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The Relationship between Glycemia, Insulin and Oxidative Stress in Hereditary Hypertriglyceridemic Rat

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Summary

The aim of this study was to determine the effects of insulin infusion on oxidative stress induced by acute changes in glycemia in non-stressed hereditary hypertriglyceridemic rats (hHTG) and Wistar (control) rats. Rats were treated with glucose and either insulin or normal saline infusion for 3 hours followed by 90 min of hyperglycemic (12 mmol/l) and 90 min of euglycemic (6 mmol/l) clamp. Levels of total glutathione (GSH), oxidized glutathione (GSSG) and total antioxidant capacity (AOC) were determined to assess oxidative stress. In steady states of each clamp, glucose infusion rate (GIR) was calculated for evaluation of insulin sensitivity. GIR ($mg.kg^{-1}.min^{-1}$) was significantly lower in hHTG in comparison with Wistar rats; 25.46 (23.41 - 28.45) vs. 36.30 (27.49 - 50.42) on glycemia 6 mmol/l and 57.18 (50.78 - 60.63) vs. 68.00 (63.61 - 85.92) on glycemia 12 mmol/l. GSH/GSSG ratios were significantly higher in hHTG rats at basal conditions. Further results showed that, unlike in Wistar rats, insulin infusion significantly increases GSH/GSSG ratios in hHTG rats: 10.02 (9.90 - 11.42) vs. 6.01 (5.83 - 6.43) on glycemia 6 mmol/l and 7.42 (7.15 - 7.89) vs. 6.16 (5.74 - 7.05) on glycemia 12 mmol/l. Insulin infusion thus positively influences GSH/GSSG ratio and that way reduces intracellular oxidative stress in insulin-resistant animals.

Key words

Clamp • Oxidative stress • Insulin • Rat

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Introduction

Oxidative stress is a common pathogenic factor for the dysfunction of β-cells and endothelial cells. There is evidence that β-cell dysfunction results from prolonged exposure to high glucose, elevated free fatty acids level, or a combination of both (Evans *et al.* 2003). β-cells are particularly sensitive to reactive oxygen species (ROS) due to inadequate expression of free-radical quenching (antioxidant) enzymes such as catalase, glutathione peroxidase, and superoxide dismutase (Tiedge *et al.* 1997). The capability of oxidative stress to damage mitochondria and ultimately decrease insulin secretion is therefore obvious (Robertson *et al.* 2003). It has been demonstrated that oxidative stress generated by short exposure of β-cell preparations to H_2O_2 increases production of $p21^{phox}$ and decreases flux of insulin mRNA, cytosolic ATP, and calcium into cytosol and mitochondria (Maechler *et al.* 1999). The key role of increased glucose metabolism in the impairment of β-cell function through oxidative stress has been recently confirmed. Intracellular ROS increased 15 min after the exposure to high glucose level, and this effect was blunted by inhibitors of the mitochondrial function (Sakai *et al.* 2003). Glucose-induced insulin secretion was also suppressed by H_2O_2, a chemical substitute for ROS (Sakai *et al.* 2003). Interestingly, the first phase of glucose-induced insulin secretion could be suppressed by 50 μM H_2O_2, H_2O_2 or high glucose suppressed the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme, and inhibitors of the mitochondrial function abolished the latter effects. These

data suggest that high glucose concentrations induce mitochondrial ROS, which suppresses the first phase of glucose-induced insulin secretion, at least in part, through the suppression of GAPDH activity (Sakai *et al.* 2003). These results have been confirmed *in vivo*. In subjects with normal glucose tolerance, glutathione infusion failed to affect β-cell response to glucose (Paolisso *et al.* 1992). In contrast, glutathione significantly potentiated glucose-induced insulin secretion in patients with impaired glucose tolerance (Paolisso *et al.* 1992). Furthermore, when the latter group was studied in the condition of hyperglycemic clamp, glutathione infusion significantly potentiated the β-cell response to glucose when plasma glucose levels varied between 10 and 15 mmol/l (Paolisso *et al.* 1992). Indeed, many studies show that high glucose concentrations induce endothelial dysfunction. *In vitro*, the direct role of hyperglycemia has been suggested by the fact that arteries isolated from normal animals and subsequently exposed to exogenous hyperglycemia exhibit attenuated endothelium-dependent relaxation (Bohlen and Lash 1993). Consistently, *in vivo* studies have also shown that hyperglycemia directly induces endothelial dysfunction in diabetic and non-diabetic subjects (Giugliano *et al.* 1997, Kawano *et al*. 1999). The role of free radical generation in production of the hyperglycemia-dependent endothelial dysfunction is suggested by studies showing that both *in vitro* (Tesfamariam and ohen1992) and *in vivo* (Marfella *et al*. 1995, Ting *et al.* 1996) the acute effects of hyperglycemia are counterbalanced by antioxidants. Recent studies have demonstrated that superoxide overproduction by the mitochondrial electron transport chain induced by hyperglycemia seems to play a key role in the activation of all other pathways involved in the pathogenesis of endothelial dysfunction (Nishikawa *et al.* 2000, Garcia Soriano *et al.* 2001).

Prague hereditary hypertriglyceridemic rats (hHTG) were originally derived from Wistar rat colony (Vrána and Kazdová 1990). The hHTG rat is a strain expressing hereditary hypertriglyceridemia as in human familiar hypercholesterolemia and associates hyperinsulinemia and hypertension (Vrána and Kazdová 1990, Reaven 1993). This rat strain is a relevant model of familiar hypercholesterolemia and metabolic syndrome, available for the assessment of lipoprotein metabolism and insulin resistance (Ueno *et al.* 2004, Zicha *at al.* 2006).

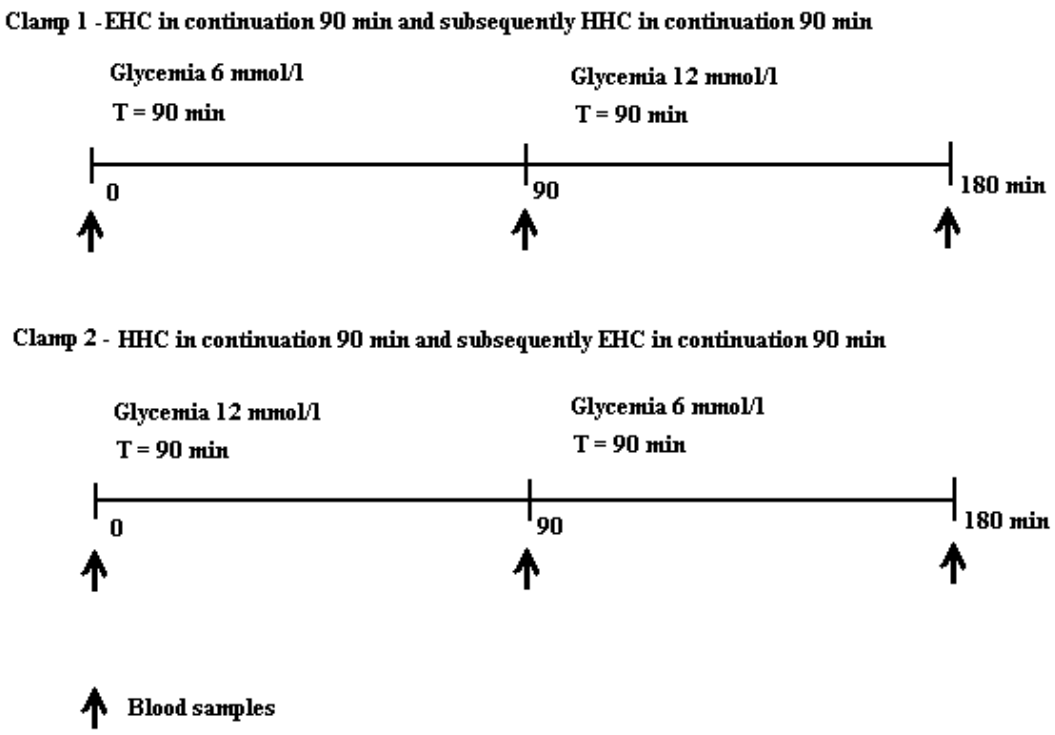
The aim of our study was to assess whether increased production of reactive oxygen species induced by different glycemia levels, could be reversed by exogenously delivered insulin. Prague hereditary hypertriglyceridemic rats with metabolic syndrome and age-matched healthy control Wistar rats were used for this purpose.

Material and Methods

Male Wistar rats (n=20) were provided by Charles River Breeding Laboratories and hHTG rats (n=20) were provided by Dr. L. Kazdová (IKEM, Prague). The rats were maintained at a constant temperature (23±1 ºC), with a fixed 12-h artificial light period. They were housed in stainless steel cages (6 rats per cage) with free access to water and standard chow. At the beginning of the experiment, the rats were aged 3 months. The euglycemic-hyperinsulinemic glucose clamp technique (EHC) and hyperglycemic hyperinsulinemic clamp (HHC) were used to study insulin sensitivity. All procedures and experimental protocols were approved by the Animal Ethics Committee of the Faculty of Medicine Charles University in Pilsen.

The euglycemic clamp procedure was performed according to previously described technique (De Fronzo *et al.* 1979). After intraperitoneal anesthesia (ketamin 100 mg/kg i.p. + xylazin 16 mg/kg i.p.), a small incision was made 0.5 cm from the cervical midline and at the level of the forelegs, and the internal jugular vein was exposed. After superior ligation, the vessel was catheterized with silastic tubing, carotid artery was catheterized on the same side. The catheters were tunneled subcutaneously and emerged on the dorsal side of the neck. All skin incisions were closed with a 3-0 thread and the catheters were filled with a heparin (concentration 100 U/ml). The catheters required no more care before the study.

The metabolic experiment was performed 48 h after surgery in food-deprived conscious rats. At the beginning of the experiment, two successive blood samples were taken at time –10 min and 0 min for measurement of basal glycemia. Insulin (Actrapid, Novo Nordisk, Denmark) and 30 % glucose were then infused; the rate of glucose infusion was corrected manually every 5 min to maintain desired level of glycemia, the rate of insulin infusion was 0.05 $IU.kg^{-1}.min^{-1}$. Each glycemia was maintained for 90 min. In control group normal saline instead of insulin was delivered. Blood samples of 1 ml each were taken at time 0 min, 90 min and 180 min.



hHTG (n=20)		Wistar (n=20)	
Insulin infusion (n =5)	Clamp 1	Insulin infusion (n =5)	Clamp 1
Insulin infusion (n =5)	Clamp 2	Insulin infusion (n =5)	Clamp 2
Normal saline infusion (n =5)	Clamp 1	Normal saline infusion (n =5)	Clamp 1
Normal saline infusion (n =5)	Clamp 2	Normal saline infusion (n =5)	Clamp 2

Fig. 1. Study design.

A total of 3.2 ml of blood (including laboratory measurements and glycemia testing) was withdrawn during the experiment for all measurements. Glycemia measurements were performed by the glucose oxidase method using a glucose analyzer (HemoCue, Sweden). During insulin administration glucose infusion rate (GIR in $mg.kg^{-1}.min^{-1}$) was calculated for evaluation of insulin sensitivity. Rats were divided into eight groups of 5 rats each and underwent study experiment as described in Figure 1.

Measurement of plasma triglycerides, insulin, albumin, uric acid

Plasma triglyceride levels were measured by the auto analyzer (BM Hitachi, 717, Meylan, France) using a kit Dialab D96386 (Dialab, Czech Republic). Plasma insulin was estimated by rat ELISA kit (Lincoplex, USA). Albumin was measured using Human 10560 kit (Human Gesellschaft, Germany). Uric acid was measured using DOT AU803 kit (DOT, Czech Republic).

Determination of antioxidant components

Total glutathione (GSH and GSSG) levels were determined colorimetrically (Bioxytech, USA). Total antioxidant capacity (AOC) was measured by Total Antioxidant Status (Randox, United Kingdom) using Hitachi 717 analyzer (λ=600nm).

Statistical analysis

Analysis of variance was used to compare multiple group means. When the data were not normally distributed, the Kruskal-Wallis test was performed to compare multiple group means, followed by the Mann-Whitney test. $P < 0.05$ value was considered statistically

Table 1. Baseline characteristics of hHTG and Wistar rats.

	Albumin (g/l)	Plasma triglycerides (mmol/l)	Uric acid (umol/l)	GSH/GSSG
hHTG - insulin	36.2 (35.6 - 36.7)	0.43 (0.41 - 0.50)	29.0 (25.0 - 43.8)	9.43 (7.60 - 10.00)
hHTG - normal saline	37.1 (36.5 - 37.4)	0.43 (0.40 - 0.49)	28.5.7 (23.8 - 41.2)	8.54 (6.75 - 9.89)
Wistar - insulin	36.3 (35.8 - 37.4)	0.28* (0.26 - 0.30)	18.5* (12.1 - 20.2)	1.85** (1.16 - 2.52)
Wistar - normal saline	36.4 (35.2 - 37.3)	0.29* (0.26 - 0.31)	17.8* (13.0 - 18.9)	1.94** (1.42 - 2.17)

*p<0.05, ** p<0.01 in comparison to corresponding hHTG group. Data are medians and interquartile range.

significant. All data are expressed as median and interquartile range.

Results

Cross-over study design was used to eliminate the impact of different glycemia sequences on measured parameters and subsequently, corresponding measurements at each glycemia level were merged. Baseline body weight and hematocrit levels were similar in all experimental groups (data not shown). Wistar and hHTG groups significantly differed in triglycerides and uric acid levels (Table 1).

Insulin sensitivity presented as glucose infusion rate (GIR) was significantly lower in hHTG rats at both glycemia levels in comparison with Wistar rats (Fig. 2). GIR ($mg.kg^{-1}.min^{-1}$) was 25.46 (23.41 - 28.45) vs 36.30 (27.49 - 50.42) on glycemia 6 mmol/l and 57.18 (50.78 - 60.63) vs. 68.00 (63.61 - 85.92) on glycemia 12 mmol/l suggesting that hHTG rats were insulin-resistant without prior high-fructose diet stimulation.

Antioxidant capacity (AOC) did not differ between hHTG and Wistar rats at basal conditions. Moreover, different glycemia levels during clamp did not increase AOC in individual groups nor caused any statistically significant changes in AOC between hHTG and Wistar rats (Table 2).

GSH/GSSG ratios were significantly higher in all hHTG groups as compared to controls (Table 3). Insulin infusion significantly increased GSH/GSSG ratio in hHTG group on glycemia 6 mmol/l, and this effect was partially diminished by increasing glycemia level to 12 mmol/l (Table 3). There were no significant changes in Wistar groups (Table 3). Quotients of GSH/GSSG ratios were used to distinguish between insulin and glycemia effects on redox state, the individual quantities were measured under insulin and normal saline infusions along with corresponding glycemia. As shown in Table 4, GSH/GSSG ratio improved almost twice in hHTG group on glycemia 6 mmol/l treated with insulin, while this effect was not observed in Wistar group. The decrease of GSH/GSSG ratio induced by hyperglycemia 12 mmol/l in hHTG group was significantly reversed by insulin infusion and again, this effect was not observed in Wistar group (Tables 3 and 4). Insulin infusion did not have any significant effect regardless of glycemia levels in the control Wistar group (Table 4).

Discussion

Prague hereditary hypertriglyceridemic rats represent a model of nonobese metabolic syndrome with hypertension, hypertriglyceridemia and insulin resistance (Vrána and Kazdová 1990, Štolba *et al.* 1992). All these metabolic changes can be aggravated by high carbohydrate intake. hHTG rats were insulin-resistant according to measured GIR during clamps even without prior high-fructose diet stimulation. Insulin resistance of hHTG rats was also followed by higher baseline triglyceride and uric acid levels.

Total antioxidant capacity (AOC) did not significantly change during clamps in both Wistar and hHTG strains and did not depend on glycemia nor insulin infusion. These results are in agreement with our previous findings (Kyselová e*t al.* 2002).

Plasma lipid peroxidation intermediates were not

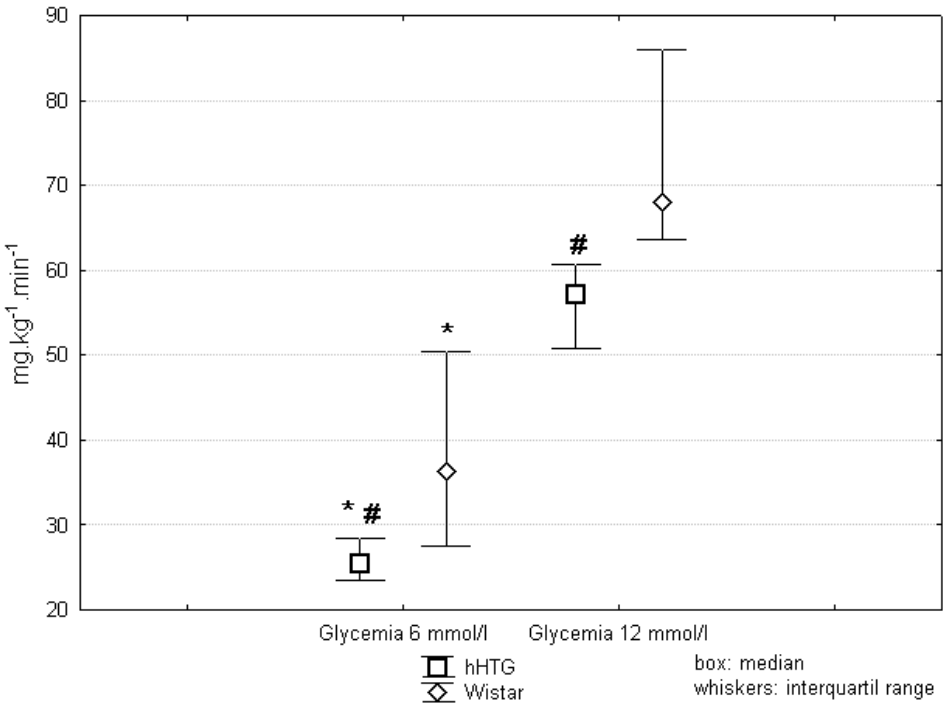


Fig. 2. Glucose infusion rate measured under euglycemic and hyperglycemic conditions during hyperinsulinemic clamp in hHTG and Wistar rats. * $p<0.01$ in comparison to glycemia 12 mmol/l. # $p<0.05$ in comparison to Wistar rats. Data are medians (boxes) and interquartile range (whiskers).

Table 2. Total antioxidant capacity (AOC) mmol/l.

	AOC		
Group	**Basal conditions**	**Glycemia 6 mmol/l**	**Glycemia 12 mmol/l**
HTG – insulin infusion	0.83 (0.80 - 0.89)	0.81 (0.77 - 0.90)	0.77 (0.72 - 0.89)
Wistar – insulin infusion	0.85 (0.80 - 0.99)	0.81 (0.75 - 0.91)	0.88 (0.79 - 0.93)
HTG – normal saline infusion	0.86 (0.82 - 0.89)	0.83 (0.80 - 0.86)	0.84 (0.81 - 0.86)
Wistar – normal saline infusion	0.89 (0.83 - 0.96)	0.86 (0.81 - 0.95)	0.90 (0.86 - 0.94)

Data are medians and interquartile range.

measured because only minimal changes in TBARS (thiobarbituric acid reactive substances) levels during clamps were observed in our previous study (Kyselová e*t al.* 2002). Non-significant changes of malondialdehyde levels were also observed in type 2 diabetics during hyperinsulinemic clamp (Bravi *et al.* 2006).

Baseline GSH/GSSG ratios as a marker of intracellular oxidative stress in hHTG rats were significantly higher than those in control Wistar rats. These changes are probably caused by chronic activation of antioxidant mechanisms in insulin-resistant animals in which higher production of reactive oxygen species is present. GSH/GSSG ratios remained higher in hHTG rats during the clamps regardless of insulin infusion. The insulin infusion in hHTG rats was capable to increase GSH/GSSG ratio much higher above values reached without insulin. Moreover, insulin infusion was capable of reversing observed decrease of GSH/GSSG ratio induced by hyperglycemia. On the contrary, no significant changes in GSH/GSSG ratios were observed in Wistar rats with normal metabolism.

These changes could be explained by insulin infusion, because insulin suppresses proinflammatory transcription factors (Dandona *et al.* 2001, Aljada *et al.* 2002), ROS generation, and the expression of $p47^{phox}$ which is a key component of NADPH oxidase, enzyme that generates the superoxide radicals (Dandona *et al.* 2001, Van den Berghe *et al.* 2001). Other important effects of insulin are vasodilatation and inhibition of platelet aggregation (Steinberg *et al.* 1994, Trovati *et al.* 1994, Grover *et al.* 1995). These effects are mediated by an increase in nitric oxide (NO) release and NO synthase activity in the endothelium and the platelet (Zeng and Quon 1996, Aljada *et al.* 2000).

On the other hand, glucose has a proinflammatory effect, which occurs even without increasing of plasma

Table 3. Reduced/oxidized glutathione ratio (GSH/GSSG).

Group	GSH/GSSG ratio			
	Glycemia 6 mmol/l		Glycemia 12 mmol/l	
HTG – insulin infusion	10.02 (9.90 - 11.42)$^{*,\#}$	P=0.002	7.42 (7.15 - 7.89)$^{\#}$	P=0.003
HTG – normal saline infusion	6.01 (5.83 - 6.43)$^{\#}$		6.16 (5.74 - 7.05)$^{\#}$	
Wistar – insulin infusion	3.18 (1.44 - 3.69)	NS	2.11 (1.69 - 2.93)	NS
Wistar- normal saline infusion	2.93 (2.24 - 3.42)		3.08 (2.10 - 5.30)	

* p<0.01 in comparison to glycemia 12 mmol/l. # p<0.01 in comparison to corresponding Wistar group. Data are medians and interquartile range.

Table 4. Quotients of GSH/GSSG ratios measured under insulin and normal saline infusions.

Group	Quotients of GSH/GSSG ratios	
	Glycemia 6 mmol/l	Glycemia 12 mmol/l
HTG	1.76 (1.57 - 1.93)$^{*,\#}$	1.14 (1.09 - 1.36)$^{\#}$
Wistar	0.84 (0.69 - 1.40)	0.62 (0.44 - 1.15)

* p<0.05 in comparison to glycemia 12 mmol/l. # p<0.01 in comparison to corresponding Wistar group. Data are medians and interquartile range

glucose concentrations into the pathological range and in spite of endogenous insulin secretion (Mohanty *et al.* 2000, Dhindsa *et al.* 2004). Glucose activates key proinflammatory transcription factors suppressed by insulin (Van den Berghe *et al.* 2001, Dhindsa *et al.* 2004) and induces an increase in ROS generation and an increase in $p47^{phox}$ expression, which is consistent with an increase in NADPH oxidase (Mohanty *et al.* 2000). The bioavailability of NO is reduced due to the increased level of superoxide radical, which transform NO to peroxynitrite. Thus, hyperglycemia induces oxidative stress, inflammation, vascular constriction, platelet hyperaggregability, and thrombosis.

Kuneš *et al.* (2004) proved a greater degree of relative NO deficiency in male hHTG rats compared to normotensive Lewis controls. It is well known, that oxidative stress is accompanied by superoxide radical overproduction. Rapid reaction of NO with superoxide radicals effectively decreases intracellular level of NO below levels needed for activation of guanylate cyclase and that way changes superoxide function to NO antagonist (Fridovich 1995, Beckman and Koppenol 1996). In such situations where superoxide radicals exceed NO level, peroxynitrite or hydroxyl radical formation could be initiated (Brune *at al.* 1990). Stimulation of NO synthase by insulin infusion with increased NO levels could explain increased GSH/GSSG ratio in hHTG rats.

These results let us conclude, that insulin is capable of reducing intracellular oxidative stress through increased GSH/GSSG ratio. Bravi *et al.* (2006) showed, that insulin in patients with type 2 diabetes mellitus is capable of increasing GSH/GSSG ratio in erythrocytes after 2 hours of incubation with insulin, and *in vivo* during euglycemic hyperinsulinemic clamp. Our findings of increased GSH/GSSG ratio in hHTG rats infused with insulin are in agreement with this suggestion. Thus, insulin infusion *per se,* rather then normoglycemia, positively influences GSH/GSSG ratio and that way reduces intracellular oxidative stress in insulinoresistant animals.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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