Hypercholesterolaemia, signs of islet microangiopathy and altered angiogenesis precede onset of type 2 diabetes in the Goto–Kakizaki (GK) rat

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Abstract

Aims/hypothesis The adult non-obese Goto–Kakizaki (GK) rat model of type 2 diabetes, particularly females, carries in addition to hyperglycaemia a genetic predisposition towards dyslipidaemia, including hypercholesterol-

aemia. As cholesterol-induced atherosclerosis may be programmed in utero, we looked for signs of perinatal lipid alterations and islet microangiopathy. We hypothesise that such alterations contribute towards defective pancreas/islet vascularisation that might, in turn, lead to

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decreased beta cell mass. Accordingly, we also evaluated islet inflammation and endothelial activation in both prediabetic and diabetic animals.

Methods Blood, liver and pancreas were collected from embryonic day (E)21 fetuses, 7-day-old prediabetic neonates and 2.5-month-old diabetic GK rats and Wistar controls for analysis/quantification of: (1) systemic variables, particularly lipids; (2) cholesterol-linked hepatic enzyme mRNA expression and/or activity; (3) pancreas (fetuses) or collagenase-isolated islet (neonates/adults) gene expression using Oligo GEArray microarrays targeted at rat endothelium, cardiovascular disease biomarkers and angiogenesis, and/or RT-PCR; and (4) pancreas endothelial immunochemistry: nestin (fetuses) or von Willebrand factor (neonates).

Results Systemic and hepatic cholesterol anomalies already exist in GK fetuses and neonates. Hyperglycaemic GK fetuses exhibit a similar percentage decrease in total pancreas and islet vascularisation and beta cell mass. Normoglycaemic GK neonates show systemic inflammation, signs of islet pre-microangiopathy, disturbed angiogenesis, collapsed vascularisation and altered pancreas development. Concomitantly, GK neonates exhibit elevated defence mechanisms.

Conclusions/interpretation These data suggest an autoinflammatory disease, triggered by in utero programming of cholesterol-induced islet microangiopathy interacting with chronic hyperglycaemia in GK rats. During the perinatal period, GK rats show also a marked deficient islet vascularisation in conjunction with decreased beta cell mass.

Keywords Beta cell mass · Cholesterol · Dyslipidaemia · Fetus · Goto–Kakizaki · Islet microangiopathy · Liver · Programming · Type 2 diabetes

Abbreviations

AOD	Antioxidant defence
APOA1	Apolipoprotein A1 (PGI ₂ -stabilising factor)
CCL2	CC-chemokine ligand-2 or monocyte chemoattractant protein-1 MCP-1
CCL3	CC-chemokine ligand-3 or macrophage inflammatory protein-1 α (MIP-1 α)
CVD	Cardiovascular disease
CXCL1	CXC-chemokine ligand-1 or chemokine GRO1/KC (murine IL-8 equivalent)
E21	Embryonic day 21
ECM	Extracellular matrix
GK	Goto–Kakizaki
GSH	Glutathione
IL-1Ra	Interleukin-1 receptor antagonist
LCAT	Lecithin:cholesterol acyltransferase
MMP	Matrix metalloproteinase
NF κ B	Nuclear factor- κ B
OS	Oxidative stress

PGI ₂	Prostaglandin I ₂ (or prostacyclin)
PON-1	Paraoxonase-1
PTGIS	PGI ₂ synthase or cytochrome P450 8A1 (CYP8A1)
RANKL	Receptor activator of nuclear factor- κ B ligand or osteoprotegerin ligand (OPGL)
ROS	Reactive oxygen species
VWF	von Willebrand factor
ZDF	Zucker diabetic fatty

Introduction

The adult Goto–Kakizaki (GK) rat is a spontaneous model of non-obese type 2 diabetes. In addition to hyperglycaemia, the GK rat shows various lipid anomalies such as high circulating NEFA levels, hypertriacylglycerolaemia and hypercholesterolaemia [1]. While hyperglycaemia and hyperlipidaemia induce endothelial dysfunction and inflammation, hypercholesterolaemia alone specifically triggers atherosclerosis [2, 3]. Recently, we demonstrated the presence of inflammation in pancreatic islets of human type 2 diabetic patients and various rodent models of the disease [4, 5]. We suggested that endothelial activation might underlie inflammation in type 2 diabetic islets, as it does in the eye (retinopathy), kidney (glomerulopathy) and nerves (neuropathy). To test this hypothesis, we treated GK rats with the soluble IL-1 receptor antagonist (IL-1Ra): 1 month of IL-1Ra treatment from onset of diabetes onwards reduced markers of islet endothelial cell activation/dysfunction, inflammation and fibrosis [5, 6]. In vivo, IL-1Ra normalised islet capillaries with decreased evidence of microangiopathy and reduced hyperglycaemia in GK rats as observed in type 2 diabetic patients [7].

Adult GK females are more hypercholesterolaemic than males [1]. In addition maternal cholesterolaemia increases during gestation and a placental gradient of cholesterol is formed between the mother and the fetus [8, 9]. In humans, even temporary maternal hypercholesterolaemia may trigger fatty streak lesions in fetal arteries and accelerate progression of atherosclerosis in normocholesterolaemic children [2]. Such in utero programming has been shown in experimental models of atherosclerosis [3]. We now hypothesise that GK rats present with hypercholesterolaemia combined with an early islet microangiopathy-like reaction during the perinatal period.

In addition, as GK rats exhibit more than a 50% decrease in beta cell mass from fetal life onwards [10, 11], we hypothesise that defective pancreatic vascularisation is associated with perinatal beta cell mass deficit. Indeed, vessel development, a major component of pancreatic islet development and function, is particularly active around

birth [12–14] and altered programming of vascularisation has been shown to affect the development and function of various organs [15–17].

To gain further insight into GK dyslipidaemia, we analysed various systemic variables, including different classes of lipids and lipid-related variables at three different ages: embryonic day 21 (E21, hyperglycaemic fetuses) [10], 7 days (normoglycaemic neonates) and 2.5 months after birth (diabetic and insulin-resistant adults) [18]. We also analysed islet and pancreatic gene expression, using Oligo GEArray membranes targeted at rat endothelium, angiogenesis and cardiovascular disease (CVD) biomarkers. Finally, vascularisation was quantified by immunochemistry for endothelial markers.

Methods

Animals All animal experiments were conducted on age-matched GK and Wistar (control) rats from our local colony (Paris, France), in accordance with accepted standards of animal care established by the French National Centre for Scientific Research. GK rats were bred in our own colony at the University Paris-Diderot, together with Wistar control rats from the GK strain derived after backcrossing of animals selected at the upper limit of normal distribution for glucose tolerance [19]. Characteristics of GK rats have been previously described [1, 4–6, 10, 11, 18–21]. At the indicated age, rats were weighed, bled at time of decapitation and blood, liver and pancreas collected for analysis. Fetuses were taken from pregnant rats fed ad libitum with a standard diet (diet 133; Usine d’Alimentation Rationnelle, Villemoisson-sur-Orge, France) at 21 days of gestation (E21) and processed as already described [10, 11] after maternal anaesthesia with pentobarbital sodium (35 mg/kg body weight). At E21, the number of fetuses per litter was similar in Wistar and GK rats, 9.0 ± 0.9 ($n=9$) and 8.2 ± 0.9 ($n=8$), respectively, as were the fetal body weights 3.7 ± 0.1 g ($n=55$) and 3.6 ± 0.1 g ($n=59$), respectively. The body weight of male GK rats was significantly decreased vs Wistar at both 7 days (9.1 ± 0.2 g, $n=26$ vs 15.9 ± 0.1 g, $n=36$; $p<0.001$) and 2.5 months of age (271 ± 3 g, $n=22$ vs 402 ± 12 g, $n=14$; $p<0.001$).

Systemic variables Basal morning glycaemia was determined with a glucometer. Serum insulin was assayed by ELISA (rat insulin ELISA, cat 10-1124-01, Mercodia, Uppsala, Sweden). Serum NEFA levels were quantified using an enzymatic colorimetric assay (NEFA C, Wako Chemicals, Neuss, Germany). Triacylglycerol, total cholesterol and HDL-cholesterol serum levels were determined by colorimetric assays (Pentra Triglycerid CP kit, Pentra Cholesterol CP kit and HDL-cholesterol direct kit, respectively; ABX Diagnostics, Montpellier, France). Serum

ketone levels were measured by quantification of β -hydroxybutyrate, using the β -hydroxybutyrate LiquidColor kit (Stanbio Laboratory, Boern TX, USA). Serum alkaline phosphatase activity was quantified using an enzymatic colorimetric assay (Alp CP kit, ABX Diagnostics). Serum leptin was measured using Luminex technology (Millipore, Zug, Switzerland) [5]. Plasma paraoxonase-1 (PON-1) activity and lecithin:cholesterol acyltransferase (LCAT) activity were determined, as previously described [6, 22].

Liver variables *Lcat*, *Pon1* and *Apoa1* (encoding apolipoprotein A-1 [APOA1]) mRNA levels were determined by quantitative RT-PCR on a Light Cycler 480 device (Roche Diagnostics, Mannheim, Germany) and PON-1 activity was determined, as previously described [6, 23]. Primers are listed in electronic supplementary material (ESM) Table 1.

Pancreatic islet isolation and mRNA analysis Analysis of mRNA was performed on pooled pancreases from E21 fetuses. Islets from 7-day-old rats were isolated from the pancreas by a collagenase digestion procedure originally developed for adult rat pancreas and adapted to neonates (see **Methods** in ESM). Total islet RNA was extracted according to standard protocols using the RNeasy minikit from Qiagen (RNeasy Kit 74104, Hombrechtikon, Switzerland). Islet RNA recovery was around 30–50 μ g per ten rats. RNA was amplified (two cycles) and biotin-labelled cRNA probes were synthesised using Bioarray High Yield RNA transcript labelling reagents from Enzo Diagnostics (Microsynth, Balgach, Switzerland). Three Oligo GEArray membranes (SABiosciences, Frederick, MD, USA, 128 genes each) were used for hybridisation and were targeted at: (1) rat endothelium (ORN-015.2: permeability and vessel tone, angiogenesis, endothelial cell activation and cell injury); (2) rat cardiovascular disease biomarkers (ORN-037: cell adhesion, immune response, apoptosis, extracellular matrix [ECM] molecules, blood coagulation, circulation, lipid metabolism and genes associated with heart disease); and (3) rat angiogenesis (ORN-024.2: angiogenic factors and other factors involved in angiogenesis, such as cytokines, chemokines, growth factors, adhesion molecules, protease inhibitors, ECM proteins and transcription factors). The procedure was according to protocols from SuperArray Bioscience Corporation (www.superarray.com/). Differential gene expression was confirmed by quantitative RT-PCR (RT: SuperScript II Reverse Transcriptase 11752–050, Invitrogen Life Sciences, Basel, Switzerland and PCR: qPCR Cyber Green kit: Eurogentec RT-SN10-05, Seraing, Belgium) and quantification performed after normalisation to housekeeping genes as indicated. Primers are listed in ESM Table 1. Further gene classification was performed using investigators’ expertise and the PubMed website (www.ncbi.nlm.nih.gov/gene).

Pancreas immunohistochemistry for endothelial markers Rat islet endothelial proliferation is very high around birth but declines rapidly during the first week postpartum [12]. Nestin was thus used to stain fetal proliferative endothelial cells [24] and von Willebrand factor (VWF) to stain postnatal mature endothelial cells [4, 6]. For nestin immunohistochemistry, fetal pancreas was fixed in aqueous Bouin's solution and embedded in paraplast. Pancreas sections (6 μ m) were exposed to mouse anti-nestin (1/50, SC-33677, Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with biotinylated anti-mouse IgG (1/200, Vector Laboratories, Burlingame, CA, USA) and processing with the Vectastain ABC kit PK6100 (Vector Laboratories). VWF immunochemistry was performed on neonatal pancreas cryosections (7 μ m) with rabbit anti-human VWF (1/100, Dako, Glostrup, Denmark) and swine anti-rabbit secondary antibody (1/100, Dako). Immunostained areas were quantified using an Olympus BX40 microscope as previously described by Lacraz et al. [6].

Statistical analysis Data are presented as means $\pm$ SEM. Statistical analyses used the Student's *t* test for unpaired data. Significance was defined as $p<0.05$ and statistical tendency as $0.05\leq p\leq 0.10$.

Results

Systemic metabolic and hormonal anomalies in GK rats as a function of age While GK rats are well known to be normoglycaemic from birth to around weaning at postnatal day 28 [18], GK fetuses present hyperglycaemia at E21 of age (Fig. 1) [10]. E21 GK fetuses were also hypoinsulinaemic, as were 7-day-old neonates, contrary to hyperinsulinaemic and insulin-resistant adult GK rats. As expected, leptinaemia followed the same pattern as insulinaemia [25]. Circulating NEFA levels were similarly low in E21 Wistar and GK fetuses but abnormally elevated in 7-day-old neonates and

Fig. 1 Systemic metabolic and hormonal variables in Wistar and GK rats as a function of age. All variables were determined in serum of Wistar (white columns) and GK (black columns) rats under fed conditions, except PON-1 activity [23] and fetal insulin measured in plasma as previously described [10]. Values are means $\pm$ SEM for 6–71

fetuses, 10–25 neonates and 4–10 adults in each group of animals. Statistically significant difference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) and statistical tendency († $0.05\leq p\leq 0.10$) vs age-matched Wistar rats, as analysed by Student's *t* test for unpaired data

adult GK rats compared with Wistar controls. Systemic triacylglycerol levels followed the same pattern. Circulating levels of the hepatic β -oxidation marker, β -hydroxybutyrate, were lower in E21 GK than Wistar fetuses but higher in normoglycaemic 7-day-old GK neonates and identical in adult rats. Importantly, total cholesterol was abnormally elevated in GK rats regardless of age. Although systemic GK and Wistar HDL-cholesterol levels were similar in hyperglycaemic fetuses, HDL-cholesterol levels were decreased in normoglycaemic GK neonates but increased in diabetic GK adults. Finally, alkaline phosphatase levels, reflecting liver and/or bone anomalies, were increased in GK rats regardless of age. Therefore, dyslipidaemia, in particular systemic cholesterol anomalies, already exists in hyperglycaemic fetuses and normoglycaemic GK neonates.

Systemic and hepatic disturbances of HDL-linked variables in GK rats as a function of age To gain understanding of the very early cholesterol anomalies in GK rats, we measured the evolution of HDL-related variables at different ages (Table 1). Hyperglycaemic E21 GK fetuses exhibited decreased systemic activity of LCAT, the critical enzyme involved in HDL maturation, but normal hepatic *Lcat* expression. In addition, they also exhibited a drastic increase in hepatic *ApoA1* mRNA compared with Wistar fetuses. *ApoA1* encodes the predominant HDL protein, but there was no difference in blood- and liver-HDL-associated lipo-lactamase PON-1 (at the level of its activity and/or mRNA level) between GK and Wistar fetuses. By contrast, normoglycaemic 7-day-old GK neonates showed increased systemic LCAT activity with no change in hepatic gene expression. Hepatic *ApoA1* expression was downregulated

in this group. Circulating and hepatic PON-1 activities were both lowered as compared with age-matched Wistar, with no change in *Pon1* expression. Finally, hyperglycaemic 2.5-month-old GK rats were characterised by a normal systemic LCAT activity despite a lowered hepatic *Lcat* expression. Adult GK rats also displayed elevated circulating PON-1 activity. In liver, this variable was normal despite downregulation of its gene expression. Hepatic *ApoA1* expression was unchanged in GK adults.

Altogether, these data highlight a pronounced post-transcriptional up/downregulation of hepatic HDL-linked genes and a highly variable pattern of systemic and hepatic HDL-linked variables in GK rats as a function of age and/or metabolic state.

Signs of pancreas or islet inflammation and/or endothelial alterations in GK rats from perinatal life onwards We assessed islet gene expression in Wistar and GK neonates using Oligo GEArray membranes targeted at rat endothelium, CVD and angiogenesis. Over a total of 384 genes, 38 were found to be differentially expressed in 7-day-old prediabetic GK vs control Wistar islets (ESM Table 2). Quantitative RT-PCR confirmed a significant or strong tendency for modulation of 20 genes that we classified into four different groups (Table 2). The first group includes genes involved in inflammation/atherosclerosis that exert mostly deleterious effects with only a few beneficial effects. Most of these genes were upregulated (*Casp1*, *Cc12*, the rodent *Il8* analogue *Cxcl1*, *Il15*, *Mmp19*) or tended towards upregulation (*ApoA1*, *Cpb2*, *Flt4*, *Fn1*, *Il18*, *Mmp9*, *Ptgis*), while *F3*, *Npy* and *Tnfrsf11b* were downregulated and *Nfkb* (also known as *Nfkb1*) tended to be downregulated.

Table 1 Circulating and hepatic HDL-linked variables in Wistar and GK rats as a function of age

Tissue	E21 fetuses		7-day-old neonates		2.5-month-old adults	
	Wistar	GK	Wistar	GK	Wistar	GK
Blood						
PON-1 activity (%)	98.4 $\pm$ 4.7 (n=5)	98.7 $\pm$ 5.8 (n=3)	100.0 $\pm$ 3.8 (n=6)	85.5 $\pm$ 1.9** (n=6)	94.8 $\pm$ 2.8 (n=3)	117.9 $\pm$ 7.3* (n=4)
LCAT activity (%)	100.0 $\pm$ 5.6 (n=4)	67.2 $\pm$ 3.6** (n=4)	100.0 $\pm$ 4.5 (n=6)	140.3 $\pm$ 9.7** (n=4)	100.0 $\pm$ 7.4 (n=3)	89.0 $\pm$ 2.4 (n=3)
Liver						
<i>Pon1</i> expression (%)	100.0 $\pm$ 64.0 (n=3)	98.1 $\pm$ 7.7 (n=3)	103.1 $\pm$ 16.9 (n=3)	61.3 $\pm$ 13.3 (n=5)	100.7 $\pm$ 7.3 (n=4)	55.1 $\pm$ 3.8** (n=4)
PON-1 activity (%)	100.0 $\pm$ 5.1 (n=3)	93.4 $\pm$ 11.8 (n=3)	100.7 $\pm$ 10.7 (n=3)	66.8 $\pm$ 0.5* (n=4)	100.0 $\pm$ 4.8 (n=7)	94.7 $\pm$ 7.5 (n=8)
<i>Lcat</i> expression (%)	100.0 $\pm$ 41.3 (n=3)	161.2 $\pm$ 12.0 (n=4)	105.5 $\pm$ 21.6 (n=4)	198.1 $\pm$ 36.8 (n=5)	101.4 $\pm$ 9.4 (n=4)	55.9 $\pm$ 9.4* (n=4)
<i>ApoA1</i> expression (%)	100.0 $\pm$ 75.0 (n=3)	505.4 $\pm$ 96.3* (n=4)	101.1 $\pm$ 10.8 (n=3)	62.3 $\pm$ 9.2* (n=5)	101.7 $\pm$ 13.1 (n=3)	127.4 $\pm$ 5.8 (n=3)

All variables were determined under fed conditions. Concerning liver gene data, hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was used as a housekeeping gene (ESM Table 1): no variation was observed between Wistar and GK samples

Values are mean $\pm$ SEM for the number (n) of rats and expressed relative to the mean corresponding value recorded within the same experiments in Wistar rats

* p <0.05, ** p <0.01 vs age-matched Wistar group, as analysed by Student's *t* test for unpaired data

Table 2 Gene expression in islets from prediabetic (7-day-old) male GK rats vs age- and sex- matched Wistar controls using three different Oligo GEArray microarrays and quantitative RT-PCR

Gene symbol, Oligo GEArray reference and name of encoded protein	Relative mRNA levels (GK vs Wistar)	Properties and roles of encoded molecules	
		First group: inflammation, microangiopathy, atherosclerosis	Second group: angiogenesis
<i>ApoA1</i> ORN-037 APOA1 or PGI ₂ -stabilising factor	3.2±0.9 [†] (n=3)	Antioxidant/anti-inflammatory/ anti-atherosclerotic	
<i>Casp1</i> ORN-015.2 Caspase-1 or IL-1-converting enzyme (ICE)	4.9±1.7 [*] (n=7)	Bioactive IL-1β and IL-18 processing/ proinflammatory	Possibly proangiogenic via bioactive IL-1β and IL-18 processing
<i>Ccl2</i> (<i>Mcp1</i>) ORN-015.2, ORN-024.2, ORN-037 CCL2 or monocyte chemoattractant protein-1 (MCP-1)	4.4±1.0 [*] (n=3)	Monocyte and neutrophil chemotactism/ proatherosclerotic	Proangiogenic
<i>Ctgfa</i> ORN-015.2 Chromogranin A	0.2±0.0 ^{****} (n=3)		Vessel protection/proangiogenic
<i>Cpb2</i> ORN-015.2 Plasma carboxypeptidase B-2 (CPB-2) or thrombin-activatable fibrinolysis inhibitor (TAFI)	1.9±0.3 [†] (n=3)	Coagulation and fibrinolysis coupling/ involvement in hypo-fibrinolysis, obesity, insulin resistance and type 2 diabetes/ modulator of inflammation	
<i>Cxcl1</i> (<i>Il8</i>) ORN-015.2, ORN-024.2, CXCL1 or chemokine GRO1/KC (murine analogue of IL-8)	3.9±0.5 ^{***} (n=3)	Monocyte and neutrophil arrest/ proatherosclerotic	Proangiogenic/endothelial cell recruitment
<i>F3</i> ORN-015.2, ORN-037 Tissue factor or factor 3 (F3)	0.4±0.1 ^{***} (n=3)	Initiator of blood coagulation/vascular smooth muscle cell (VSMC) migration and proliferation/expressed in atherosclerotic lesions	Proangiogenic/expression during normal pancreas development/ produced by islet endocrine cells
<i>Flt4</i> (<i>Legfr3</i>) ORN-015.2 Fms-related tyrosine kinase (FLT4) or VEGFR3	1.7±0.3 [†] (n=3)	Expressed in macrophage within atherosclerotic lesions	Involved in pancreatic tumour development
<i>Fnl</i> ORN-015.2 Fibronectin-1	2.3±0.5 [†] (n=3)	ECM component/interaction with oxidised LDL and macrophages/upregulated in atherosclerosis	Possibly via proangiogenesis
<i>Il15</i> ORN-015.2 IL-15	1.4±0.1 [*] (n=3)	Monocyte, macrophage and T cell recruitment/induced by VLDL/upregulated in endothelial activation and atherosclerosis/ modulation of intimal thickening	Proangiogenic
<i>Il18</i> ORN-024.2, ORN-037 IL-18	3.9±1.2 [†] (n=3)	Activated by caspase-1/macrophage production/present in atherosclerotic lesions	Dual effect: mostly proangiogenic
<i>Mmp9</i> ORN-015.2, ORN-024.2, ORN-037 MMP-9	1.7±0.3 [†] (n=3)	Collagen degradation/expressed in macrophages and granulocytes/upregulated in atherosclerosis	Dual effect: pro- or anti-angiogenic

Induced by VLDL

<i>Mmp19</i> predicted ORN-024.2 MMP-19	1.4±0.1* (n=3)	Collagen degradation/expressed in macrophages and granulocytes/upregulated in atherosclerosis	Proangiogenic (deficient proangiogenesis in <i>Mmp19</i> - knockout mice)	Expressed in normal rat pancreas development, concomitantly with islet formation, remodelling and maturation	Triggered by NEFA via toll-like receptors and cholesterol via angiotensinogen
<i>Msln</i> ORN-037 Mesothelin	0.2±0.0**** (n=3)				
<i>Nkx1</i> ORN-037 Nuclear factor-κB-1 (NFκB-1)	0.6±0.1† (n=3)	Transcription factor inducible in all cell types/ major role in coordinating innate and adaptive immunity, cellular proliferation, apoptosis and development/activated by proinflammatory cytokines, pathogen- associated molecular patterns and H ₂ O ₂	Complex involvement in angiogenesis		
<i>Npy</i> ORN-037 Neuropeptide Y (NPY)	0.1±0.0**** (n=3)	Vascular mitogen/present in atherosclerotic lesions	Proangiogenic		
<i>Nrp1</i> ORN-024.2 Neuropilin-1 (NRP-1)	0.6±0.0**** (n=3)		Co-receptor to a tyrosine-kinase receptor for VEGF/proangiogenic		
<i>Pdgfr</i> ORN-024.2 Platelet-derived growth factor-β (PDGF-β)	0.5±0.2† (n=3)		Involved in blood vessel formation and early haematopoiesis	Involved in islet development, beta cell proliferation and neogenesis	
<i>Ptgis</i> ORN-015.2 PGI ₂ synthase	1.4±0.2† (n=3)	Vasodilator/inhibitor of platelet aggregation/ inhibitor of VSMC proliferation/ anti-atherosclerotic			
<i>Tnfrsf11b</i> ORN-015.2 TNF receptor superfamily, member-11b (TNFRSF-11b) or osteoprotegerin	0.3±0.1** (n=3)	RANKL (receptor activator of NFκB ligand) decoy receptor/inhibitor of endothelial cell calcification/upregulated in atherosclerosis but anti-atherosclerotic	Proangiogenic		

Different housekeeping genes were tested and we used translation elongation factor-1a (*Ef1a*) (ESM Table 1)

For references, see ESM Table 2

Values are mean±SEM for the number (n) of determinations in distinct islet preparations, as measured by quantitative RT-PCR

ORN-015.2, Oligo GEArray Rat Endothelial Cell Biology Microarray; ORN-024.2, Oligo GEArray Rat Angiogenesis Microarray; ORN-037, Oligo GEArray Rat Cardiovascular Disease Biomarker Microarray

Student's *t* test for unpaired data: * $p<0.05$; ** $p<0.01$; *** $p<0.005$; **** $p<0.001$; † $0.05\leq p\leq 0.10$ (statistical tendency)

The second group includes angiogenesis-related genes, classified as follows: (1) downregulated proangiogenic genes: *Chga*, *F3*, *Npy*, *Nrp1*, *Pdgfβ* (also known as *Pdgfb*) and *Tnfrsf11b*; and (2) upregulated proinflammatory but also proangiogenic genes: *Casp1*, *Ccl2*, *Cxcl1*, *Il15*, *Il18*, *Fnl* and *Flt4*. The third group includes genes involved in pancreas development: *F3*, *Msln*, and *Pdgfβ* were downregulated, while *Flt4* and *Fnl* tended to be upregulated. The fourth group includes *Apoa1* and *Il15*, encoding molecules involved in lipid metabolism or being stimulated by lipids.

Next, we investigated whether the expression of endothelium-related genes is already disturbed in the fetal GK pancreas. We selected four inflammatory genes that were upregulated in neonatal GK islets (*Casp1*, *Il18*, *Il15*, *Cxcl1*) and two downregulated proangiogenic genes (*Nrp1* and *Npy*) to compare with fetal pancreas gene expression (ESM Fig. 1). All the genes selected, whether up- or downregulated in neonates, were a priori similarly expressed in fetal Wistar and GK pancreases. However, the proangiogenic gene-expression pattern appears to be reversed in fetal GK pancreas and neonatal islets.

As a 'pre-microangiopathy-like state' appears to already characterise islets of normoglycaemic/hypercholesterolaemic GK neonates, we measured the neonatal islet expression of other endothelial activation-related genes which were mostly overexpressed in islets of diabetic GK adults [6]. A comparison of 33 endothelial activation-related genes in islets of normoglycaemic GK neonates and diabetic adults is shown in ESM Fig. 2. More than 50% of the genes were similarly expressed in neonatal Wistar and GK islets. Six genes were found to be upregulated in neonatal GK islets (*Casp1*, *Ccl2*, *Cxcl1*, *Il15*, *Il18* and *Ptgis*) and seven downregulated or with a trend towards downregulation (*Cox2*, *Il6*, *Hif1α* [also known as *Hif1a*], *Nfkb*, *Nos2*, *Npy*, and *Nrp1*). By contrast, diabetic GK adults vs Wistar controls showed islet upregulation of almost all endothelial activation-related genes investigated, as well as the expected panel of inflammatory cytokine/chemokine genes (ESM Fig. 2).

In summary, GK neonates exhibit signs of islet pre-microangiopathy, altered angiogenesis and deficient pancreas development, while signs of islet inflammation, endothelial activation and microangiopathy are aggravated by post-weaning hyperglycaemia [5, 6].

Deficient islet vascularisation in GK fetuses and neonates
Altered vascularisation programming might strongly affect organ development and function [15–17] and GK rats present deficient beta cell mass from fetal life onwards [10, 11]. Rat islet endothelial proliferation is very high around birth but declines rapidly during the first week postpartum [12]. We therefore quantified pancreas and islet vascularisation using nestin and VWF labelling of

fetal proliferative and postnatal mature endothelial cells, respectively. Figure 2a–e shows that total pancreas section area, total nestin-positive pancreas area, total nestin-positive pancreas area/total pancreas section area and insular nestin-positive area were significantly decreased in GK vs Wistar E21 fetuses (by 29%, 62%, 56% and 55% respectively). In GK neonates, total VWF-positive pancreas area was 43% lower than in Wistar controls, total VWF-positive pancreas area/total pancreas section area was unchanged and insular VWF-positive area was drastically decreased (by 90%) in GK vs Wistar neonates (Fig. 2f–j). Reduction in beta cell mass was also confirmed at both ages (data not shown).

Thus, a marked deficient islet vascularisation is concomitantly observed with decreased beta cell mass in GK rats during the perinatal period.

Discussion

In this report we show that GK fetuses and neonates exhibit circulating and hepatic lipid anomalies, particularly abnormal cholesterol metabolism. In addition, neonatal GK islets exhibit a pre-microangiopathy-like pattern, associated with signs of altered angiogenesis and disturbed pancreas development. Finally, marked decreased islet vascularisation and beta cell mass are present in GK fetuses and neonates.

Dyslipidaemia from fetal life onwards in GK rats
As previously published, hyperglycaemic GK fetuses and normoglycaemic 7-day-old neonates have low basal insulinaemia [10, 11, 18]. GK neonates are normoglycaemic until weaning, because of transiently increased whole body insulin sensitivity [18]. Once GK rats are shifted to a carbohydrate-enriched diet, they exhibit chronic mild hyperglycaemia followed by insulin resistance (hyperinsulinaemia) [18].

A major new finding is that GK rat dyslipidaemia starts in the perinatal period. Increased neonatal NEFA and to a lesser extent triacylglycerol levels result mainly from perinatal GK hypoinsulinaemia. Systemic cholesterol anomalies might result from maternal–fetal GK hyperglycaemia [10, 11]: indeed, a substantial body of data provides a link between glucose and cholesterol metabolism [26, 27]. Moreover, low perinatal GK leptinaemia might also contribute to cholesterol anomalies by lowering the expression/activity of acyl-coenzyme A:cholesterol acyltransferase-1, thus reducing cholesteryl ester accumulation in macrophages and foam cell formation [28]. These perinatal data add to the knowledge on cholesterol metabolism in diabetic GK adults and their gene linkage [1].

Fig. 2 Pancreas immunochemistry for endothelial markers in Wistar and GK E21 fetuses and 7-day-old neonates. **a** Nestin immunochemical staining of fetal proliferative endothelial cells [24]; in particular in Wistar islets, note that nestin labelled peri- and intra-islet vascularisation as described elsewhere [12, 24] and numerous capillaries are also observed in the mesenchyme or near the acini. Scale bar, 50 μm . **b–e** Immunolabelled pancreas or islet area for nestin in Wistar (white columns) and GK (black columns) rats was quantified for each pancreas section and expressed relative to the corresponding pancreas section area or total islet area, respectively ($n=4$ fetuses): (**b**) pancreas section area; (**c**) nestin-positive area; (**d**) nestin-positive/pancreas section area; (**e**) insular nestin-positive area. **f** VWF immunochemical staining

of postnatal mature endothelial cells [4, 6]. Magnification $\times 250$. **g–j** Immunolabelled pancreas or islet area for VWF in Wistar (white columns) and GK (black columns) rats was quantified for each pancreas section and expressed relative to the corresponding pancreas section area or total islet area, respectively ($n=5–6$ neonates per group): (**g**) pancreas section area; (**h**) VWF-positive area; (**i**) VWF-positive/pancreas section area; (**j**) insular VWF-positive area. In pancreas sections stained for endothelial markers, the islet border is defined by a dashed line. Values are means $\pm$ SEM. * $p<0.05$ and *** $p<0.001$ and statistical tendency ($^{\dagger}0.05 \leq p \leq 0.10$) vs age-matched Wistar rats, as analysed by Student's *t* test for unpaired data

Early disturbances in HDL metabolism in GK rats GK fetuses are hyperglycaemic because of maternal diabetes [10, 11]. Hyperglycaemia induces deleterious protein glycation, as described for the predominant HDL protein APOA1 or HDL-maturation protein LCAT [29, 30]. Hyperglycaemia also induces reactive oxygen species (ROS) production, which is responsible for oxidative stress (OS), and therefore represents the pathogenic mechanism unifying diabetes and CVD [31]. Thus, in GK fetuses, glycation of LCAT might decrease its systemic activity with attempted compensation through increased hepatic mRNA levels. Similarly, glycation of APOA1 may cause the sharp in vivo increase in liver *Apoa1* expression, while high glucose concentrations in vitro reduce *Apoa1* expression in hepatocytes [27]. The absence of modification of blood and liver HDL-associated lipo-lactamase PON-1 is probably due to the very low PON-1 activity in rodents before birth. Indeed, PON-1 activity develops rapidly from birth to 21 days of age [32].

In 7-day-old normoglycaemic GK rats, the decrease in both systemic and hepatic PON-1 activities might result from prenatal PON-1 glycation, only visible after birth [32, 33]. In addition, inhibition of liver PON-1 activity is an

early biochemical change linked to increased lipid peroxidation and liver damage [34]. Notably, oxysterols increase total liver peroxides, IL-8 and CC-chemokine ligand-2 (CCL2) [35]. Free cholesterol decreases hepatic glutathione (GSH) levels, sensitises hepatocytes to TNF- α and leads to inflammation [36]. The findings of maternal/fetal hyperglycaemia and concomitant fetal hypercholesterolaemia lead us to suggest that OS is at work in perinatal GK liver. This hypothesis is reinforced by the existence of systemic low erythrocyte GSH content and elevated CCL2, CC-chemokine ligand-3 (CCL3) and CXC-chemokine ligand-1 (CXCL1) levels in GK neonates [20].

Low neonatal GK circulating HDL-cholesterol levels and high total cholesterol/HDL-cholesterol ratio might result from decreased circulating fetal LCAT activity. However, return to normoglycaemia after birth and attempts to increase liver *Lcat* mRNA levels may improve LCAT activity in GK neonates. The postnatal liver *Apoa1* down-regulation that parallels low systemic HDL levels in normoglycaemic GK neonates might reflect a delayed effect of fetal hyperglycaemia [27]. Such perinatal adaptive regulation of GK liver variables highlights an early ‘programmed’ and deleterious interaction of carbohydrates

and lipids on hepatic offspring lipid metabolism, as is observed with maladapted maternal diets [37].

In GK adults systemic signs of OS, as assessed by elevated oxidised GSH levels in erythrocytes, develop after diabetes onset [20]. However, high circulating PON-1 activity and α -tocopherol concentrations suggest that diabetic GK adults have installed systemic antioxidant defences (AODs). This may consequently return systemic GK chemokine levels to age-matched Wistar levels [6]. Similarly, normalisation of total cholesterol/HDL-cholesterol ratio in diabetic GK adults might result from stimulation of neonatal systemic GK LCAT activity. Indeed, adult GK rats overexpressed some AOD genes in liver (Lacraz, unpublished data) and overall defence involving islet antioxidant/cAMP/antiapoptotic pathway, which confers beta cell protection by decreasing ROS and apoptosis [20, 21]. ESM Table 3 shows that systemic HDL-cholesterol levels and total cholesterol/HDL-cholesterol ratio correlated negatively and positively, respectively, with pancreas/islet expression of *Casp1*, *IL18* and *IL15*. These data are in agreement with the well-recognised anti-inflammatory effects of HDL [38].

Signs of defective islet angiogenesis, development and vascularisation in GK fetuses and/or neonates A major feature of GK rats from perinatal life onwards (except surprisingly at E21) is a markedly lower body weight than Wistar controls, despite maternal/fetal hyperglycaemia [10, 11, 18]. However, low perinatal levels of trophic factors, like insulin, IGFs and leptin, might be responsible for low postnatal GK body weight [11, 39–41]. Low perinatal GK leptinaemia is associated with a low fat/body mass percentage [18]. Furthermore, a defective IGF2 and IGF1 receptor protein production in GK pancreatic rudiment as early as E13.5 has been reported [11]. Insulin, IGFs and leptin also stimulate angiogenesis, a crucial process for adapted development [39–41]. Our neonatal data highlight deficient GK islet expression of genes, such as *F3*, *Msln*, *Pdgfr β* , *Chga*, *Npy* and *Nrp1*, encoding molecules involved in pancreas development and/or angiogenesis. Lammert hypothesised that changes within the vascular system may act very early in triggering type 2 diabetes. This is based on the role of blood vessels in islet development and the vascular-related functions of most genes involved in human disease [14]. Accordingly, we show that beta cell-mass-deficient GK fetuses and neonates also exhibit marked islet vascularisation defects, as assessed by nestin and VWF immunostaining, respectively [10, 11, 24]. The concomitant downregulation of *Npy* and *Nrp1* and collapse of vascularisation in neonatal GK islets should be noted. Indeed, systemic GK glucose and alkaline phosphatase levels correlated positively with the pancreas/islet expression of both *Nrp1* and *Npy*, while NEFA levels correlated negatively with the expression of both these factors (ESM

Table 3). These data suggest a link between metabolic disturbances and angiogenesis. Adult GK rats are unable to adapt beta cell mass and vascularisation after beta cell depletion [42]. This might partly result from GK rats being unable to upregulate islet *Vegf*, in contrast to 7–12-week-old Zucker diabetic fatty (ZDF) rats [43]. The expression/signalling of angiogenic factor is also abnormal in GK myocardial microvasculature [44]. Thus, the non-obese GK rat model resembles human non-obese type 2 diabetic patients, who exhibit a genetic basis for low birthweight and deficient vascularisation [14, 45].

'Pre-microangiopathy-like' state in islets of normoglycaemic GK neonates We show here that several genes involved in inflammation and atherosclerosis are overexpressed in neonatal GK islets. Importantly this is concomitant with high islet ROS levels, elevated GSH oxidised state and altered *Gpx1*, *Gsr* and *Trx* [also known as *Txn1* or *Txn*] expression, as previously described [20]. These islet conditions might result from the proinflammatory effects of fetal hyperglycaemia and perinatal hypoinsulinaemia and dyslipidaemia, particularly proatherosclerotic hypercholesterolaemia. However, the neonatal islet inflammation/endothelial activation is markedly lower than in diabetic adult GK islets. Indeed, after weaning, GK islets are boosted by chronic hyperglycaemia and hyperlipidaemia, leading to full blown islet endothelial activation, inflammation, fibrosis and beta cell deterioration [4–6, 20]. The positive correlation between systemic insulin levels and pancreatic/islet *Cxcl1* expression (ESM Table 3) might reflect decreased anti-inflammatory effects of insulin due to emergence of insulin resistance. While fetal GK hypercholesterolaemia might trigger an in utero cholesterol-induced disease programming as in humans [3], taken together these data highlight a complex programming of metabolically induced endothelial and islet alterations. Moreover, it has been recently suggested that atherosclerosis and type 2 diabetes are inflammasome-mediated autoimmune-inflammatory diseases [46, 47]. The overexpression of genes encoding caspase-1, IL-1, IL-18, nuclear factor- κ B (NF κ B), and many inflammatory proteins in neonatal and/or adult GK rat islet physiopathology reinforces this hypothesis.

Attempts to compensate perinatal deleterious effects in islets of prediabetic GK neonates First, neonatal islets are the site of: upregulation of most genes encoding proinflammatory cytokines and chemokines with proangiogenic properties, possibly reflecting a repair process; and upregulation or trends towards upregulation of *Flt4*, which participates in the recruitment of VEGF-A-secreting macrophages involved in angiogenesis [48], *F3* and *Mmp19* [49, 50]. Second, those islets show a trend towards upregulation of genes involved in vascular protection, like those encoding prostaglandin I_2

(PGI₂) synthase (PTGIS), which synthesises the vasodilator agent PGI₂, and APOA1, also known as PGI₂-stabilising factor [51]. Intriguingly, *Apoa1* expression is assumed to occur in liver and intestine only, but has also been observed in human fetal pancreas [52]. Third, neonatal GK islets exhibit a downregulation of inflammatory NFκB-related genes, such as *Cox2*, *Il6*, *Nos2* and *Nfkb* itself [53]. The mechanism of this phenomenon deserves further investigation. At a later stage, as mentioned above, islets of adult diabetic rats partly protect themselves against OS through large adaptive AOD upregulation [20].

In conclusion, dyslipidaemia and elevated systemic/hepatic cholesterol levels are already present in hyperglycaemic GK fetuses and normoglycaemic GK neonates. This is associated with signs of neonatal islet pre-microangiopathy, altered angiogenesis and pancreas development and deficient islet vascularisation. GK rats also present various forms of defence mechanisms potentially able to limit disease progression, while deficient angiogenesis might prevent full beta cell recovery. This pathophysiological sequence may help to understand why low birthweight infants, who exhibit several similar metabolic disorders [54], are at risk of developing insulin resistance, type 2 diabetes and CVD [3, 55].

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