Metformin Attenuates Myocardium Dicarbonyl Stress Induced by Chronic Hypertriglyceridemia

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Summary

Reactive dicarbonyls stimulate production of advanced glycation endproducts, increase oxidative stress and inflammation and contribute to the development of vascular complications. We measured concentrations of dicarbonyls – methylglyoxal (MG), glyoxal (GL) and 3-deoxyglucosone (3-DG) – in the heart and kidney of a model of metabolic syndrome – hereditary hypertriglyceridemic rats (HHTg) and explored its modulation by metformin. Adult HHTg rats were fed a standard diet with or without metformin (300 mg/kg b.w.) and dicarbonyl levels and metabolic parameters were measured. HHTg rats had markedly elevated serum levels of triacylglycerols ($p<0.001$), FFA ($p<0.01$) and hepatic triacylglycerols ($p<0.001$) along with increased concentrations of reactive dicarbonyls in myocardium (MG: $p<0.001$; GL: $p<0.01$; 3-DG: $p<0.01$) and kidney cortex (MG: $p<0.01$). Metformin treatment significantly reduced reactive dicarbonyls in the myocardium (MG: $p<0.05$, GL: $p<0.05$, 3-DG: $p<0.01$) along with increase of myocardial concentrations of reduced glutathione ($p<0.01$) and glyoxalase 1 mRNA expression ($p<0.05$). Metformin did not have any significant effect on dicarbonyls, glutathione or on glyoxalase 1 expression in kidney cortex. Chronically elevated hypertriglyceridemia was associated with increased levels of dicarbonyls in heart and kidney. Beneficial effects of metformin on reactive dicarbonyls and glyoxalase in the heart could contribute to its cardioprotective effects.

Key words

Hypertriglyceridemia • Dicarbonyl stress • Methylglyoxal • Glyoxalase • Metabolic syndrome • Metformin

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Introduction

The protein glycation caused by reactive dicarbonyls stimulates the production of advanced glycation end products (AGEs) and subsequently contributes to the development of chronic vascular complications, in particular in patients with diabetes (Schalkwijk *et al.* 2015). Under normal conditions, the excessive protein glycation is prevented through glutathione-dependent glyoxalase detoxification. An impaired balance between the generation of dicarbonyls and the efficiency of their scavenger pathways leads to dicarbonyl stress (Rabbani *et al.* 2015). Both of these processes are impaired in diabetic patients, where dicarbonyl generation is increased and glyoxalase activity including glutathione status is decreased (Maessen *et al.* 2015). Dicarbonyl stress is involved in the pathogenesis of metabolic syndrome, as well as in diabetic macro- and microvascular complications. Higher plasma levels of methylglyoxal are observed in type 1 and 2 diabetic patients (Fleming *et al.* 2012) and in obese patients with metabolic syndrome (Uribarri *et al.* 2015). In addition, it has been reported that methylglyoxal administration induces endothelial dysfunction, oxidative

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stress and impaired vasodilatation (Sena *et al.* 2012), and increases macrophage infiltration in adipose tissue in experimental studies (Matafome *et al.* 2012). An excessive generation of dicarbonyl species such as methylglyoxal (MG) is typically associated with hyperglycemia and high glucose variability (Maessen *et al.* 2015); nevertheless its other possible inductors include also dyslipidemia and insulin resistance (Tenenbaum *et al.* 2014).

Metformin, the most widely prescribed glucose-lowering agent for the treatment of type 2 diabetes, has been proposed as a scavenger of reactive dicarbonyl species. It has been previously demonstrated that metformin, through the guanidine group, can bind to methylglyoxal (Kinsky *et al.* 2016), and that metformin treatment is able to reduce plasma methylglyoxal levels in patients with type 2 diabetes (Kender *et al.* 2016). We have previously demonstrated in a rat model of chronic inflammation that metformin administration decreased methylglyoxal levels in heart (Malinska *et al.* 2016).

In the current study we measured concentrations of dicarbonyls in the heart and the kidney of a rodent model of metabolic syndrome – non-obese hereditary hypertriglyceridemic rats. This strain originating from Wistar rats is characterized by severe hypertriglyceridemia, insulin resistance, hyperinsulinemia, hepatic steatosis and oxidative stress with an absence of obesity and hyperglycemia thus representing an experimental model of metabolic syndrome (Kazdova *et al.* 1997, Zicha *et al.* 2006). We hypothesized that severe hypertriglyceridemia and insulin resistance will be associated with increased dicarbonyl levels even in the absence of hyperglycemia and that metformin treatment will reduce dicarbonyls in both the heart and the kidney.

Methods

Animals and diet

All experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997) and were approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine.

Six-month old Wistar male rats obtained from Charles River Laboratories (controls) and the non-obese hereditary hypertriglyceridemic strain of rats (HHTg) were used in this study. The rats were fed a standard laboratory diet with or without metformin at a dose of 300 mg/kg b.w. for 4 weeks. At the end of experiments, animals were sacrificed in a postprandial state.

Analytic methods/Biochemical analyses

Serum levels of triacylglycerols, glucose, total cholesterol, HDL-cholesterol and FFA were measured using commercially available kits (Erba Lachema, Brno, Czech Republic and Roche Diagnostics, Mannheim, Germany). Serum insulin and carboxymethyl lysine (CML) concentrations were determined using a Mercodia Rat Insulin ELISA kit (Mercodia AB, Uppsala, Sweden) and a Rat CML ELISA kit (Mybiosource, San Diego, USA). Plasma and urine lactate were analyzed electrochemically using ion-selective electrodes (Radiometer, Czech Republic). β-hydroxybutyrate and acetoacetate plasma concentrations were determined using an enzymatic method, as previously described (Galán *et al.* 2001).

For the oral glucose tolerance test (OGTT), blood glucose was determined after a glucose load (3 g of glucose/kg b.w.) administered intragastrically after overnight fasting. The blood glucose concentration were determinated through analysis of blood samples collected from the tail at 0, 30, 60, 120 min after glucose loading. The area under curve (AUC) for glucose was calculated over the 120 min period.

For determination of tissue triacylglycerols, samples were extracted in chloroform/methanol and further processed as described previously (Malinska *et al.* 2015).

Levels of reduced (GSH) and oxidized (GSSG) forms of glutathione were determined using a high-performance liquid chromatography method with fluorescent detection in accordance with the HPLC diagnostic kit (Chromsystems, Gräfelfing, Germany).

Dicarbonyl stress parameters

Dicarbonyl concentrations were determined after derivatization with 1,2-diamino-benzene and using the HPLC method with fluorescence detection according to Fleming and Bierhaus (Thornalley *et al.* 1999).

Glo-1 activity was analyzed using the method described by Arai *et al.* (2014). Red blood cells were collected by centrifugation of blood (EDTA) samples and washed 3 times with 0.01 M PBS (pH 7.4). Washed cells were lysed using cold deionized water. Hemoglobin concentrations were determined according to the Drabkin´s assay (Sigma-Aldrich, Prague, Czech Republic).

Glyoxalase 1 mRNA expression

Total RNA was isolated from the kidney cortex and left ventricle using RNA Blue (Top-Bio, Praha,

Czech Republic). Reverse transcription and quantitative real-time PCR analyses were performed using the TaqMan RNA-to C_T 1-Step Kit and TaqMan Gene Expression Assay (Applied Biosystems, Foster City, USA) and carried out using a ViiA™ 7 Real Time PCR System (Applied Biosystems, Foster City, USA). Relative expression of *Glo-1* was determined after normalization against *β-actin* as an internal reference and calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell cultures, treatment

Confluent Human Kidney HEK293 cells were cultivated in a control medium (DMEM, Hyclone, USA) supplemented with 10 % FBS, Biochem, Germany) and treated with either 0.5 mM metformin or a combination of 0.5 mM metformin and 10 mM lactate (Sigma-Aldrich, Prague, Czech Republic) for 18 h. Cells were then trypsinized and methylglyoxal content was determined in aliquots containing $15*10^6$ cells according to the method described above.

Statistical analysis

Statistical analysis was performed using either a one-way ANOVA Kruskal-Wallis test with multiple comparisons or a Mann-Whitney test. A value of $p<0.05$ was considered to be statistically significant. The Pearson correlation was calculated to determine the relationship between glutathione and methylglyoxal in the myocardium. Data are presented as mean ± SEM with 95 % CI.

Results

The effect of hypertriglyceridemia on basal metabolic parameters

Compared with controls, hypertriglyceridemic rats exhibited markedly elevated serum levels of triacylglycerols, FFA and ectopic triacylglycerol accumulation in the liver and muscle, impaired glucose tolerance, hyperinsulinemia and increased AGE product carboxymethyl lysine (CML) and ketone bodies (Table 1).

Table 1. The effects of hypertriglyceridemia and metformin on metabolic parameters.

	Wistar	HHTg	p1<	HHTg + metformin	p2<
Body weight (g)	480.00±22.00	483.00±23.00	NS	450.00±12.00	0.05
Serum triglycerides (mmol/l)	1.37±0.23	4.78±0.43	0.01	2.39±0.13	0.02
FFA (mmol/l)	0.19±0.03	0.83±0.06	0.01	0.70±0.08	0.05
Cholesterol (mmol/l)	1.72±0.10	1.54±0.10	NS	1.91±0.33	NS
HDL-C (mmol/l)	1.24±0.05	0.75±0.03	0.01	1.23±0.08	0.02
Triglycerides in the liver (μmol/g)	4.32±0.70	13.87±2.23	0.01	9.20±1.22	0.05
Triglycerides in muscle (μmol/g)	4.96±1.95	8.43±1.64	0.05	8.55±1.70	NS
Fasting glucose (mmol/l)	3.86±0.13	5.30±0.27	0.05	4.49±0.26	NS
Insulin (pmol/l)	469.00±30.00	580.00±83.00	0.05	225.00±28.00	0.01
AUC_{0-120} (mmol/l)	674.00±9.00	787.00±19.00	0.05	818.00±44.00	NS
β-hydroxybutyrate (μmol/l)	45.50±2.60	91.60±2.90	0.01	127.90±6.30	0.01
Acetoacetate (μmol/l)	27.10±4.90	44.30±6.60	0.01	39.10±4.90	NS
CML (ng/ml)	104.70±1.00	131.00±6.50	0.05	130.80±1.10	NS
GSH/GSSG in myocardium	4.01±0.16	2.15±0.09	0.05	4.65±0.26	0.01
GSH/GSSG in kidney cortex	20.22±0.87	20.04±0.26	NS	18.48±0.13	NS

Data are mean ± SEM. n=8. P1 – HHTg vs. Wistar. P2 – HHTg + metformin vs. HHTg.

In hypertriglyceridemic rats we observed markedly increased serum levels of methylglyoxal (1.802±0.121 vs. 0.662±0.161 nmol/ml, $p<0.01$). Concentrations of individual reactive dicarbonyls in the myocardium and kidney cortex were significantly elevated in HHTg rats (Fig. 1) compared to normotriglyceridemic controls.

Hypertriglyceridemia was also associated with impaired glutathione metabolism in the myocardium as shown in Figure 2A. The reduced form of glutathione was decreased and the oxidized form of glutathione was increased in the myocardium of HHTg rats.

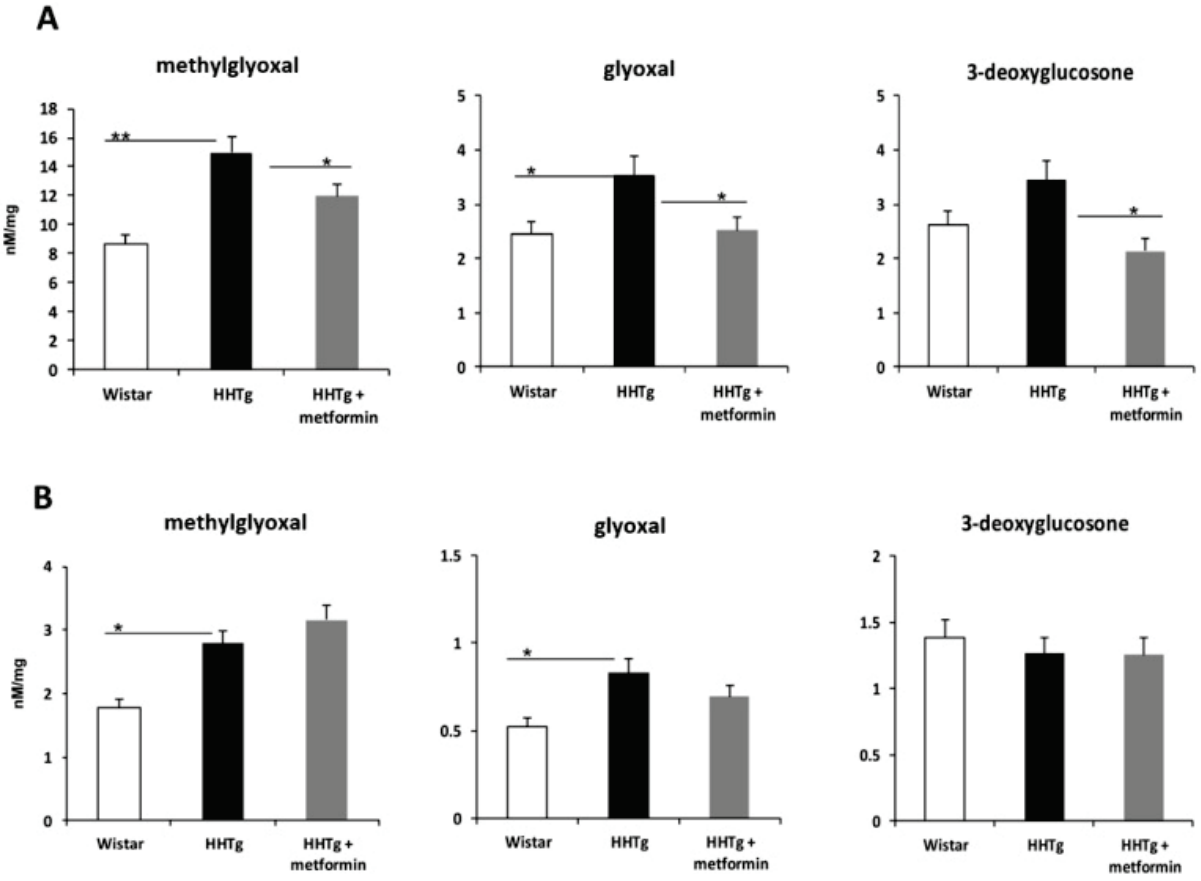

Fig. 1. The effects of hypertriglyceridemia and metformin on dicarbonyl levels in myocardium (**A**) and kidney cortex (**B**). Data are expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$.

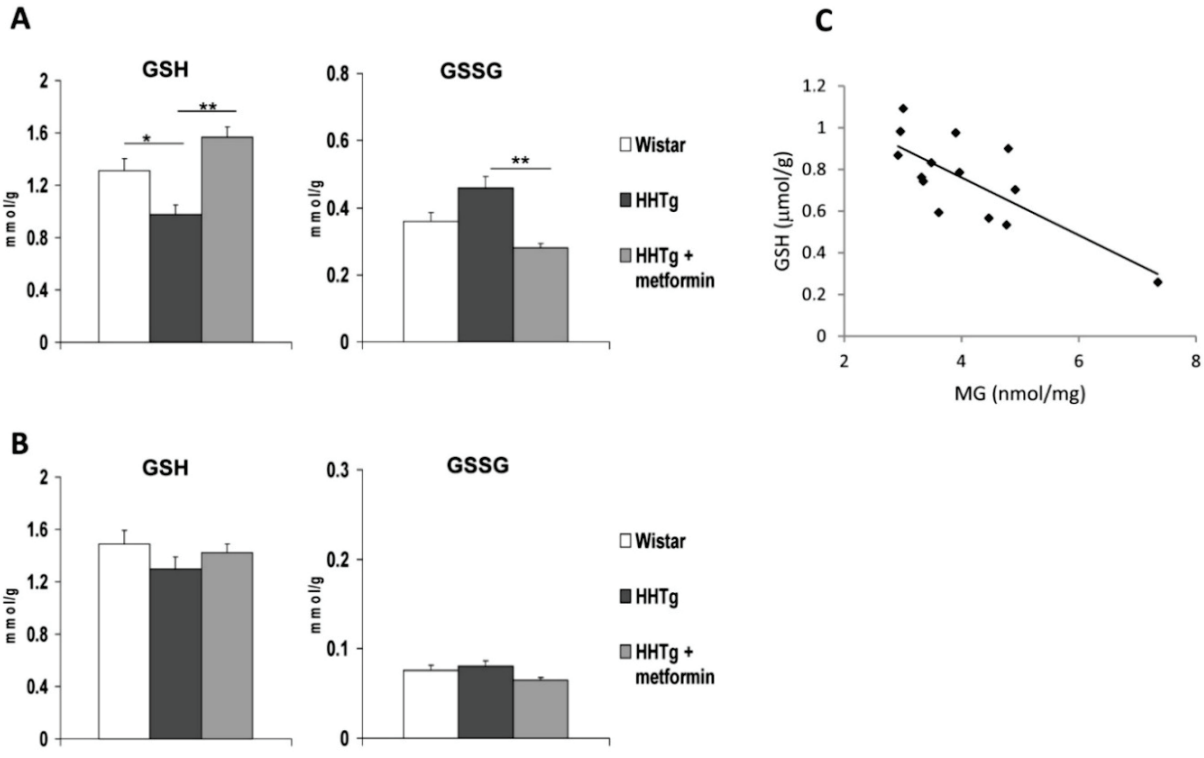

Fig. 2. The effects of hypertriglyceridemia and metformin on glutathione in myocardium (**A**) and kidney cortex (**B**) and the relationship between methylglyoxal and glutathione in myocardium (**C**), Spearman's correlation coefficient $R^2=0.5882$, $p<0.05$. Data are expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$.

The effect of metformin

Metformin administration to HHTg rats mildly reduced body weight and had a positive effect particularly on lipid metabolism compared to untreated HHTg rats (Table 1).

As regards carbonyl stress, metformin treatment significantly reduced serum levels of methylglyoxal (0.915±0.219 vs. 1.802±0.121 nmol/ml, $p<0.01$), but other dicarbonyls in the serum did not change. As shown in Figure 1, metformin treatment was associated with significantly reduced levels of all measured dicarbonyls in the myocardia of HHTg rats. However, there was no significant effect of metformin on dicarbonyl concentrations in the kidney cortex (Fig. 1).

Concentrations of hydroxybutyrate, lactate and acetoacetate in plasma and urine were significantly elevated in metformin-treated HHTg rats compared to untreated rats (Fig. 4).

Incubation with metformin significantly reduced the concentration of MG in the human kidney HEK293 cell culture. However, the presence of lactate in the medium reduced the effect of metformin on MG in isolated kidney cells (Fig. 4).

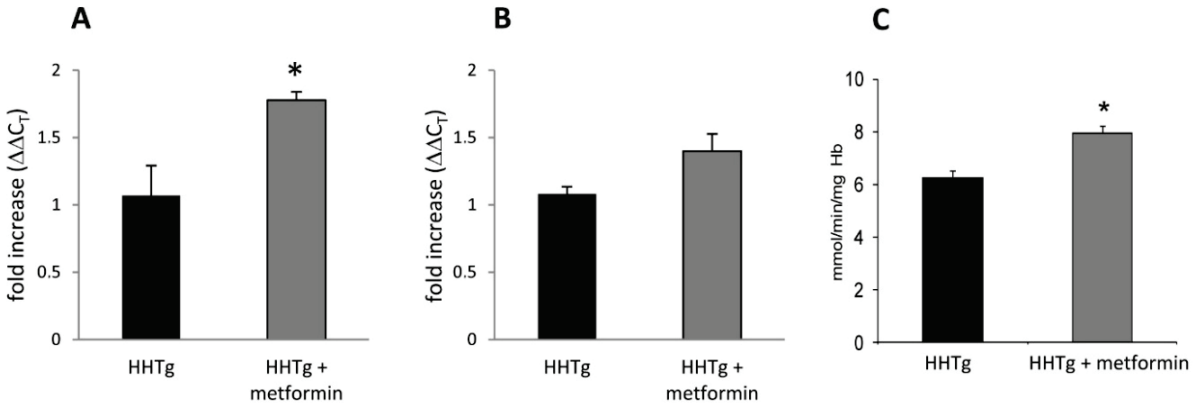

Fig. 3. The effect of metformin on glyoxalase 1 mRNA expression in myocardium (**A**) and kidney cortex (**B**) and on glyoxalase 1 activity in erythrocytes (**C**). Values are presented as mean ± SEM. * $p<0.05$ compared to HHTg.

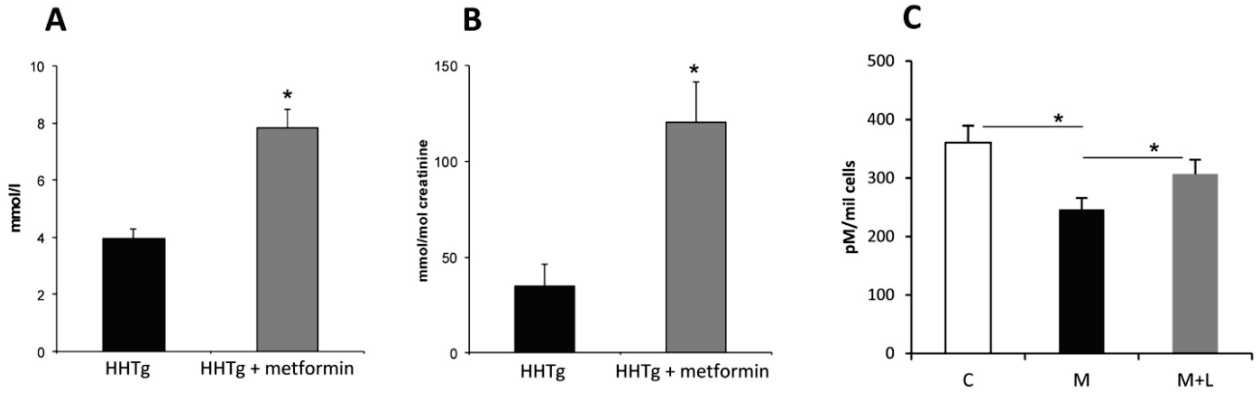

Fig. 4. The effect of metformin on lactate in plasma (**A**) and urine (**B**) and *in vitro* on human kidney cells (**C**). C – control, M – metformin, M+L – metformin + lactate. Data are expressed as mean ± SEM. * $p<0.05$.

The effect of metformin on glutathione

In the myocardium we observed improved glutathione metabolism in HHTg metformin-treated rats (Fig. 2), an elevation in the reduced form of glutathione and a decrease in the oxidized form of glutathione. This effect of metformin on glutathione was not observed in the kidney cortex (Fig. 2). A direct relationship between methylglyoxal and reduced glutathione in the myocardium was confirmed by negative correlation (Fig. 2C).

The effect of metformin on glyoxalase 1 expression and activity

Gene expression of mRNA Glo-1 was increased in the myocardium (left ventricle) after metformin treatment, whereas mRNA Glo-1 expression in the kidney cortex did not differ between metformin-treated

and -untreated HHTg rats (Fig. 3). Metformin administration also significantly increased glyoxalase 1 activity measured in red blood cells compared to untreated rats.

Discussion

One of the unifying hypotheses connecting diabetes with its chronic complications suggests that enhanced metabolic flux and the deleterious effects of high glucose levels are mediated by the generation of toxic metabolites (Fleming *et al.* 2012). Of these, reactive dicarbonyls are among the most important (Rabbani *et al.* 2015). Interestingly, increased dicarbonyls production has also been described in patients with metabolic syndrome and dyslipidemia without overt diabetes suggesting their possible involvement in the increased risk in cardiovascular complications in these patients (Rabanni *et al.* 2016). The results of our study demonstrate for the first time that chronically elevated triglyceride and FFA levels, in the absence of obesity are associated with increased production of reactive dicarbonyl species, in particular methylglyoxal. In addition to its increased circulating levels, we observed also markedly elevated tissue levels of dicarbonyls. Previous studies have shown that MG and GL can be produced from oxidized lipids, both within their degradation and during lipoperoxidation (Turk *et al.* 2011) or by increased glyceroneogenesis in triacylglycerols/FFA cycle (Masania *et al.* 2016). Although lipid metabolism in myocardium and kidney is slightly different, the elevation of dicarbonyls in these tissues in HHTg rats is nearly the same so is implausible to significantly influence the creation of dicarbonyls. Other possible mechanisms of hypertriglyceridemia-induced dicarbonyl accumulation include increased oxidative stress, increased ketogenesis and subsequent AGE formation (Dornadula *et al.* 2015). Our experimental results in hypertriglyceridemic rats support the increasing evidence that chronically increased lipids can be as important as carbohydrates in the stimulation of excessive reactive dicarbonyl species production.

The massive accumulation of dicarbonyls in the myocardium of hypertriglyceridemic rats in our study was associated with an impaired balance of GSH status. It has been shown that adequate levels of the reduced form of glutathione are important for optimal activity of the glyoxalase system, which is involved in the detoxification of MG and GL (Rabanni *et al.* 2016). An inverse relationship between MG and reduced glutathione in the myocardium suggests a possible direct relationship. One the mechanisms could be a MG-induced deactivation of the antioxidant enzyme glutathione reductase thus further enhancing the potential for oxidative stress damage. Other studies have shown that high serum and adipose tissue levels of MG are closely related to insulin resistance in fructose-fed rats (Jia *et al.* 2007), and MG treatment *in vitro* impairs insulin-signaling activation in skeletal muscle cells (Riboulet-Chavey *et al.* 2006) through increased oxidative stress and direct effects on insulin signaling pathway (Nigro *et al.* 2014).

In our current study, we focused on the effects of metformin treatment on dicarbonyl levels and its metabolic consequences. Previous studies have shown that metformin may have numerous beneficial effects independent of its glucose lowering properties including cardioprotective effects (Rena *et al.* 2013). Our previous study in SHR rats with transgenic expression of human CRP (Malinska *et al.* 2016) demonstrated metformin-induced decrease of methylglyoxal in the heart. Here we focused on the possible mechanisms that could explain metformin effects on dicarbonyl stress. In our current study in hypertriglyceridemic rats, metformin treatment reduced dicarbonyl accumulation and increased Glo-1 expression in the myocardium. Both of these changes could have contributed to and partly explain the cardioprotective effects of metformin seen in clinical practice. Other studies have shown that metformin improves the GSH/GSSG balance in the myocardium and prevents dicarbonyl accumulation as a cofactor of the glyoxalase system (Ashour *et al.* 2012, Foretz *et al.* 2014). Metformin has also been proposed as a scavenger of methylglyoxal (Rena *et al.* 2013, Kinsky *et al.* 2016).

Our data show that metformin can decrease MG directly through the activation of its key detoxification enzyme, Glo-1. Another important mechanism involves the interaction and activation of redox-sensitive transcription factors such as Nrf2, AP1 and NFκB, which can again upregulate Glo-1 transcription (Xue *et al.* 2012). At the transcriptional level, apart from Glo-1, metformin has been also shown to restore key antioxidant defense enzymes such as glutathione-S-transferase and catalase (Kender *et al.* 2014).

In our study, untreated HHTg rats had elevated circulating levels of ketone bodies, which were further increased by metformin treatment. Metformin is capable to readdress fatty acid metabolism from lipogenesis towards fat oxidation and ketone body production, so increased β-hydroxybutyrate after metformin

administration can associated with increased fatty acid oxidation. Although the development of severe lactate acidosis is perceived as a negative consequence associated with metformin administration (DeFronzo *et al.* 2016) recent trials with novel antidiabetic drugs gliflozins have suggested that moderate ketone bodies elevation could have the potential to improve myocardium metabolism (Ferrannini *et al.* 2016). Recent studies have reported that the failing heart relies on ketone bodies as a significant alternative fuel, when the fatty acids utilization is diminished (Aubert *et al.* 2016). Accumulation of ketone bodies in the myocardium occurs as a compensatory response against oxidative stress (Nagao *et al.* 2016). It is thus tempting to speculate that increased ketone bodies seen in our study can also generally contribute to cardioprotective effect of metformin.

Interestingly, while we observed a significant metformin-induced attenuation of dicarbonyl stress in the heart no such effects could be seen in the kidney. In our study, an incubation of isolated human kidney cell cultures with metformin rapidly reduced MG concentrations, but this effect was abolished in the presence of lactate. Likewise, the presence of lactate reduced the effect of metformin on dicarbonyl stress in kidney cells. Taken together our data suggest that the lack of improvement of dicarbonyl stress in the kidney as compared to myocardium could be due to high levels of lactate in the kidney that abolish metformin effects.

In summary, our results indicate that chronically elevated hypertriglyceridemia and FFA are associated with increased levels of methylglyoxal in serum and with markedly elevated reactive carbonyls in the heart and kidney. The beneficial effect of metformin administration on reactive dicarbonyls and glyoxalase 1 in the heart could contribute to the cardioprotective effect of metformin independently of its antihyperglycemic effect. It remains to be shown whether similar organ-specific effects of metformin on dicarbonyl stress can also be detected in humans.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

AGE – advanced glycation end product, CML – carboxymethyl lysine, FFA – free fatty acids, GSH – reduced form of glutathione, GSSG – oxidized form of glutathione, TBARS – thiobarbituric acid reactive substance, TAG – triacylglycerol, MG – methylglyoxal, GL – glyoxal, 3-DG – 3-deoxyglucosone, Glo-1 – glyoxalase 1.

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