Glycoxidised LDL isolated from subjects with impaired glucose tolerance increases CD36 and peroxisome proliferator–activator receptor γ gene expression in macrophages

J. Graessler · J. Pietzsch · T. Westendorf · U. Julius ·
S. R. Bornstein · S. Kopprasch

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Abstract

Aims/hypothesis Glycoxidised LDL has been implicated in the pathogenesis of atherosclerosis, a major complication of diabetes. Since atherogenesis may occur at an early stage of diabetes, we investigated whether circulating LDL isolated from subjects with IGT ($n=20$) showed an increased glycooxidation status and explored the proatherogenic effects of LDL samples on macrophages.

Subjects and methods We investigated LDL modifications using GC-MS. Murine macrophages were incubated with LDL samples for 1 h, and then mRNA expression rates of the scavenger receptors CD36 and scavenger receptor class B type 1 (SCARB1, formerly known as SR-BI) and transcription factor peroxisome proliferator–activator receptor γ (PPAR γ) were quantified by real-time RT-PCR.

Results The GC-MS experiments revealed that oxidative modifications of proline, arginine, lysine and tyrosine

residues in apolipoprotein B100 were three- to fivefold higher in LDL samples from IGT subjects compared with those from NGT subjects ($n=20$). Moreover, LDL glycooxidation estimated by both N^ϵ -(carboxymethyl)lysine (CML) and N^ϵ -(carboxyethyl)lysine (CEL) residues was increased more than ninefold in LDL from IGT subjects compared with samples from NGT subjects. Compared with NGT LDL, IGT LDL elicited a significantly higher CD36 ($p<0.05$) and PPAR γ ($p<0.05$) gene expression, whereas SCARB1 mRNA expression was not affected.

Conclusions/interpretation These data suggest that IGT is associated with increased glycooxidation of circulating LDL, which might contribute to the conversion of macrophages into a proatherogenic phenotype.

Keywords Advanced glycosylation end products · CD36 · Gene expression · Glycooxidation · Humans · Impaired glucose tolerance · LDL · Macrophage · PPAR γ scavenger receptor

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J. Graessler and J. Pietzsch contributed equally to this study.

J. Graessler (✉) · T. Westendorf · U. Julius · S. R. Bornstein ·
S. Kopprasch
Department of Internal Medicine,
Division of Pathological Biochemistry,
Carl Gustav Carus Medical School, University of Technology,
Fetscherstrasse 74,
D-01307 Dresden, Germany
e-mail: Juergen.Graessler@uniklinikum-dresden.de

J. Pietzsch
Department of Radiopharmaceutical Biology,
Institute of Radiopharmacy,
Research Centre Dresden-Rossendorf,
Dresden, Germany

Abbreviations

α ASA	α -aminoadipic semialdehyde
apoB100	apolipoprotein B100
CEL	N^ϵ -(carboxyethyl)lysine
CML	N^ϵ -(carboxymethyl)lysine
ECEE	$N(O)$ -ethoxycarbonyl ethyl ester
ECEE-F ₃	$N(O)$ -ethoxycarbonyl trifluoroethyl ester
γ GSA	γ -glutamyl semialdehyde
HACA	6-hydroxy-2-aminocaproic acid
HAVA	5-hydroxy-2-aminovaleric acid
MSRI/II	macrophage scavenger receptor types I and II
MCP-1	monocyte chemoattractant protein-1
MPO	myeloperoxidase

<i>m/z</i>	mass to charge ratio
oxLDL	oxidised low-density lipoprotein
PPAR γ	peroxisome proliferator-activated receptor γ
SCARB1	scavenger receptor class B type 1

Introduction

Macrovascular disease with accelerated atherosclerosis is a common and severe complication in patients with type 2 diabetes mellitus. Recent evidence suggests that vascular complications are triggered prior to overt manifestation of disease, and may occur in conjunction with IGT. The oxidation of LDL observed in prediabetic states [1] has been identified as a crucial step in atherosclerotic lesion formation [2]. The seemingly unregulated uptake of modified LDL by monocytes/macrophages, with subsequent foam cell formation, is mediated by a variety of scavenger receptors, including macrophage scavenger receptor types I and II (MSRI/II, also known as SR-AI/II), CD36, scavenger receptor class B type 1 (SCARB1, also known as SR-BI), CD68 and lectin-type oxidised LDL receptor 1 (LOX-1). Previous studies have implicated peroxisome proliferator-activated receptor γ (PPAR γ) in the transcriptional regulation of CD36 by oxidised LDL (oxLDL) [3]. Nagy et al. [3] demonstrated that oxLDL upregulates the expression of the gene encoding PPAR γ (*PPARG*), followed by increased transcription of *CD36* and enhanced scavenger receptor-mediated cellular uptake of oxLDL via a positive feedback loop. Potential anti-atherogenic consequences of activating PPAR γ include the ability of PPAR γ agonists to inhibit expression of the genes encoding TNF α , IL-1 β , IL-6, and C–C chemokine receptor 2 [4, 5].

In addition to oxidation, LDL can also be modified by glycation processes, thus forming AGEs, such as *N* $^{\epsilon}$ -(carboxymethyl)lysine (CML) and *N* $^{\epsilon}$ -(carboxyethyl)lysine (CEL), under hyperglycaemic conditions [6]. However, the exact mechanisms of AGE generation in vivo are not fully understood. Glucose is an important AGE precursor, but lipids, reactive oxygen species and amino acids might also significantly contribute to the complex process of AGE formation [7, 8]. Previous in vitro studies revealed that both oxidised [9–11] and glycooxidised LDL [12] are able to increase *CD36* mRNA expression in macrophages. Moreover, glycation of LDL, in the absence of oxidation, gives rise to lipid accumulation in macrophages [13]. Thus, both LDL glycation and oxidation alone are sufficient for the formation of lipid-laden cells.

Since atherosclerosis starts in prediabetic states, such as IGT [1, 14], the aim of the present study was to determine whether LDL obtained from IGT subjects is modified by glycooxidation, and to investigate the potential proathero-

genic effects of the LDL samples on *CD36*, *SCARB1* and *PPARG* mRNA expression in macrophages.

Subjects and methods

Subjects Forty subjects (20 with NGT, 20 with IGT) who were at risk of developing diabetes owing to a family history of type 2 diabetes mellitus, obesity and/or hyper-/dyslipoproteinaemia were examined. Clinically overt diabetes and medication affecting glucose tolerance were exclusion criteria. All subjects consented to participate in the study, which was approved by the local ethics committee.

NGT and IGT were diagnosed based on the results of an OGTT (75 g oral glucose challenge) according to the following WHO guidelines and criteria: 2 h post-challenge plasma glucose concentration of 7.8–11.1 mmol/l for IGT, and >11.1 mmol/l for type 2 diabetes mellitus. During the OGTT, plasma glucose levels were determined at 0, 30, 60, 90 and 120 min. The baseline clinical and biochemical characteristics of the study group are given in Table 1.

LDL isolation and biochemical characterisation Immediately prior to the oral glucose challenge, plasma samples were collected in EDTA-coated tubes for LDL preparation.

Table 1 Baseline clinical and metabolic parameters of NGT and IGT subjects

	NGT (<i>n</i> =20)	IGT (<i>n</i> =20)	<i>p</i> value
Age (years)	38.8 $\pm$ 7.4	42.1 $\pm$ 5.7	
Sex (male/female)	6/14	6/14	
BMI (kg/m ²)	28.9 $\pm$ 7.7	28.2 $\pm$ 4.9	
WHR	0.88 $\pm$ 0.10	0.89 $\pm$ 0.08	
RR systolic (mmHg)	132.5 $\pm$ 17.2	132.9 $\pm$ 14.5	
RR diastolic (mmHg)	87.8 $\pm$ 10.6	88.7 $\pm$ 10.5	
Plasma glucose (mmol/l) ^a			
0 min	5.1 $\pm$ 0.5	5.7 $\pm$ 0.7	<0.01
30 min	8.6 $\pm$ 1.0	9.6 $\pm$ 1.3	<0.05
60 min	7.9 $\pm$ 1.8	10.5 $\pm$ 1.8	<0.001
90 min	6.7 $\pm$ 1.5	10.0 $\pm$ 1.5	<0.001
120 min	6.0 $\pm$ 1.0	9.0 $\pm$ 1.0	<0.001
HbA _{1c} (%)	4.8 $\pm$ 0.3	5.1 $\pm$ 0.8	
Triacylglycerol (mmol/l)	1.3 $\pm$ 0.7	1.7 $\pm$ 0.9	
Total cholesterol (mmol/l)	5.4 $\pm$ 0.8	5.4 $\pm$ 0.8	
HDL-cholesterol (mmol/l)	1.5 $\pm$ 0.4	1.4 $\pm$ 0.4	
LDL-cholesterol (mmol/l)	3.3 $\pm$ 0.9	3.3 $\pm$ 0.7	
NEFA (mmol/l)	0.54 $\pm$ 0.24	0.49 $\pm$ 0.16	
Leucocyte count (Gpt/l)	6.1 $\pm$ 1.5	5.9 $\pm$ 1.6	
C-reactive protein (mg/l)	3.3 $\pm$ 4.2	3.6 $\pm$ 4.1	

Data are means $\pm$ SD

Gpt giga-particles

^a For plasma glucose the values at various time points after oral glucose challenge are shown.

To 1.5 ml plasma, 15 µl conservation medium was added, which contained 70 g sucrose, 50 mg phenylmethylsulfonyl fluoride, 20 mg streptomycin sulfate, 20 mg DL-dithiothreitol and 20 mg sodium azide per 100 ml water. After addition of conservation medium, plasma samples were shock-frozen in liquid nitrogen and stored at -80°C until analysis. LDL (density 1.006–1.063 g/ml) was isolated by very fast ultracentrifugation, as described previously [15]. The lipid constituents of the plasma and lipoprotein fractions were measured as previously described [15]. LDL apolipoprotein B100 (apoB100) was measured by immunoelectrophoresis (Sebia, Issy-les-Moulineaux, France). The extent of LDL apoB100 modification was evaluated by sensitive GC-MS analysis of highly specific products of the following modifications of amino acid side chain residues: (1) oxidation: γ -glutamyl semialdehyde (γ GSA), α -amino adipic semialdehyde (α ASA) and 3-chlorotyrosine; and (2) glycation: CML and CEL. Of note, γ GSA and α ASA form, by reduction, the analytes 5-hydroxy-2-aminovaleric acid (HAVA) and 6-hydroxy-2-aminocaproic acid (HACA), which are suitable for the method employed [16]. LDL samples were processed in subdued light to prevent photo-oxidation. All buffers and solutions were degassed and stored under argon. Delipidation and reduction of LDL and enzymatic hydrolysis of apoB100 using non-specific protease type XIV were performed as previously described [17]. Within the 24-h hydrolysis period, no self-digestion of the protease was observed. To determine the stability and recovery of the analytes, known amounts of HAVA, HACA and CML standard substances were incubated with protease type XIV, with and without native apoB100 for 24 h. The percentage recovery, determined from three independent measurements for each experiment, was $90.2 \pm 5.4\%$ for HAVA and $92 \pm 4.1\%$ for HACA (all data are means $\pm$ SD), respectively, indicating sufficient stability of the analytes during enzymatic hydrolysis. The free amino acids from protein hydrolysates were then isolated by cation exchange chromatography using Dowex AG-50W-X4 resin (H^+ , 100–200 mesh; Bio-Rad Laboratories, Richmond, CA, USA). The purified and dried protein amino acids were derivatised either to *N*(*O*)-ethoxycarbonyl trifluoroethyl ester (ECEE- F_3) derivatives (for analysis of HAVA, HACA and 3-chlorotyrosine) or to *N*(*O*)-ethoxycarbonyl ethyl ester (ECEE) derivatives (for analysis of CML and CEL) and analysed following the GC-MS protocols described previously by this group of investigators [17]. All samples were run in triplicate. Under the mass spectrometric conditions employed (electron impact ionisation; electron energy 70 eV) the most abundant and characteristic ions of the ECEE- F_3 derivatives of HAVA (M_r 287), HACA (M_r 301) and 3-chlorotyrosine (M_r 441) were the $[\text{M}-127]^+$ ion (base peak) resulting from the loss of $^{\bullet}\text{CO}_2\text{CH}_2\text{CF}_3$, and the

$[\text{M}-89]^+{\bullet}$ ion from the loss of $\text{NH}_2\text{CO}_2\text{Et}$ from the corresponding molecular ions ($[\text{M}]^+{\bullet}$), respectively. Prominent and highly characteristic ions of the ECEE derivatives of CML (M_r 404) and CEL (M_r 418) were the $[\text{M}-73]^+$ ion resulting from the loss of $^{\bullet}\text{CO}_2\text{Et}$ and $[\text{M}-46]^+$ ion formed by ejection of EtOH from the molecular ion ($[\text{M}]^+{\bullet}$). Diagnostically useful ions for HAVA (at a mass to charge ratio $[m/z]$ 160 and m/z 198), HACA (at m/z 174 and m/z 212), 3-chlorotyrosine (at m/z 314 and m/z 352), CML (at m/z 331 and m/z 358), CEL (at m/z 345 and m/z 327), L-norleucine (at m/z 158 and m/z 196), and L-*p*-chlorophenylalanine (at m/z 226 and m/z 264) were monitored in the selected ion monitoring (SIM) mode under the control of the HP-ChemStation data system (Hewlett-Packard, Palo Alto, CA, USA) [17]. For quantification of HAVA, HACA, 3-chlorotyrosine, CML and CEL, calibration curves were obtained by plotting the peak area ratio for each characteristic oxidation marker : internal standard ion pair (HAVA, HACA, CML and CEL vs L-norleucine; 3-chlorotyrosine vs L-*p*-chlorophenylalanine) vs the mass ratio of the analytes and the internal standard, respectively. Calibration curves were analysed by unweighted least-squares linear regression analysis and were found to be linear over the range studied (2–600 nmol/l; $R^2 > 0.99$) and of good quality (intra-assay CV $< 4.5\%$, inter-assay CV $< 6.1\%$). In terms of signal reproducibility, there was $< 8\%$ variation in the peak areas being observed for the diagnostic ions used. The limit of determination of all analytes was 1 nmol/l (1 fmol per injection) by this method. By strictly using reductive conditions, the adverse formation of oxidation/glycation markers during sample processing could be inhibited. Furthermore, control experiments in the presence of aminoguanidine showed no additional formation of oxidation/glycation markers during sample processing (data not shown in detail).

Cell culture Murine macrophages (RAW 264.7 cell line; LGC Promochem, Teddington, Middlesex, UK) were grown in RPMI 1640 Medium (Biochrom, Berlin, Germany) containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere under 5% CO_2 . After reaching 90% confluence (approximately 7 days of culture), LDLs were added to macrophages in the same culture medium at a concentration of 30 µg protein/ml. In control experiments, PBS was added instead of LDL. Following the addition of lipoprotein suspensions, cells were incubated for 1 h. Before cell harvest, the supernatant fraction was removed, aliquoted and stored at -75°C for analysis of monocyte chemoattractant protein-1 (MCP-1) with the commercial Quantikine M mouse JE/MCP-1 immunoassay kit (R&D Systems, Minneapolis, MN, USA). After LDL exposure, cell viability was demonstrated by trypan blue exclusion; no differences were observed between control and LDL experiments.

RNA preparation and quantitative RT-PCR RNA preparation and real-time quantitative PCR were performed as previously described [11]. Real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. LightCycler TM-polymerase chain reaction (PCR) assays for *GAPDH* (the gene encoding glyceraldehyde-3-phosphate dehydrogenase), *CD36* and *SCARB1* were based on SYBR Green I detection (FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals). Approximately 20 ng of cDNA, 3 mmol/l of $MgCl_2$, and 1 μ mol/l of primers were used in the following cycling protocol: denaturation 600 s at 95°C, 40 cycles of 10 s at 95°C, 10 s at primer-specific temperature, and product-specific elongation time at 72°C. Because of its low copy number, the transcript level of *PPARG* was analysed by specific hybridisation probes (0.2 μ mol/l, FastStart DNA Master Hybridization Probes; Roche Molecular Biochemicals). The specificity of each of the amplified PCR products was verified by melting point analyses and subsequent agarose gel electrophoresis. Primers and hybridisation probes were purchased from Tibmolbiol (Berlin, Germany). The sequences of primers and hybridisation probes, the PCR conditions, and product length are shown in Electronic supplementary material Table 1.

The identity of each of the PCR products was confirmed by direct sequencing (ALF Express; Amersham Biosciences, Uppsala, Sweden). For normalisation of the transcript levels of *CD36*, *SCARB1* and *PPARG*, the house-keeping gene *GAPDH* was quantified in parallel.

The copy number of the *GAPDH*, *CD36*, *SCARB1* and *PPARG* mRNAs was calculated in relation to the amplification product amounts of external standards. LightCycler

capillaries coated storage-stable with amounts of 10^1 – 10^6 molecules of HPLC-calibrated mouse *CD36*, *SCARB1*, *PPARG* and *GAPDH* fragments (Roboscreen, Leipzig, Germany) were used as standards for quantification. Transcript amounts were calculated applying the second derivative maximum method of the LightCycler software 3.1.

Statistical analysis Differences between subjects with NGT and those with IGT, in terms of clinical characteristics, composition of LDL particles and effects of LDL on gene expression, were assessed by univariate ANOVA. Spearman's rank correlation coefficient (ρ) was calculated between glycoxidation parameters of LDL apoB100 and plasma glucose levels during OGTT and for the correlation analysis of MCP-1 levels in supernatant of macrophages with *PPARG* gene expression. All data were analysed using the SPSS statistical package (v. 12.0 for Windows; SPSS, Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant.

Results

Table 1 shows the clinical and biochemical characteristics of the NGT and IGT subjects. Plasma glucose levels during the OGTT differed significantly between the two groups. Those with IGT showed a tendency towards higher HbA_{1c} and triacylglycerol levels and lower HDL-cholesterol levels as compared with NGT subjects, although these differences were not statistically significant. As indicated by the blood leucocyte count and serum levels of C-reactive protein, IGT subjects did not exhibit greater systemic inflammation compared with those with NGT.

Table 2 Lipid and protein composition of LDL and oxidation/glycation characteristics of apoB100 from NGT and IGT subjects

Parameter	Control-LDL ^a (n=20)			<i>p</i> value	
		NGT-LDL (n=20)	IGT-LDL (n=20)	Control subjects vs NGT	NGT vs IGT
Triacylglycerol (%)		6.40±2.06	7.48±2.33		NS
Cholesteryl esters (%)		36.17±3.76	36.04±2.82		NS
Non-esterified cholesterol (%)		8.69±1.40	7.92±0.86		NS
Proteins (%)		28.18±3.62	29.22±3.65		NS
Phospholipids (%)		20.57±4.96	19.33±2.99		NS
HAVA ^b	0.38±0.09	0.33±0.23	1.05±0.55	NS	0.001
HACA ^c	0.03±0.02	0.16±0.14	0.76±0.41	<0.001	<0.001
3-Chlorotyrosine ^d	0.02±0.01	0.05±0.04	0.16±0.14	<0.01	<0.001
CML ^c	0.18±0.03	1.56±0.65	14.54±6.61	<0.001	<0.001
CEL ^c	0.03±0.01	0.17±0.10	1.59±0.97	<0.001	<0.001

Data are means±SD

^a For a description of control values see Table 3

^b Residues per 10,000 arginine plus proline residues

^c Residues per 10,000 lysine residues

^d Residues per 10,000 tyrosine residues

After LDL preparation, the lipid and protein constituents of the lipoprotein particles were assessed (Table 2). LDL from NGT and IGT subjects showed no significant differences in the percentage of triacylglycerol, cholesteryl ester, non-esterified cholesterol, phospholipids and protein contents. Investigation of apoB100 glycoxidation parameters, however, revealed substantial differences between LDL from NGT subjects and samples from IGT subjects. HAVA, proposed to be a specific oxidation marker of proline and arginine side chain residues, and HACA, a primary oxidation product of lysine side chain residues, were increased by threefold and fivefold, respectively, in LDL samples from IGT subjects compared with those from NGT subjects. Moreover, 3-chlorotyrosine, a specific oxidation product of myeloperoxidase (MPO)-derived hypochlorous acid, was elevated by threefold in LDL from the IGT group compared with the NGT group. Of interest, the most pronounced differences were observed in CML and CEL content, being 9.3-fold and 9.5-fold higher in IGT subjects than in NGT subjects (see Table 2). For comparison, data for a healthy control population were obtained (see Tables 2 and 3). Of note, levels of the lysine and tyrosine modifications, but not the arginine and proline modifications, in LDL from the NGT group significantly exceeded those in the LDL from the healthy control population (see Table 2).

To test whether LDL glycoxidation parameters were related to the metabolic status of NGT and IGT subjects, a correlation analysis was performed between glycoxidation markers and plasma glucose levels estimated during OGTT.

Table 3 Oxidation/glycation characteristics of apoB100 from a healthy normolipidaemic and normoglycaemic control population

Marker	mol/mol apoB100	Residues per 10,000
HAVA	0.012±0.004	0.38±0.09 ^a
HACA	0.001±0.001	0.03±0.02 ^b
3-Chlorotyrosine	0.0003±0.0001	0.02±0.01 ^c
CML	0.006±0.001	0.18±0.03 ^b
CEL	0.001±0.001	0.03±0.01 ^b

Data are means±SD

The glycoxidation marker content of all LDL samples was normalised to the content of LDL apoB100 (as mol of measured analyte per mol of apoB100) and is expressed in the results as modified residues per 10,000 residues of the corresponding parent amino acid. Mature apoB100 consists of a single polypeptide chain of 4,536 amino acids, and there is only one copy of the protein on each LDL particle. ApoB100 (M_r 516,000, without carbohydrate content) contains 148 arginine, 170 proline, 148 tyrosine and 357 lysine residues [42].

All subjects (ten men and ten women, 23–41 years old) were non-smokers and were free of renal, hepatic, haematological and thyroid abnormalities. No subject was taking antioxidants such as probucol or vitamins A and E

^a Arginine plus proline residues

^b Lysine residues

^c Tyrosine residues

Table 4 Correlation analysis of apoB100 oxidation/glycation markers before OGTT and plasma glucose levels during OGTT

	Time point (min)				
	0	30	60	90	120
CML					
ρ	0.484	0.484	0.616	0.822	0.881
p value	0.002	0.002	0.000	0.000	0.000
CEL					
ρ	0.497	0.509	0.615	0.829	0.883
p value	0.001	0.001	0.000	0.000	0.000
HAVA					
ρ	0.231	0.250	0.463	0.713	0.726
p value	0.152	0.124	0.003	0.000	0.000
HACA					
ρ	0.294	0.351	0.543	0.787	0.795
p value	0.066	0.028	0.000	0.000	0.000
3-chlorotyrosine					
ρ	0.453	0.421	0.489	0.623	0.694
p value	0.003	0.008	0.001	0.000	0.000

ρ Spearman's rank correlation coefficient

$n=40$ at each time point

As shown in Table 4, each of the apoB100 modifications was significantly positively associated with plasma glucose levels at the different time points of the OGTT. Remarkably, for all parameters the strongest association was achieved 120 min after the oral glucose challenge. Furthermore, compared with HAVA, HACA and 3-chlorotyrosine, CML and CEL showed a stronger correlation with plasma glucose profile (see Table 4).

Glycoxidative apoB100 modifications are thought to ultimately result in the formation of new epitopes that are specifically recognised by scavenger receptors [18]. After establishing that there is a higher degree of LDL glycoxidation in IGT subjects, we investigated whether these LDL samples are able to stimulate biological activities with proatherogenic properties that differed from LDL samples from NGT subjects. For this purpose, murine macrophages were exposed to isolated LDL at a concentration of 30 μ g protein/ml. After an incubation time of 1 h, macrophage *CD36*, *SCARB1* and *PPARG* mRNA expression was quantified by RT-PCR using the LightCycler instrument. *CD36* showed the highest expression of all genes tested (5.84% relative to *GAPDH*, our housekeeping gene), followed by *SCARB1* (2.08%) and *PPARG* (0.11%). The very low expression rate of *PPARG* (on average, approximately 200 cDNA molecules/1 μ l synthesised cDNA) emphasises its function as a transcriptional regulator. The necessary methodological sensitivity for *PPARG* cDNA detection was achieved using specific hybridisation probes in combination with coated LightCycler capillaries as external standards. The expression rates of *CD36*, *SCARB1*

Fig. 1 Box plots showing mRNA expression rates of *CD36* (a), *PPARG* (b) and *SCARB1* (c) normalised to *GAPDH* and PBS controls in murine macrophages after 1 h incubation with LDL obtained from NGT and IGT subjects. In these plots, horizontal lines within boxes represent median values; the upper and lower horizontal lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. * $p < 0.05$ for NGT vs IGT subjects. Data were adjusted for age, sex, WHR, BMI and fasting plasma glucose

and *PPARG* are shown in Fig. 1. To demonstrate the variations within the groups, the data are given by box plots showing medians and 10th, 25th, 75th and 90th percentiles. Most importantly, *CD36* and *PPARG* mRNA expression was significantly higher in LDL from IGT subjects than in LDL from NGT subjects, whereas *SCARB1* mRNA levels did not differ between the two groups. A significant positive correlation between *CD36* and *PPARG* expression was found both in the NGT ($\rho = 0.485$, $p < 0.05$) and IGT ($\rho = 0.705$, $p < 0.001$) groups, underlining the close regulatory associa-

tion in macrophages, as previously proposed [19]. Moreover, *PPARG* expression was significantly negatively related to MCP-1 release from macrophages when treated with LDL from IGT subjects ($\rho = 0.572$, $p = 0.013$) but not NGT subjects. No correlation was observed between mRNA expression rates for any of the analysed genes and glycoxidation parameters of isolated LDL.

Discussion

The main finding of this study is that the apoB100 of circulating LDL isolated from subjects with IGT was substantially more glycoxidised compared with that in LDL particles from NGT subjects. Furthermore, LDL isolated from IGT subjects induced a more pronounced expression of the scavenger receptor *CD36* and the transcription factor *PPARG* in murine macrophages, while *SCARB1* expression was not affected. Using a specific and sensitive GC-MS methodology, the present study revealed a three- to fivefold increase in apoB100 arginine, proline, lysine and tyrosine oxidation, and a ninefold increase in the glycoxidation markers CML and CEL in subjects with IGT compared with healthy normolipidaemic and normoglycaemic individuals. Of note, the NGT subjects used as reference group in this study should also be considered to be at risk of development of diabetes owing to a family history of type 2 diabetes mellitus, obesity and/or hyper-/dyslipoproteinaemia. This could explain the higher level of both apoB100 CML and CEL residues found in NGT subjects compared with reference values for CML obtained from healthy volunteers, for example, in the study reported by Fu et al. [20], and in a small group of healthy volunteers investigated by us. Nevertheless, the CML and CEL values in IGT subjects were remarkably higher than those in NGT subjects.

The present findings are consistent with data showing increased HAVA content in circulating LDL recovered from hypercholesterolaemic subjects who were at high atherosclerotic risk [21]. In addition, the specific amino acid oxidation products γ GSA, α ASA and 3-chlorotyrosine have been identified in LDL apoB100 in atherosclerotic lesions, thus providing additional support for the involvement of oxidatively modified LDL apoB100 amino-acid side-chain residues in the development of atherosclerosis [17].

Of interest, the apoB100 in LDL samples from IGT subjects has a markedly higher 3-chlorotyrosine content compared with that in samples from the NGT subjects. 3-Chlorotyrosine is a specific marker of oxidation by MPO, an enzyme that is abundant in activated proinflammatory cells such as polymorphonuclear leucocytes and monocytes [22]. Recently, clinical evidence has been provided for a close association between MPO levels in the circulation and

existing cardiovascular disease. Moreover, the MPO levels can be predictive of future cardiac events and outcome [23]. In the present study, blood leucocyte count and high-sensitivity C-reactive protein levels were not enhanced in IGT subjects compared with NGT subjects, suggesting the absence of acute inflammatory processes. Thus, elevated 3-chlorotyrosine levels in IGT subjects might indicate an enhanced chronic low-grade inflammatory burden that favours atherogenesis.

The origin of glycoxidised LDL in the circulation is as yet unknown. It has been widely assumed that circulating blood is not a site of LDL oxidation, since serum has strong anti-oxidative abilities [24]. It is possible that glycoxidised LDL is formed in the subendothelial space of the vessel and enters the circulation by backward diffusion from stable atherosclerotic lesions, or by release from unstable atherosclerotic plaques. To what extent hyperglycaemic conditions that are present in IGT and diabetes mellitus contribute to increased rates of LDL glycation and/or oxidation within the bloodstream *in vivo* remains to be established. In this context, it has to be considered that oxidation and glycation processes forming covalent modifications of the LDL protein and lipid moiety occur at markedly different rates under different conditions *in vitro* [25]. It is assumed that, under anabolic conditions, these different rates of formation of covalent modifications of LDL constituents may contribute in a very heterogeneous manner to steady-state levels of LDL glycoxidation markers.

The strong correlation of LDL glycoxidation markers with plasma glucose levels after oral glucose challenge in the present study indicates that temporary or sustained hyperglycaemia may be causally related to glycoxidative modification of circulating LDL.

Oxidation and glycation processes, both stand-alone and combined, result in the conversion of LDL into a form that is recognised by the scavenger receptors of macrophages, a fundamental mechanism of atherogenesis [2]. However, the nature of the LDL modifications required for cellular recognition and unregulated uptake are poorly understood.

Very recently, animal experiments using dynamic small-animal positron emission tomography showed an extremely fast and complete blood clearance of oxidatively modified LDL in rats *in vivo*, which has been explained by the concerted action of various scavenger receptors on resident macrophages and endothelial cells in liver, spleen and kidney [26]. Conversely, previous studies published by others, and our own *in vivo* data in animal models, demonstrate a significantly delayed catabolism of glycated LDL [27]. Dynamic small-animal positron emission tomography studies in rats revealed that glycated LDL had a lower affinity for LDL receptors and enhanced interactions with receptors for AGEs responsible for higher plasma retention time of glycated LDL *in vivo* (Pietzsch J,

Hoppmann S, Richter S, Haase C, unpublished results). With respect to the present data, it can be assumed that, under catabolic conditions, the different interaction of modified LDL with LDL receptors, receptors for AGEs, and the efficient scavenger apparatus may discriminate between two types of modified LDL particles: those that show fast plasma clearance ($\text{ox}_{\text{high}}/\text{glyc}_{\text{high}}\text{LDL}$; $\text{ox}_{\text{high}}/\text{glyc}_{\text{low}}\text{LDL}$); and those that show low plasma clearance ($\text{ox}_{\text{low}}/\text{glyc}_{\text{high}}\text{LDL}$; $\text{ox}_{\text{low}}/\text{glyc}_{\text{low}}\text{LDL}$). This is consistent with our findings showing circulating LDL with lower concentrations of oxidation markers but higher levels of glycoxidation markers in subjects with IGT. Furthermore, it is likely that oxidatively modified LDL in the circulation comprise particles with a still lower extent of oxidation, including the so-called minimally modified LDL, which are in part still recognised via the LDL receptor.

Considering the heterogeneity of oxidative processes of sizable physiological relevance, such as transition metal-catalysed oxidation, MPO-catalysed reactions, nitrosative or glycativ stress, additional work is needed to understand the nature of the original oxidative insult. In this respect, further investigations are necessary to provide information on threshold values of *in vivo* oxidation and glycation to discriminate the metabolic fate of modified LDL particles *in vivo*. Knowledge of such threshold values should significantly support the interpretation of concentration measurements of circulating modified LDL particles, specific products of LDL oxidation and glycation, or antibodies against modified LDL in plasma samples in various diseases, thus allowing the critical evaluation of their actual relevance as potential indicators of oxidative or glycativ stress or atherosclerotic risk [26].

Previous *in vitro* studies have shown that the two scavenger receptors CD36 and MSRI/III mediate the majority of lipid accumulation in macrophages exposed to oxLDL [28, 29]. Furthermore, several studies, although not all, have demonstrated that targeted deletion of either *MSRI* (which codes for both types of the receptor) or the *CD36* gene locus in the hyperlipidaemic mouse substantially decreases atherosclerosis [30–32]. Macrophage expression of *CD36*, but not *MSRI*, is under the strong control of the nuclear transcriptional regulator PPAR γ [3, 33]. Because of the complex effects of PPAR γ on lipid metabolism and inflammatory processes, its role in atherosclerosis remains controversial. Potential atherogenic effects of PPAR γ include the facilitated uptake of oxLDL by macrophages via enhanced *CD36* expression, while anti-atherogenic consequences of PPAR γ activation include inhibition of inflammatory cytokine expression and induction of the ABCA1 transporter protein by liver X receptor α , which promotes macrophage cholesterol efflux [34].

In the present study, glycoxidised LDL obtained from patients with IGT stimulated expression of *CD36* and

PPARG in macrophages to a significantly greater extent than LDL from NGT subjects. Together with the fact that we found a strong positive correlation between *CD36* and *PPARG* expression, these data demonstrate that in vivo-modified LDL induce a feed-forward loop, previously demonstrated in macrophages in response to incubation with in vitro oxidised LDL [3, 11]. In addition to the positive association between *CD36* and *PPARG* gene expression, we found a significant negative correlation between *PPARG* expression rates and MCP-1 release from macrophages after exposure to LDL from IGT subjects but not from NGT subjects. Since MCP-1 enhances the recruitment of monocytes into the arterial wall [35], this result points to the anti-inflammatory effect of PPAR γ activation. The consequences of PPAR γ activation may thus be both proatherogenic and anti-atherogenic. The net effect is likely to reflect a balance between local effects in the arterial wall and systemic effects on lipid metabolism and inflammation [19]. Studies in LDL receptor-deficient mice using the most prominent group of PPAR γ activators, glucose-lowering glitazones, showed an inhibition of atherosclerotic lesion formation [36, 37].

CD36 and *SCARB1* belong to the same class B family of scavenger receptors and share considerable structural homology. They share many ligands, including HDL, LDL, VLDL, oxLDL and anionic phospholipid vesicles, but differ markedly in tissue distribution, preferred binding partners and function in lipid metabolism. *SCARB1* is expressed in macrophages in human and murine atherosclerotic lesions [38, 39]. Theoretically, in macrophages, *SCARB1* may protect against atherosclerosis by stimulating cholesterol efflux and preventing foam cell formation. Alternatively, its function in the uptake of modified lipoproteins might promote foam cell formation, rendering macrophage *SCARB1* a proatherogenic factor. Previous in vitro studies have shown that oxLDL-induced changes in *SCARB1* mRNA expression may be dependent on the differentiation status of the investigated cells, with transcription upregulated in the early differentiation stage [40, 41] and downregulated in fully differentiated macrophages [11, 40]. The lack of difference in the rates of *SCARB1* expression stimulated by LDL from NGT and IGT subjects in the present study suggests that, in contrast to *CD36*, macrophage expression of *SCARB1* is less sensitive to changes in LDL glycoxidation status.

In summary, LDL obtained from subjects with IGT exhibited an increased apoB100 glycoxidative status compared with LDL from normoglycaemic subjects. Chemical LDL modifications were closely correlated with temporary hyperglycaemia after oral glucose challenge. As a possible consequence of apoB100 modification, *CD36* and *PPARG* mRNA expression in murine macrophages was stimulated to a significantly greater extent by LDL from IGT subjects than

by samples from NGT subjects, while *SCARB1* expression was not affected. Therefore, IGT is associated with early events in the pathogenesis of cardiovascular complications in diabetic patients. These data further underline the importance of early preventive treatment and strategies.

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Duality of interest None of the authors is aware of any duality of interest.

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