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

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 H2O2 increases production of p21<sup>fl</sup> 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 H2O2, a chemical substitute for ROS (Sakai et al. 2003). Interestingly, the first phase of glucose-induced insulin secretion could be suppressed by 50 μM H2O2, H2O2 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 Ohen 1992) 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 et 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 °C), 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.
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 ($\lambda=600$nm).

**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 significant.

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**Fig. 1.** Study design.
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 et al. 2002).

Plasma lipid peroxidation intermediates were not

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**Table 1. Baseline characteristics of hHTG and Wistar rats.**

<table>
<thead>
<tr>
<th></th>
<th>Albumin (g/l)</th>
<th>Plasma triglycerides (mmol/l)</th>
<th>Uric acid (umol/l)</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar - insulin</td>
<td>36.3</td>
<td>(26.2 - 37.4)</td>
<td>0.28*</td>
<td>18.5**</td>
</tr>
<tr>
<td>Wistar - normal saline</td>
<td>36.4</td>
<td>0.29*</td>
<td>17.8*</td>
<td>1.94**</td>
</tr>
<tr>
<td>hHTG - insulin</td>
<td>36.2</td>
<td>(35.6 - 36.7)</td>
<td>0.43</td>
<td>9.43</td>
</tr>
<tr>
<td>hHTG - normal saline</td>
<td>37.1</td>
<td>(36.5 - 37.4)</td>
<td>0.43</td>
<td>8.54</td>
</tr>
</tbody>
</table>

* denotes p<0.05, ** denotes p<0.01 in comparison to corresponding hHTG group. 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 et 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 p47phox 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 2. Total antioxidant capacity (AOC) mmol/l.

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal conditions</th>
<th>Glycemia 6 mmol/l</th>
<th>Glycemia 12 mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTG – insulin infusion</td>
<td>0.83 (0.80 - 0.89)</td>
<td>0.81 (0.77 - 0.90)</td>
<td>0.77 (0.72 - 0.89)</td>
</tr>
<tr>
<td>Wistar – insulin infusion</td>
<td>0.85 (0.80 - 0.99)</td>
<td>0.81 (0.75 - 0.91)</td>
<td>0.88 (0.79 - 0.93)</td>
</tr>
<tr>
<td>HTG – normal saline infusion</td>
<td>0.86 (0.82 - 0.89)</td>
<td>0.83 (0.80 - 0.86)</td>
<td>0.84 (0.81 - 0.86)</td>
</tr>
<tr>
<td>Wistar – normal saline infusion</td>
<td>0.89 (0.83 - 0.96)</td>
<td>0.86 (0.81 - 0.95)</td>
<td>0.90 (0.86 - 0.94)</td>
</tr>
</tbody>
</table>

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 p47phox 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 transforms 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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References
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