Lipasin/Betatrophin Is Differentially Expressed in Liver and White Adipose Tissue Without Association With Insulin Resistance in Wistar and Goto-Kakizaki Rats

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Summary

Lipasin is a recently identified lipokine expressed predominantly in liver and in adipose tissue. It was linked to insulin resistance in mice and to type 1 and type 2 diabetes (T1D, T2D) in humans. No metabolic studies concerning lipasin were performed yet in rats. Therefore, we used rat model of T2D and insulin resistance, Goto-Kakizaki (GK) rats, to determine changes of lipasin expression in liver and in white adipose tissue (WAT) over 52 weeks in the relation to glucose tolerance, peripheral tissue insulin sensitivity and adiposity. GK rats were grossly glucose intolerant since the age of 6 weeks and developed peripheral insulin resistance at the age of 20 weeks. Expression of lipasin in the liver did not differ between GK and Wistar rats, declining with age, and it was not related to hepatic triacylglycerol content. In WAT, the lipasin expression was significantly higher in Wistar rats where it correlated positively with adiposity. No such correlation was found in GK rats. In conclusion, lipasin expression was associated neither with a mild age-related insulin resistance (Wistar), nor with severe genetically-based insulin resistance (GK).

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Introduction

Growing evidence suggests that reduction of functional β-cell mass together with insulin resistance leads to the development of overt type 2 diabetes (Liebowitz *et al.* 2011). Evidence of a circulating factor inducing β-cell proliferation in an insulin-resistant state was provided by Flier, who reported markedly increased proliferation rate in isolated pancreatic islets after transplantation under the kidney capsule of insulin-resistant mice (IRS1/insulin receptor double KO and *ob/ob*) (Flier *et al.* 2001). Yi *et al.* (2013) suggested a link between insulin resistance and recently described lipokine lipasin (betatrophin/RIFL/TD26) (Zhang and Abou-Samra 2013, Quagliarini *et al.* 2012, Ren *et al.* 2012), by showing a marked increase of lipasin transcription after few weeks of insulin-resistance induced by insulin receptor antagonist S961 in mice (Yi *et al.* 2013). Under the basal conditions, lipasin was found predominantly expressed in liver and adipose tissue. Ren *et al.* (2012) found metabolic regulation when

demonstrated dramatically increased lipasin mRNA expression after refeeding in mice. Yi *et al.* (2013) observed increased β-cell proliferation associated with lipasin over-expression and proposed it as the mediator of β-cell regeneration in mice, hence the new name betatrophin. Similar observation was reported in rats (Chen *et al.* 2015). However, the claim of lipasin as the important mediator of β-cell regeneration in mice was later disproved (Gusarova *et al.* 2014, Kaestner *et al.* 2014, Yi *et al.* 2014). Nevertheless, lipasin as a regulatory lipokine (Stephens 2012, Zhang and Abou-Samra 2014) remains of a great interest and a number of studies tried to understand its role and its regulation in the metabolic disturbances during the development of insulin-resistance and diabetes in humans. Trebotic *et al.* (2015) reported increased levels of circulating lipasin in both normal pregnancy and in gestational diabetes. Elevated serum lipasin concentration was found in obesity, T2D and T1D patients (Fu *et al.* 2014b, Yamada *et al.* 2015). Tokumoto *et al.* (2015) suggested that in humans lipasin serum concentrations positively correlated with the duration of T2D and inversely with insulin secretory capacity. Chen *et al.* (2015) reported positive correlation between serum levels of lipasin and the homeostasis model assessment of insulin resistance index. This group also found inverse correlation between serum levels of lipasin and the quantitative insulin-sensitivity check index in newly diagnosed T2D patients. On the other hand, Gomez-Ambrosi *et al.* (2014) reported reduced serum lipasin concentrations in human obesity and in obesity-associated insulin resistance. Fenzl *et al.* (2014) found correlation of circulating lipasin with atherogenic lipid profiles in high-risk obesity or type 2 diabetes, but no association with β-cell function and glucose homeostasis. In summary, while a number of studies support positive association between lipasin plasma levels and insulin-resistance, diabetes and adiposity, conflicting findings remain unresolved. The studies in humans were confined to the protein levels in serum, for practical reasons. Animal models of type 2 diabetes allow discerning the regulation of lipasin in individual tissues. So far, rodent metabolic studies were published only on mice, where an increase of mRNA of lipasin expression in liver and fat were reported in *ob/ob*, *db/db* and in pregnant mice (Yi *et al.* 2013). No metabolic studies concerning lipasin were performed yet in rats.

Goto-Kakizaki (GK) rats represent a widely used model of T2D. It was established by breeding Wistar rats selected at the upper limit of normal glucose tolerance. In GK rats early glucose intolerance and peripheral tissue insulin resistance develop spontaneously, their beta-cell mass and function gradually deteriorate, and overt hyperglycemia ensue (Portha *et al.* 2012). Aim of our study was to determine age-related changes in lipasin expression in the liver and white adipose tissue (WAT) over the period of one year and in the relation to the above mentioned characteristics. The data were compared to the Wistar rats as age-related controls.

Methods

Animals and experimental design

Four weeks old Goto-Kakizaki rats (GK/MolTac, Aarhus colony) and Wistar Han rats were purchased from Taconic Farms, Inc., Hudson, NY and kept in the IKEM breeding facility in a temperature-controlled environment with a 12 h light/dark cycle. The animals were randomized into 4 subgroups each comprising 6-8 animals and assayed at the age of 6, 20, 40, and 52 weeks. The animals had free access to the standard laboratory chow diet, and to the tap water. All tests including weighing, blood sampling, and glucose tolerance tests were initiated between 8-9 a.m. on random fed animals. Blood glucose concentration was measured by Accu-chek glucometer, insulin concentrations were determined using Rat Insulin kit (Mercodia, Sweden). Adiposity was defined as the wet weight of epididymal fat pads normalized per 100 g body weight (Sedova *et al.* 2004). Triacylglycerol content in the liver was determined as described previously (Cahova *et al.* 2004). All experiments were performed in accordance with the Animal Protection Law of the Czech Republic 311/1997, which is in compliance with the Principles of Laboratory Animal Care (NIH Guide to the Care and Use of Laboratory Animals, 8th edition, 2013) and were approved by the Ethical Committee of IKEM (permission no. 26/2012).

Glucose tolerance test

Glucose tolerance in 6 weeks old animals was measured by intraperitoneal glucose tolerance test (IPGTT). Fasted rats were administered glucose 3 g/kg b.w. intraperitoneally and blood glucose concentration was measured in 30 min intervals for next 120 min. In older animals (20, 40 and 52 weeks), intravenous glucose tolerance test (IVGTT) was employed. Fasted rats were injected with 20 % glucose into tail vein (1 g/kg b.w.)

and blood glucose was measured at the 10 min time intervals up to 60 min.

Determination of muscle and adipose tissue insulin sensitivity in vitro

The procedure was performed as described previously (Cahova *et al.* 2004). Tissue sensitivity to insulin was measured *in vitro* by ^{14}C-glucose incorporation into lipids (adipose tissue) or glycogen (musculus soleus). Distal parts of epididymal adipose tissue (approx. 150±25 mg) and musculus soleus (160±20 mg) were rapidly dissected. Soleus muscles were attached to a stainless steel frame *in situ* at *in vivo* length. The tissues were incubated 2 h at Krebs-Ringer bicarbonate buffer with 5 mmol/l glucose; 0.1 mCi (U-^{14}C)-glucose/ml (UVVR, Czech Republic) and 2 % (adipose tissue) or 0.5 % (m. soleus) bovine serum albumin, gaseous phase 95 % O_2 and 5 % CO_2 in the presence (250 mU/ml) or absence of insulin in incubation medium. All the incubations were carried out at 37 ºC in sealed vials in shaking water bath. Neutral lipids were extracted to chloroform/methanol (2:1) and the radioactivity was counted by scintillation counting. ^{14}C-glucose accumulation into glycogen in soleus was determined after tissue digestion by boiling in 30 % KOH and precipitation in 96 % ethanol.

Isolation and cultivation of rat pancreatic islets

Pancreatic islets were isolated using collagenase digestion followed by separation in a discontinuous density gradient, as previously described (Berkova *et al.* 2005). Briefly, rat pancreata were cannulated through the bile duct and filled with 15 ml of collagenase solution (1 mg/ml) (Sevapharma, Czech Republic). The excised pancreata were incubated for 18 min at 37 °C with moderate shaking. Collagenase digestion was stopped by several washing steps with cold Hank´s Balanced Salt Solution (supplemented with 1 % fetal bovine serum), and the tissue suspension was filtered through a 500 µm mesh. Pancreatic islets were separated using Ficoll (Sigma-Aldrich) discontinuous density gradient yielding in 600-700 purified islets per rat.

Glucose-stimulated insulin secretion (GSIS) test

Freshly isolated islets from individual animals were divided into three aliquots of 20 islets, pipetted on polyester membranes with 8 µm pores (Transwell cell culture inserts, Corning, NY, USA), placed into six well plate and incubated for 18 h at 5 % CO_2 and 37 °C in 4 ml of islet culture media (CMRL supplemented with 10 % FBS, 10 mM HEPES, 2 mM Glutamax, 100 U/ml Penicillin, and 100 µg/ml Streptomycin). The GSIS assay medium consisted of Krebs-Ringer bicarbonate buffers equilibrated with 5 % CO_2 at 37 °C, and supplemented with either 3 mM (low) or 22 mM (high) glucose. Throughout the assay care was taken to minimize mechanical stress on the islets. All incubations were carried on at 37 °C and 5 % CO_2, in the volume of 4 ml. Islets were equilibrated for 15 min in low glucose GSIS assay medium. Glucose-stimulated insulin secretion was then tested over 60 min at three subsequent concentrations of glucose (low, high and again low). After each incubation period, insulin from the vicinity of the islets was gently flushed into the corresponding lower chamber, and mixed medium was sampled. After the assay, albumin was washed from the islets with PBS and islets were collected and lysed using proteinase K, EDTA and SDS. Total DNA content in each assayed islet aliquot was determined using dsDNA specific assay, PicoGreen kit (Invitrogen, USA).

Immunohistochemistry

Immunohistochemical detection of PCNA (mouse monoclonal, Dako, Denmark) was performed on 4 µm-thick paraffin sections using a two-step indirect method. After deparaffinization in xylene and rehydration in graded ethanol, endogenous peroxidase was blocked. Primary antibody anti PCNA was applied for 30 min. The antibody was detected by Histofine Simple Stain Rat MAX PO (Nichirei, Japan). Finally visualization was performed with the Dako Liquid DAB+ Substrate-Chromogen System (Dako, Denmark) and counterstaining with Harris's hematoxylin.

Real-time quantitative PCR analysis (RT-qPCR)

Total RNA was extracted from rat liver and adipose tissue using an RNeasy Mini Kit (Qiagen, France). 500 ng of total RNA was reverse-transcribed using a High Capacity Reverse Transcription Kit (Life Technologies, Forrest City, CA) and RT-qPCR was performed (viiA7, Life Technologies, Forrest City, CA) using EvaGreen qPCR Mix (Solis BioDyne, Estonia). Lipasin expression in the liver and WAT was determined by RT-qPCR using specific primers (NM_001271710.1) Forward: GGAGATTCAGGCAGAAGAGGAC, Reverse: GTGAGAGCCCACAAGAGGTG, product size 213 bp;) and normalized to B2m (NM_012512.2) Forward: CCATTCAGAAAACTCCCCAAATTC,

Reverse: GGCTCCTTCAGAGTGACGTG, product size 271 bp) and Cyc1 (NM_001277194.1) Forward: GGGACTCAAGATGTTGTTGATG, Reverse: GTTCCTGGTCTGCTGGAGAGC, product size: 172 bp). Calculations were based on the comparative cycle threshold Ct method ($2^{-\Delta\Delta Ct}$), the group of 6 weeks old Wistar rats was taken as the calibrator (Livak and Schmittgen *et al.* 2001).

Statistical analysis

Data are presented as a mean ± SD of multiple determinations. Statistical significance of the differences among groups was tested using one-way ANOVA with Bonferroni correction for multiple comparisons. Differences were considered statistically significant at the level of $p < 0.05$. The correlation between adiposity and lipasin expression in adipose tissue was evaluated using Pearson´s correlation coefficient.

Results

Metabolic characterization of Goto-Kakizaki and Wistar rats

GK rats were growth-retarded throughout the entire experiment as compared to Wistar rats (Fig. 1A). Adiposity steadily increased in Wistar rats, but it remained unchanged in GK rats (Fig. 1A). Triacylglycerols accumulated in the liver of Wistar rats along with age (more than four-fold over 52 weeks), but the lipid content did not increase over the same time period in the liver of GK rats (Fig. 1B). Serum triglycerides remained within the range 0.8-1.6 mmol/l and did not significantly differ between the two strains (data not shown). Blood glucose concentration steadily

rose over 52 weeks in GK rats, reaching overt hyperglycemia already by the age of 20 weeks (Fig. 1C). In Wistar rats it remained within the normal range throughout the whole year (Fig. 1C). Glucose tolerance and its two critical components, insulin sensitivity and insulin secretion, were evaluated *in vivo* and *in vitro*. GK rats were glucose-intolerant to a high degree already at the age of 6 weeks and they remained so throughout the study (Fig. 2A). Wistar rats became glucose-intolerant to a mild degree only at the age of 52 weeks (Fig. 2A). Insulin-sensitivity of peripheral tissues was assessed *in vitro* for muscle and WAT. The ability of insulin to stimulate incorporation of glucose into glycogen (muscle) or into neutral lipids (WAT) did not differ between the two strains at the age of 6 weeks and it dropped by the age of 20 weeks in both strains (Figs 2B and 2C). However, the decrease of insulin-sensitivity on both tissues was three-fold deeper in GK than in Wistar rats. In GK rats, serum insulin rose two-fold by the age of 20 weeks, but its concentration gradually declined below the original level (Fig. 3A). In Wistar rats, insulin increased three-fold by the age of 20 weeks and remained relatively stable for 52 weeks (Fig. 3A). *In vitro*, glucose-stimulated insulin secretion by isolated islets rose by the age of 20 weeks in both strains. While it gradually declined in GK rats, it remained stable in Wistar rats (Fig. 3B). Histological examination showed morphological distortion and diminution of the GK islets, including stellar shaped islets, by 20 weeks of age which progressed further on. Proliferation activity visualized in pancreatic islets by immunohistochemical presence of "proliferating cell nuclear antigen" (PCNA) responsible for DNA replication and repair was lower in GK rats as compared to age-matched Wistar controls (Fig. 4).
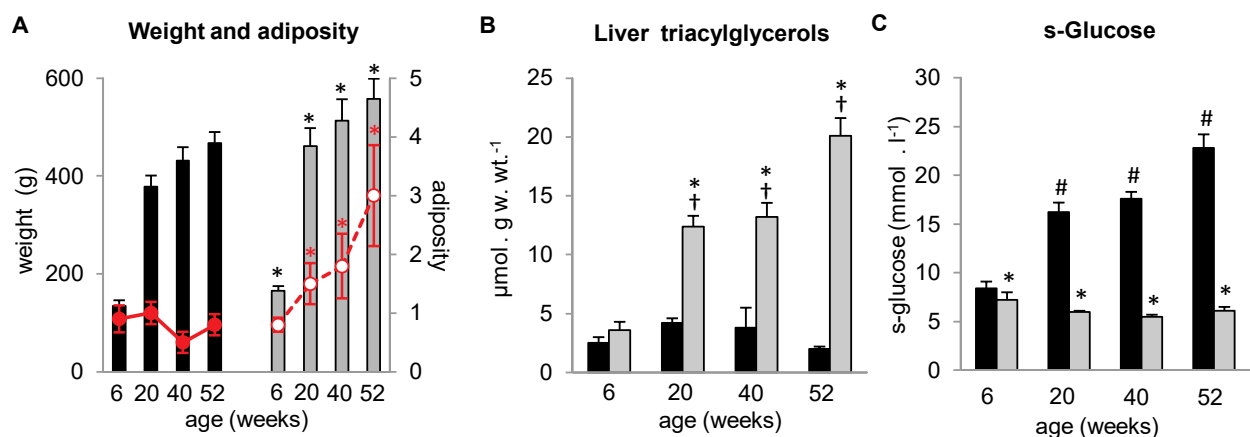
Fig. 1. Characterization of GK model and the age-matched Wistar controls. **A)** weight and adiposity of GK/Tac a Wistar rats during the experiment. Black bars – GK rats, grey bars – Wistar rats, red lines indicate adiposity expressed as the ratio of epididymal fat pads weight per 100 g body weight; **B)** liver triacylglycerol content; **C)** blood glucose (fed). Statistical significances were evaluated using one way ANOVA with Bonferroni correction: * p<0.05 Wistar vs. Goto, # p<0.05 GK vs. 6-week-old GK, † p<0.05 Wistar vs. 6-week-old Wistar.
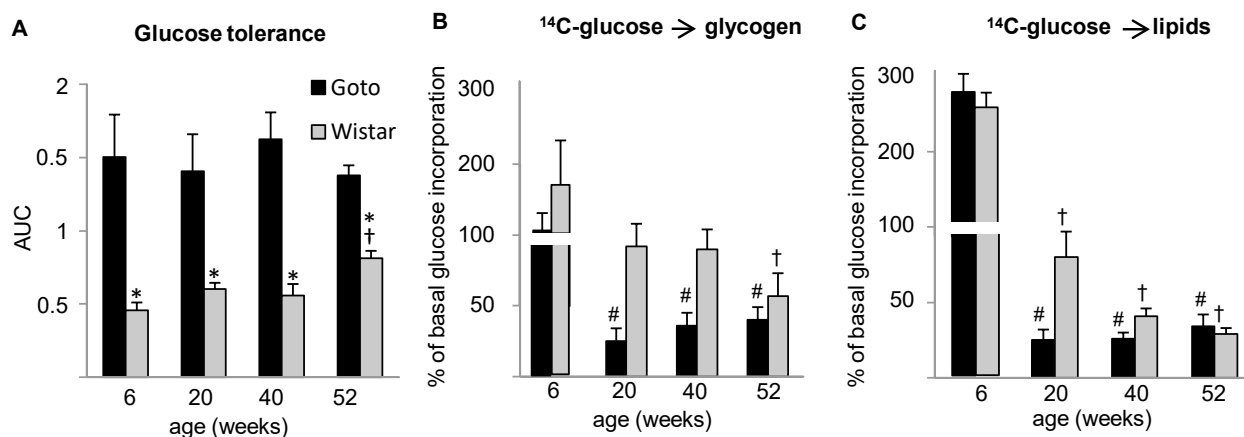
Fig. 2. Glucose tolerance and peripheral tissue insulin sensitivity in GK model and the age-matched Wistar controls. **A**) glucose tolerance measured as IPGTT (6 weeks) or IVGTT (20, 40 and 52 weeks), data are expressed as area under curve (AUC) in mol glucose per 60 and 120 min, resp.; **B**) effect of insulin on the incorporation of glucose into glycogen (µmol glucose . g w.wt.$^{-1}$. 60 min^{-1}) in m. soleus *in vitro* expressed as the insulin-stimulated increase over the basal incorporation (%); **C**) effect of insulin on the incorporation of glucose into lipids (µmol glucose . g w.wt.$^{-1}$. 60 min^{-1}) in epididymal adipose tissue *in vitro* expressed as the insulin-stimulated increase over the basal incorporation (%). Statistical significances were evaluated using one way ANOVA with Bonferroni correction: * $p<0.05$ Wistar vs. Goto; # $p<0.05$ GK vs. 6-week-old GK; † $p<0.05$ Wistar vs. 6-week-old Wistar.
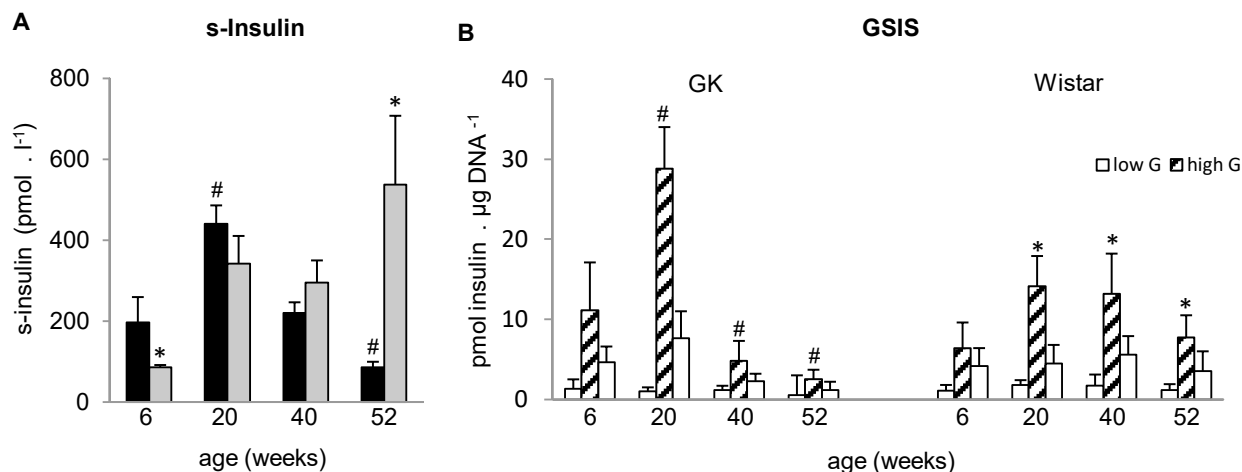
Fig. 3. Fed serum insulin (**A**) and glucose-stimulated insulin secretion (**B**) of pancreatic islets *in vitro* (low G=3 mM glucose, high G=22 mM glucose). Black bars – GK rats, grey bars – Wistar rats, white bars – low glucose, hatched bars – high glucose. All data are expressed as a mean ± SD, n=6-8. Statistical significances were evaluated using one way ANOVA with Bonferroni correction: * $p<0.05$ Wistar vs. Goto, # $p<0.05$ GK vs. 6-week-old GK.

Lipasin expression in liver and WAT

The expression of lipasin specific mRNA was evaluated at the same time points as the metabolic assessment in the two most important organs. In the liver, the expression of lipasin decreased gradually with age, and did not differ between the strains (Fig. 5A). In WAT, there was also no difference in the expression at the age of 6 weeks between GK and Wistar rats. However, since the age of 20 weeks, the expression gradually declined in the GK rats, while it steadily rose in Wistar rats (Fig. 5B). The lipasin mRNA expression correlated neither with the degree of insulin resistance nor with serum level of triglycerides. The only correlation was found between the

lipasin expression in WAT and adiposity (R=0.81) in Wistar rats (Fig. 5B). This data show that the level of lipasin expression is not related to insulin resistance in GK rats in the two most relevant organs and is differentially expressed in liver and white adipose tissue.

Discussion

We present here the first metabolic study into the expression of endogenous lipasin in GK and Wistar rats. It produced two unexpected findings. First, our data show in GK rats, that the expression of lipasin in liver and WAT remained unaffected by the presence of severe insulin

resistance (Figs 5A and 5B). Our data contrast with the findings reported in other rodent models of insulin resistance states, e.g. *ob/ob mice*, *db/db* mice, pregnant mice, and the mice treated with inhibitor of insulin receptor, where an increased levels of lipasin mRNA in liver and WAT or lipasin protein in serum were reported (Yi *et al.* 2013). One possible caveat in our study is the control animals to which the lipasin expression is compared. Ideally, the same strain without insulin resistance should be used. However, GK rats were insulin resistant already at the age of 6 week, precluding such a control. Wistar rats are an outbred strain from which GK were developed (Portha 2012), so they are genetically similar, but not identical. It is therefore plausible to assume that the two strains differ in yet unknown gene or set of genes, which regulate expression of lipasin in liver and fat. Lipasin is a metabolically regulated protein (Ren *et al.* 2012, Stephens 2012, Zhang *et al.* 2014). While our animal were fed *ad libitum* and sampled at fixed time, the authors of the reports on mice do not provide much information regarding the feeding and sampling.

The data concerning the time course of the onset of insulin resistance and beta cell failure in GK rats are not absolutely coherent throughout the literature. The important factor seems to be the origin of experimental animals, i.e. the breeding colony. Portha *et al.* (2012) using GK/Par colony demonstrated markedly reduced *in vivo* secretory beta cell response to glucose together with increased insulin sensitivity in 3 weeks old prediabetic GK/Par rats (Portha *et al.* 2012, Movassat *et al.* 2008). Two month old GK/Par rats have significantly decreased insulin-stimulated whole body glucose uptake but peripheral tissue insulin resistance was proved in some but not all tissues and there was no evidence for clear-cut decreased skeletal muscle insulin action (Berthelier *et al.* 1997). In contrast, Wallis *et al.* (2008, 2004) demonstrated enhanced insulin secretory capacity both *in vivo* and *in vitro* in 12 and 24 weeks old animals together with selective insulin resistance in peripheral tissues in animals originating from GK/Ox colony (Wallis *et al.* 2008, Wallis *et al.* 2004). Our experimental animals coming from Taconic colony (GK/Tac) exhibit severe glucose intolerance at 6 weeks of age and insulin resistance in WAT and skeletal muscle since the age of 20 weeks. In accordance with Wallis *et al.* (2008, 2004) and in contrast to data reported by Portha *et al.* (2012) and Ostenson *et al.* (1993) we found enhanced glucose-stimulated insulin secretion *in vitro* in 20 weeks old GK/Tac rats that got severely deteriorated between 20 and 40 weeks of age.

Despite the fact of currently identified delta cell hyperplasia (Alan *et al.* 2015) but in line with previously reported findings (Portha *et al.* 2010) we did not observed any significant proliferation activity in GK/Tac islets. On the other hand, generally decreased PCNA immunostaining and decrease of proliferative activity in GK islets can be explained just by the increased paracrine secretion of somatostatin from increased number of delta cells (Stewart *et al.* 1995). However, the data presented in our study does not allow identifying the primary defect leading to beta cells failure in older GK/Tac rats but point to the importance of considering the GK origin for the interpretation of the results.
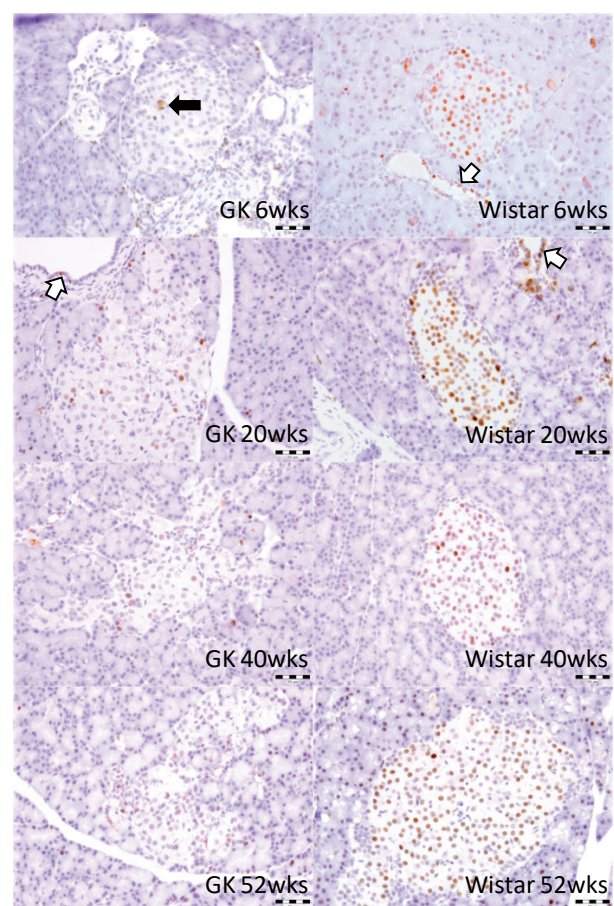
Fig. 4. Evaluation of DNA replication and repair activity visualized by immunohistochemical presence of "proliferating cell nuclear antigen" PCNA. A full range nuclear positivity of PCNA from weak to strong was a dominant observation in Wistar control islet cells at age of week 6, week 20, week 40 and week 52. In age-matched GK animals, absence of PCNA nuclear positivity was a dominant islets finding with only individual observation of weak to medium PCNA-positive nuclei. In week 6 GK sample, a cytoplasmatic positivity of PCNA was observed in presence of mitotic chromatin figure (black arrow). Since week 20, disturbance of islets shape could be observed in GK animals leading up to stellar shape of islets appearing since week 40. PCNA nuclear positivity of proliferating ductal epithelial cells serves an internal control (white arrows). Original magnification 400x.
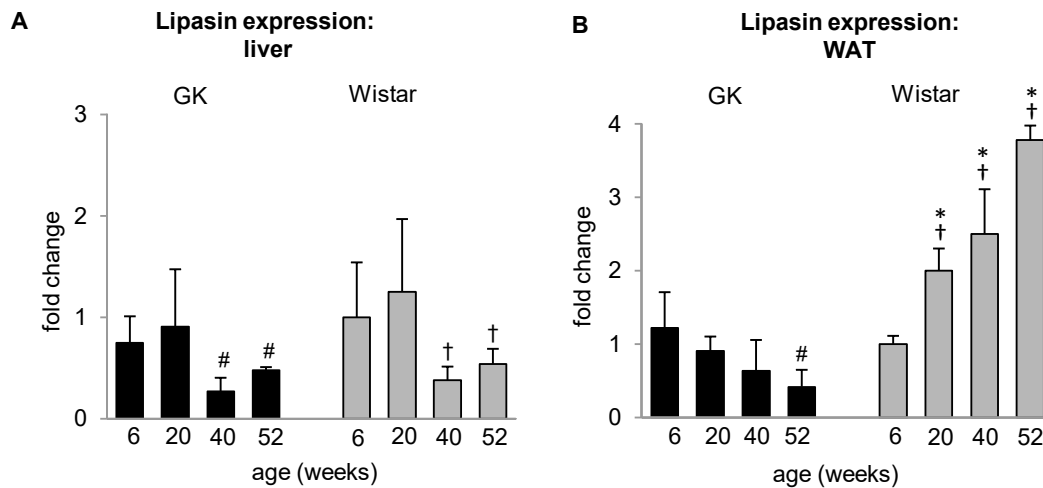
Fig. 5. Lipasin specific mRNA expression in liver (**A**) and epididymal adipose tissue (**B**). Data are expressed as fold changes relative to 6 weeks old Wistar rats (mean ± SD, n=6-8). Black bars − GK rats, grey bars − Wistar rats. Statistical significances were evaluated using one way ANOVA with Bonferroni correction: * p<0.05 Wistar vs. Goto, # p<0.05 GK vs. 6-week-old GK, † p<0.05 Wistar vs. 6-week-old Wistar.

The second unexpected finding in Wistar rats was that lipasin is differentially expressed in the liver and WAT. Wistar rats aged 52 weeks developed low degree of insulin resistance, but no association with hepatic lipasin expression was found. On the other hand, adiposity increased three-fold in one year old Wistar rats, which positively correlated with the lipasin mRNA expression in adipose tissue, suggesting a positive feedback mechanism. This finding of differential expression of lipasin has not been reported before in any species. It suggests that lipasin regulation is cell/tissue specific. Indeed it is not yet clear, by which cell type in the liver it is expressed and secreted. The finding that lipasin expression in adipose tissue but not in the liver increases proportionally with the adiposity might also explain the failure of GK rats to respond to insulin resistance with rise of lipasin. GK rat had very small amount of adipose tissue throughout the study. It is possible that a minimum amount of fat is required to mount significant response of lipasin mRNA to insulin resistance.

Some studies in humans associate increased plasma levels of lipasin with insulin resistance and obesity (Yamada *et al.* 2015, Tokumoto *et al.* 2015, Chen *et al.* 2015, Fenzl *et al.* 2014), while other authors reported the opposite findings (Gomes-Ambrosi *et al.* 2014). It is difficult to compare our results to human studies, which were all based only on levels of lipasin in serum sampled after overnight fast, which are dependent on antibody-based assays (ELISA or RIA). One explanation of the discrepancies, at least in human studies, provided Fu *et al.* (2014a) who have shown that most of the circulating lipasin lack the N-terminus and

suggested that full-length lipasin protein in serum may undergo proteolytic cleavage, resulting in release of the residual C-terminus. Importantly, some of the commercially available kits use antibodies that recognize the N-terminus, while others recognize the C-terminus. For rats, the information regarding the target sequence is not available.

Our data derived from lipasin expression in WAT are in line with reports linking lipasin to lipid metabolism but not to glucose homeostasis (Zhang *et al.* 2013, Ren *et al.* 2012, Fenzl *et al.* 2014). To our knowledge, the differential expression of lipasin specific mRNA in liver and WAT had not been reported before. It remains to be seen, whether indeed the adipose tissue, not the liver, is primarily responsible for the variation of lipasin blood levels in response to metabolic challenges.

In conclusion, our study demonstrates that hepatic expression of lipasin mRNA is associated neither with a mild age-related insulin resistance (Wistar rats) nor with severe genetically-based insulin resistance (GK rats). In normoglycemic Wistar rats we show that lipasin is differentially expressed in the liver and WAT. In WAT, we found positive correlation between lipasin mRNA expression and adiposity.

Conflict of Interest
There is no conflict of interest.

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