *ob/ob* mice compared to vehicle treatment (Hammoudi et al. 2017). An increase in Myh6 mRNA expression and also increased the alpha-MHC/beta-MHC ratio in the left ventricle of the pioglitazone and dapagliflozin + pioglitazone groups compared to the STZ group without therapy suggested favourable effects on cardiac function in our model of DM. All drug treatments decreased myocardial mRNA expression of BNP, while the mRNA level of ANP showed only a tendency to decrease compared to diabetic rats without treatment. The natriuretic peptides are established to be a remarkable biomarker to detect and monitor heart failure (Nishikimi et al. 2006). In patients with diabetic cardiomyopathy, rising ANP levels reflect the severity of cardiac dysfunction. Due to the expansion of volume and extreme pressure overload in ventricles, BNP is released from ventricular heart muscles. During hypertension, heart failure, or myocardial infarction, the elevation of BNP levels has been observed (Staudt et al. 2006). Some experimental (Liu et al. 2022) and clinical (Nesti et al. 2021) works have reported that pioglitazone and dapagliflozin can effectively improve cardiac function by influencing ANP and BNP levels in T2DM. Here, we report that they can also influence BNP in rats with T1DM. It seems that the combined therapy in our experiment could modulate diabetic cardiomyopathy on the molecular level and might provide cardioprotection. However, further experiments are needed to confirm our hypothesis.

In conclusion, pioglitazone did not influence blood glucose and significantly suppressed Ace mRNA and protein levels in the cardiac tissue of STZ-treated rats while Ace2 expression remained stable. In comparison, dapagliflozin had no impact on the Ace to Ace2 ratio, despite its significant glucose-lowering action. Notably, the combination of both compounds markedly improved blood glucose as well as cardiac damage markers but had no further impact on the Ace to Ace2 balance in cardiac tissue when compared to pioglitazone monotherapy. Conclusively, the possible cardioprotective action of pioglitazone was independent of its systemic antidiabetic and metabolic effects, and the reduction of the tissue Ace to Ace2 ratio might be the possible mechanism.

4. Experimental

4.1. Animal experiments

The experiments were performed using three-month-old male Wistar rats (220-250 g) obtained from the breeding station in Dobrá Voda (Slovak Republic). All animals were handled by certified investigators and animal keepers. The animals had free access to chow and tap water. The experimental T1DM was induced by a single dose of streptozotocin *i.p.* (STZ, 55 mg/kg, Sigma-Aldrich, St Louis, MO, USA) after overnight fasting. The STZ was dissolved in 0.1 mol/l citrate buffer, pH 4.5. After 72 hours, the developed diabetes was confirmed by glycaemia determination from a tail blood sample using an Accutrend Plus glucometer (Roche, Switzerland). Animals with a fasting blood glucose concentration 3 12 mmol/l were considered to be diabetic. Animals which did not have fasting blood glucose concentrations over 12mmol/l were excluded from the experiment. The STZ-treated rats were randomized into four groups: rats without treatment (STZ, n=10) and STZ rats with the administration of Dapa (STZ+Dapa, n=8) at a dose of 10 mg/kg of body weight, Pio (STZ+Pio, n=10) at a dose of 12 mg/kg of body weight, and their combination DapaPio (STZ+DapaPio, n=9) mixed in rat chow. The STZ-treated group received standard chow. The experiment was conducted for six weeks. Before the day of termination, the rats were placed in individual metabolic cages for 24 hours to observe their food and water consumption, as well as their urine output. The study protocol was approved by the Ethics Committee of the Faculty of Pharmacy at Comenius University in Bratislava and by the State Veterinary and Food Administration of the Slovak Republic (protocol No: Ro-1636/17-221).

4.2. Sample collection

After six weeks of follow-up, animals were sacrificed on carbon dioxide. Samples of left ventricle tissue were dissected, frozen in liquid nitrogen and stored at -80 ºC until further processing. Blood was collected from the abdominal aorta into tubes and after 1 hour centrifuged for 10 min at 4 ºC. The sera were stored at -80 ºC for subsequent biochemical analyses.

4.3. Assessment of biochemical parameters

Random and fasting blood glucose levels from tail blood samples were determined using an Accutrend Plus glucometer (Roche, Switzerland). Serum lipid levels, including total cholesterol and triglycerides (TAG), were determined via a service provided by a certified diagnostic laboratory (SYNLAB Slovakia, s.r.o., Slovak Republic).

4.4. Enzyme-linked immunosorbent assay (ELISA)

ELISAs were used to assess serum levels of Ace and Ace2 (both MyBioSource*;* Elabscience, USA), according to the manufacturer's instructions. In brief, sera samples were added into plates pre-coated with the primary antibody and incubated. Next, a secondary antibody was added and after another incubation, an enzymatic reaction with tetramethylbenzidine was analyzed spectrophotometrically. Quantification was performed using standard curves.

4.5. Real-time reverse transcription-polymerase chain reaction

Total RNA was isolated from left ventricular tissue using TRI Reagent (Sigma, USA), and its integrity was verified by agarose gel electrophoresis. Isolated RNA was reverse-transcribed into cDNA (High Capacity cDNA RT Kit with RNAse inhibitor, Applied Biosystems, USA). Real-time PCR was performed using SYBR Green (SYBR Select Master Mix, Life Technologies, USA) in the StepOne Plus Real-Time PCR System (Life Technologies, USA) according to the manufacturer's manual. The expression of the selected genes (shown in Table 3) was determined using gene-specific primers. All primers were validated as yielding a single PCR product with the predicted amplicon length. β2-microglobulin (β2m) and hypoxanthine phosphoribosyltransferase1 (Hprt1) were used as reference housekeeping genes. Mean PCR efficiency estimates per amplicon and the quantification cycle (Cq) for each sample was determined from the raw data using LinRegPCR software (version 2018.0) Efficiency corrected relative abundance was calculated by the Pfaffl-method (Pfaffl 2001). The gene expression ratio of two mRNAs was estimated by the method derived from Rutledge and Côté (2003). Briefly, the mRNA expression ratio of two genes (x_1, x_2) is the inverse ratio of the efficiency (E) corrected cycle of quantification (Cq) of genes x_2 and x_1 (respectively) multiplied by the ratio of the PCR amplicon length (A) of x_2 and x_1 (respectively), assuming the quantification of both genes was performed in the same sample with same cDNA input at the same fluorescence threshold.

$$Ratio_{X_1/X_2} \approx \frac{E_{X_2}^{Cq_{X_2}}}{E_{X_1}^{Cq_{X_1}}} \cdot \frac{A_{X_2}}{A_{X_1}}$$

4.6. SDS-PAGE and western blotting

The samples of left ventricular free walls were snap-frozen in liquid nitrogen and homogenized in a buffer containing 10 mM Tris-HCl (pH 7.4), 0.125 M sucrose, 1 mM EDTA-Na, 10% sodium dodecyl sulphate and 1 mM phenylmethylsulphonyl fluoride. Samples were subjected to sodium dodecyl sulphate- polyacrylamide gel electrophoresis on an 8% gel and transferred to a nitrocellulose membrane (Immobilon PR, Millipore Corporation, USA). Membranes were blocked with 5% PVP (ACE2) in TBST (Tris Buffer Saline + Tween 20), 5% BSA in TBST (ACE), 5% non-fat dried milk in TBST (actin) and subsequently incubated with ACE antibody (ab216476,1:500) and ACE2 antibody (ab15348, 1:1000). Actin (Sigma–Aldrich, St. Louis, MO, USA; AD2066,1:3000) was used as a loading control. Immunoreactive proteins were visualised by chemiluminescent detection (#WBLUF0, Immobilon Crescendo Western HRP substrate; Millipore Corporation, USA) and visualised and quantified using UVITEC Imaging Systems (Uvitec Limited, Cambridge, UK). These arbitrary density levels were normalized to the appropriate loading controls and calculated to the average control level to allow comparison of the protein amount between groups.

4.7. Statistical analysis

Results are expressed as the mean±standard error of the mean (SEM). The data were analysed using a one-way analysis of variance (ANOVA) followed by the LSD (least significant difference) *post hoc* test. Values were considered statistically significant when $p < 0.05$.

Acknowledgements: We thank Ms Elena Vilemova and Ms Veronika Hassova for their assistance with the experiments. The authors are grateful to Dr Gabriel Doka, for his help in calculating mRNA expression ratios. This research was funded by the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic, VEGA 2/0091/21 (EK), VEGA 1/0203/19 (PK), VEGA 1/0195/20 (JK) and by the Slovak Research and Development Agency APVV -19-0154 (EK).

Author Contributions: E.K.: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft, Supervision, Project administration, Funding acquisition. K.H.: Methodology, Validation, Investigation, Writing – Review & Editing. A. C: Methodology, Validation, Investigation, Writing – Review & Editing. J.K., P.K.: Validation, Writing – Review & Editing, Supervision, Funding acquisition.

Conflicts of interest: The authors declare no conflict of interest.

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