The novel chemokine receptor, G-protein-coupled receptor 75, is expressed by islets and is coupled to stimulation of insulin secretion and improved glucose homeostasis

Bo Liu · Zoheb Hassan · Stefan Amisten · Aileen J. King ·
James E. Bowe · Guo Cai Huang · Peter M. Jones ·
Shanta J. Persaud

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Abstract

Aims/hypothesis Chemokine (C-C motif) ligand 5 (CCL5) acts at C-C chemokine receptors (CCRs) to promote immune cell recruitment to sites of inflammation, but is also an agonist at G-protein-coupled receptor 75 (GPR75), which has very limited homology with CCRs. GPR75 is coupled to Gq to elevate intracellular calcium, so we investigated whether islets express this receptor and whether its activation by CCL5 increases beta cell calcium levels and insulin secretion.

Methods Islet CCL5 receptor mRNA expression was measured by quantitative RT-PCR and GPR75 was detected in islets by western blotting and immunohistochemistry. In some experiments GPR75 was downregulated by transient transfection with small interfering RNA. Real-time changes in intracellular calcium were determined by single-cell microfluorimetry. Dynamic insulin secretion from perfused islets was quantified by radioimmunoassay. Glucose homeostasis in lean and obese mice was determined by measuring glucose and insulin tolerance, and insulin secretion in vivo.

Results Mouse and human islets express GPR75 and its ligand CCL5. Exogenous CCL5 reversibly increased intracellular calcium in beta cells via GPR75, this phenomenon being dependent on phospholipase C activation and calcium influx. CCL5 also stimulated insulin secretion from mouse and human islets in vitro, and improved glucose tolerance in lean mice and in a mouse model of hyperglycaemia and insulin

resistance (*ob/ob*). The improvement in glucose tolerance was associated with enhanced insulin secretion in vivo, without changes in insulin sensitivity.

Conclusions/interpretation Although CCL5 is implicated in the pathogenesis of diabetes through activation of CCRs, it has beneficial effects on beta cells through GPR75 activation.

Keywords Beta cell · Chemokine (C-C motif) ligand 5 · GPR75 · Insulin secretion · Intracellular calcium · Type 2 diabetes

Abbreviations

[Ca ²⁺] _i	Intracellular calcium
CCL5	Chemokine (C-C motif) ligand 5
CCR	C-C chemokine receptor
DPP4	Dipeptidyl peptidase 4
GPCR	G-protein-coupled receptor
GPR75	G-protein-coupled receptor 75
IP ₃	Inositol 1,4,5-trisphosphate
PLC	Phospholipase C
PTX	<i>Bordetella pertussis</i> toxin
RANTES	Regulated upon activation, normal T cell expressed and presumably secreted
siRNA	Small interfering RNA

Introduction

Chemokine (C-C motif) ligand 5 (CCL5) is a 68 amino acid protein, which was formerly called Regulated upon activation, normal T cell expressed and presumably secreted (RANTES) and belongs to a family of chemotactic cytokines that play key roles in lymphocyte trafficking during inflammation [1–3]. CCL5 expression was initially thought to be T cell specific,

Bo Liu and Zoheb Hassan contributed equally to this study.

B. Liu · Z. Hassan · S. Amisten · A. J. King · J. E. Bowe ·
G. C. Huang · P. M. Jones · S. J. Persaud (✉)
Diabetes Research Group, Division of Diabetes & Nutritional
Sciences, School of Medicine, 2.9N Hodgkin Building, King's
College London, Guy's Campus, London SE1 1UL, UK
e-mail: shanta.persaud@kcl.ac.uk

but is now known to be produced by a variety of cells, including platelets, endothelial cells, smooth muscle cells and neurons [4], where it is thought to be involved in a range of biological functions. CCL5 promotes the recruitment of lymphocytes to sites of inflammation and infection through promiscuous binding to the C-C chemokine receptor (CCR)1, CCR3 and CCR5 members of the C-C motif chemokine family of G-protein-coupled receptors (GPCRs) [5]. While trafficking of immune cells is normally beneficial in reducing inflammation, in type 1 diabetes CCL5 can promote the autoimmune destruction of beta cells through lymphocyte recruitment and the secretion of inflammatory cytokines by activation of lymphocyte CCRs. This scenario is supported by the demonstration that CCL5 expression increases with the progression of diabetes in islets of NOD mice, a model of spontaneous autoimmune diabetes [6]. In addition, CCL5 is also present in islets isolated from individuals with type 1 diabetes, where it may play a role in attracting activated T cells. It is also upregulated by cytokines [7, 8] and the coxsackie B3 enterovirus [9] in islets from non-diabetic donors.

It has recently become apparent that CCL5 is also a ligand at G-protein-coupled receptor 75 (GPR75), a novel GPCR that shares only 12 to 16% sequence homology with the chemokine receptor family members [10]. GPR75 is a 540 amino acid protein with seven transmembrane domains, typical of previously characterised GPCRs [11], but its C-terminal tail is considerably longer than that of other chemokine receptors [12]; moreover, its expression has been reported to be limited to the brain, spinal cord and retinal pigment epithelium, rather than the traditional sites of chemokine receptors such as the thymus and spleen [11]. In addition, GPR75 lacks the aspartate–arginine–tyrosine motif of the transmembrane helix, which is thought to be important for CCR structure and function [12]. Furthermore, while conventional CCRs are thought to signal predominantly through the *Bordetella pertussis* toxin (PTX)-sensitive Gi family of G proteins that inhibit cAMP production [13–16], CCL5 activation of GPR75 transfected into Chinese hamster ovary cells led to phospholipase C (PLC)-mediated elevations of inositol 1,4,5-trisphosphate (IP₃) formation and intracellular calcium ([Ca²⁺]_i), both of which were inhibited by the PLC inhibitor U73122, but unaffected by PTX [10]. Consistent with this, CCL5 stimulated IP₃ formation in GPR75-transfected human embryonic kidney cells [10], confirming the Gq-dependent coupling of GPR75 activation. Treatment of GPR75-overexpressing murine hippocampal cells with CCL5 has also been shown to increase cell viability, most likely via PLC and phosphatidylinositol 3-kinase activation [10].

The exocytotic release of insulin is potentiated by incretins, activation of the parasympathetic nervous system and by numerous other secretagogues that control the secretory process by activating islet GPCRs. This stimulatory GPCR input occurs through coupling via Gs to activate adenylate cyclase

and elevate intracellular cAMP, or via Gq to stimulate PLC-mediated IP₃ and diacylglycerol accumulation. GPR75 activation is coupled to elevations of [Ca²⁺]_i, a key messenger in beta cell stimulus–response coupling, so if this novel receptor is expressed by beta cells its activation may lead to increased insulin release and improved glucose homeostasis. This study therefore examined the expression of CCL5 and GPR75 in islets of Langerhans and investigated the effects of CCL5 and GPR75 activation upon [Ca²⁺]_i and insulin secretion from mouse and human islets in vitro. The in vivo effects of CCL5 on glucose and insulin tolerance were also investigated.

Methods

Materials Culture media and supplements were purchased from Sigma-Aldrich (Poole, UK) and Invitrogen (Paisley, UK). Collagenase XI, Fura-2 AM, U73122 and anti-glucagon antibody were also provided by Sigma-Aldrich. ECL western blotting detection reagents and Rainbow molecular weight markers were from GE Healthcare (Little Chalfont, UK). RNeasy mini kits and RNase-free DNase sets were obtained from Qiagen (Manchester, UK) as were QuantiTect primers for quantitative PCR (human *CCR1*: QT00047740; *CCR3*: QT02423596; *CCR5*: QT01336601; *GPR75*: QT00201089; *GAPDH*: QT01192646; mouse *Ccr1*: QT00156058; *Ccr3*: QT00262822; *Ccr5*: QT00114569; *Gpr75*: QT02530157; *Gapdh*: QT01658692). Primers for standard PCR were from Operon Biotech (Cologne, Germany). Antibodies against somatostatin and GPR75 (ab130666) were purchased from Abcam (Cambridge, UK). The anti-insulin antibody was from Dako (Ely, UK). AlexaFluor-conjugated secondary antibodies were from Jackson ImmunoResearch (Suffolk, UK), non-interfering RNAs and small interfering RNAs (siRNAs) against GPR75 were from Thermo Scientific (Loughborough, UK). The anti-CCL5 antibody (AF478) and recombinant mouse and human CCL5 were from R&D Systems (Abingdon, UK). Accu-Chek blood glucose meters and strips were from Roche Diagnostics (Burgess Hill, UK). Ultra-sensitive mouse insulin ELISA kits were purchased from Mercodia (Uppsala, Sweden). ICR mice and *ob/ob* mice were purchased from Harlan (Bicester, UK). MIN6 beta cells were kindly provided by Junichi I. Miyazaki (University of Osaka, Osaka, Japan).

Isolation of mouse and human islets Mouse islets were isolated from male ICR mice by collagenase digestion of the exocrine pancreas [17]. Human islets were isolated from non-diabetic donors at the King's College Hospital Islet Transplantation Unit (Kings College Hospital, London, UK) with appropriate ethics approval [18]. Four separate human islet preparations were used, with a donor age range of 22 to 60 years and BMI of 20 to 37 kg/m². Islets were maintained

at 37°C in RPMI (mouse) or CMRL (human) media supplemented with fetal calf serum (10%, vol./vol.), glutamine (2 mmol/l) and penicillin-streptomycin (100 U/ml, 0.1 mg/ml).

RT-PCR RNAs extracted from MIN6 beta cells, and from mouse and human islets were reverse-transcribed into cDNAs as described previously [19]. cDNAs were then amplified by standard PCR using primers specific for mouse *Ccl5* (sense 5'-CCCTCACCATCATCCTCACT-3', antisense 5'-CCTTCGAGTGACAAACACGA-3', spanning exons 1, 2 and 3) and human *CCL5* (sense 5'-CGCTGTCATCCTCATTGCTA-3', antisense 5'-GAGCACTTGCCACTGGTGTA-3', spanning exons 1 and 2). For some analyses, quantitative PCR was performed using QuantiTect primers specific for mouse and human *GPR75*, *CCR1*, *CCR3* and *CCR5*. Relative expression of mRNAs was determined after normalisation against *GAPDH* (*Gapdh*) as an internal reference and calculated by the $2^{-\Delta\Delta C_t}$ method.

Immunohistochemistry The antibody-binding epitopes of CCL5 and GPR75 were retrieved by microwave treatment of mouse and human pancreas sections for 10 min in boiling 10 mmol/l citric acid (pH 6). Sections were immunostained overnight at 4°C with antibodies directed against CCL5, GPR75, insulin, glucagon or somatostatin (at 1:8, 1:10, 1:50, 1:50 and 1:25 dilution, respectively). Immunoreactive CCL5 and GPR75 were detected with AlexaFluor488-conjugated secondary antibodies (1:100 dilution). Islet hormones were detected with AlexaFluor594-conjugated secondary antibodies, all at 1:250 dilution. Some negative control sections were also probed with AlexaFluor secondary antibodies alone. Immunostained pancreas sections were visualised under a microscope (TE2000; Nikon, Kanagawa, Japan).

Transient transfection GPR75 was downregulated in MIN6 beta cells by transient transfection with siRNAs (150 nmol/l) directed against mouse GPR75 using previously described protocols for efficient gene knockdown [20, 21]. MIN6 beta cells transfected with non-interfering RNAs (150 nmol/l) were used as controls. The transfected cells were maintained in culture for 48 h before being collected for *Gpr75* mRNA quantification and assessment of GPR75 protein by western blotting using a rabbit anti-GPR75 antibody (1:50 dilution), with equal loading confirmed by immunoprobining with an anti- β -actin antibody (1:200 dilution). Changes in $[Ca^{2+}]_i$ in transfected cells were determined by calcium microfluorimetry.

Single cell calcium microfluorimetry Native MIN6 beta cells or MIN6 beta cells that had been transiently transfected with non-interfering RNAs or *Gpr75* siRNAs were seeded on to

acid-ethanol-washed glass coverslips before being incubated for 30 min at 37°C with the calcium fluorophore Fura-2 AM (5 μ mol/l). Cells were then perfused (1 ml/min) with a physiological salt solution containing 2 mmol/l glucose [22] in the absence or presence of recombinant mouse CCL5 (25 fmol/l to 25 nmol/l). Some of these experiments were performed in the presence of the PLC inhibitor U73122 (10 μ mol/l) or in the absence of extracellular calcium (buffers supplemented with 1 mmol/l EGTA). Real-time changes in $[Ca^{2+}]_i$ were determined by illuminating cells alternately at 340 nm and 380 nm, with the emitted light being filtered at 510 nm.

Dynamic insulin secretion The effects of CCL5 on insulin secretion from mouse and human islets were assessed using a temperature-controlled perfusion system [23, 24]. Briefly, isolated mouse or human islets were transferred to chambers containing 1 μ m pore-size nylon filters and perfused at 37°C and a flow rate of 0.5 ml/min with a physiological salt solution [22] containing 2 mmol/l or 20 mmol/l glucose in the absence or presence of recombinant mouse or human CCL5. Perifusate samples were collected at 2 min intervals for the duration of the experiments and the secreted insulin measured by radioimmunoassay [25].

Intraperitoneal glucose and insulin tolerance tests Glucose tolerance tests were performed on overnight fasted lean mice or insulin-resistant *ob/ob* mice following a single i.p. administration of glucose (2 g/kg body weight) in the absence or presence of 65 pmol recombinant mouse CCL5. Tail vein blood glucose concentrations were determined with a glucose meter. Plasma insulin following CCL5 administration in vivo was quantified using an ultra-sensitive ELISA. Insulin tolerance tests were carried out following i.p. administration of insulin (0.75 U/kg body weight). All animal procedures were approved by King's College London ethics Committee and carried out under licence, in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

Statistical analyses Numerical data are expressed as means $\pm$ SEM of multiple experiments. All statistical comparisons were made by *t* tests or ANOVA, as appropriate. Values of $p < 0.05$ were considered statistically significant.

Results

Expression and localisation of CCL5 in islets RT-PCR amplification using primers to detect mRNA encoding the chemokine CCL5 produced a product of the appropriate size (185 bp) from mouse islet cDNA, but not from MIN6 beta cell cDNA. As expected, no products were obtained when the

cDNA templates were replaced by the appropriate RNAs (Fig. 1a). Figure 1b shows that primers for human CCL5 generated a single product of 150 bp from isolated human islet cDNA and from cDNA derived from a beta cell-enriched human islet preparation [26], in which more than 97% of the cells express insulin and only 1 to 2% express glucagon and somatostatin. As with the amplifications using mouse primers, no products were obtained with the human PCR primers when cDNAs were replaced by RNAs (Fig. 1b). Sequencing of amplicons confirmed a 100% homology between predicted sequences and those of the products amplified using mouse and human CCL5 primers.

The absence of *Ccl5* mRNA expression in MIN6 beta cells suggests a non-beta cell localisation of CCL5 in mouse islets, which was confirmed by immunostaining of mouse pancreas sections with a goat anti-CCL5 antibody. Immunostaining in three individual mouse islets (Fig. 1c) shows that CCL5 immunoreactivity was confined to islet peripheral cells, suggesting non-beta cell expression, as mouse beta cells are clustered at the islet core, with alpha cells and delta cells localised towards the islet periphery. Double staining of those mouse islets with antibodies against islet hormones revealed that CCL5 co-localised with glucagon, but not with insulin or somatostatin. In human islets the expression profile of CCL5 was wider, with double staining of human pancreas sections with the CCL5 antibody and antibodies against insulin, glucagon and somatostatin indicating that some human alpha and beta, but not delta cells express CCL5 (Fig. 1d).

Expression and localisation of GPR75 in islets CCL5 activates conventional CCRs as well as GPR75, so quantitative RT-PCR was performed (1) to determine which CCL5 receptor mRNAs are expressed by mouse and human islets and (2) to quantify their relative abundance. *Gpr75* was the most abundant CCL5 receptor mRNA in mouse (Fig. 2a) and human (Fig. 2b) islets, with *CCR1*, *CCR3* and *CCR5* mRNAs being expressed at significantly lower levels or being undetectable. *Gpr75* mRNA was also present in MIN6 beta cells, but *Ccr1*, *Ccr3* and *Ccr5* mRNAs were not detectable. Parallel amplifications using cDNAs prepared from mouse acinar cells revealed that the only CCL5 receptor mRNA identified was that encoding CCR3, albeit at levels too low to quantify. No products were obtained in any of the reactions when RNA rather than cDNA was used.

Immunohistochemical analysis of wax-embedded pancreas sections was conducted to examine GPR75 cellular localisation within islets. GPR75 immunoreactive cells were dispersed throughout mouse (Fig. 2c) and human (Fig. 2d) islets. Immunostaining of consecutive pancreas sections with insulin, glucagon and somatostatin antibodies indicated that GPR75 was present in alpha and beta cells, but the lack of co-staining with the somatostatin antibody suggests that delta cells do not express this receptor. Some immunoreactive cells were also observed in the exocrine pancreas, but as immunopositive acinar cells were also present in the absence of primary antibody (data not shown), this probably reflects non-specific binding of the secondary antibody.

Fig. 1 Expression and localisation of CCL5 in mouse and human pancreas. Products of RT-PCR amplification using mouse (m) *Ccl5* (a) and human (h) *CCL5* (b) primers. Wax-embedded sections of mouse (c) and

human (d) pancreas showing expression of CCL5 (green), and insulin, glucagon and somatostatin (red), with co-localisation shown in yellow. Each image is representative of four to seven replicates

Fig. 2 Expression of CCL5 receptors in mouse and human islets. Quantification of CCL5 receptor mRNA in mouse (a) and human (b) islets, expressed as a percentage of *GAPDH* mRNA levels in the same samples; values are means $\pm$ SEM; $n=3$, $**p<0.01$ vs GPR75 in the corresponding species. Wax-embedded sections of mouse (c) and human (d) pancreas showing expression of GPR75 (green), and insulin, glucagon and somatostatin (red), with co-localisation shown in yellow. Each image is representative of five to seven replicates

CCL5 increases $[Ca^{2+}]_i$ in beta cells via activation of *GPR75* Having established that *GPR75* is the major CCL5 receptor expressed by islets, we investigated the role of CCL5 in regulating beta cell function. CCL5 stimulates $[Ca^{2+}]_i$ mobilisation in embryonic dorsal root ganglia cells [27], while kidney cells transfected with a *GPR75* plasmid show Gq-dependent elevations of $[Ca^{2+}]_i$ in response to CCL5 [10]. We therefore examined the ability of CCL5 to stimulate increases in $[Ca^{2+}]_i$ in Fura-2-loaded MIN6 beta cells. As seen in Fig. 3a, in the presence of 2 mmol/l glucose, mouse recombinant CCL5 (0.25, 2.5 and 25 nmol/l) produced rapid and reversible increases in $[Ca^{2+}]_i$ that were not concentration-dependent. The beta cells then responded to the K_{ATP} channel blocker, tolbutamide (100 μ mol/l) by producing a reversible increase in $[Ca^{2+}]_i$, indicating that beta cells were still able to maintain a membrane potential after exposure to CCL5. When beta cells were exposed to lower concentrations of CCL5, as little as 25 fmol/l induced significant elevations of $[Ca^{2+}]_i$, with concentration-dependent effects observed up to 25 pmol/l CCL5 (Fig. 3b). The requirement for PLC activation and extracellular Ca^{2+} influx in the CCL5-induced increases in beta cell $[Ca^{2+}]_i$ was determined by recordings from Fura-2-loaded MIN6 beta cells in the presence of the PLC inhibitor U73122 and in the absence of extracellular calcium, using buffers supplemented with 1 mmol/l EGTA. The response to

CCL5 (Fig. 3c) was significantly reduced in the presence of U73122 and abolished in the absence of external calcium, indicating that the elevations of calcium occur via PLC activation and Ca^{2+} influx.

In some experiments MIN6 beta cells were exposed for 48 h to *GPR75*-targeted siRNAs, which led to significant reductions of *Gpr75* mRNA expression (Fig. 3d). Western blotting (Fig. 3e) indicated that *GPR75* protein abundance in siRNA-treated cells was also greatly reduced after 48 h. In those cells in which *GPR75* had been downregulated, the stimulatory effect of all concentrations of CCL5 on $[Ca^{2+}]_i$ was absent (Fig. 3g), although it was seen in MIN6 beta cells that had been transiently transfected with non-interfering RNAs (Fig. 3f), with similar response profiles to those obtained in native MIN6 beta cells (Fig. 3a). Small and transient fluctuations of fluorescence after withdrawal of CCL5 at 0.25 and 2.5 nmol/l, but not 25 nmol/l were observed in non-interfering RNA- and *Gpr75* siRNA-transfected cells. Cells treated with siRNAs, as well as those exposed to non-interfering RNAs, showed robust responses to tolbutamide, confirming the viability of beta cells after exposure to siRNAs.

CCL5 increases insulin secretion in vitro The effect of exogenous CCL5 on dynamic insulin secretion was examined by

Fig. 3 CCL5 increases $[Ca^{2+}]_i$ in beta cells via activation of GPR75. Stimulation of intracellular Ca^{2+} by CCL5 in non-transfected MIN6 beta cells (a–c) or MIN6 cells that had been transiently transfected with 150 nmol/l non-interfering RNA (f) or with 150 nmol/l siRNA designed to downregulate *Gpr75* (g). (a, f, g) Responses to 100 μ mol/l tolbutamide (Tolb). (c) MIN6 cells were perfused with standard medium (continuous line), medium supplemented with 10 μ mol/l U73122 (dashed line), or without Ca^{2+} and supplemented with 1 mmol/l EGTA (dotted line). Calcium data are expressed as means $\pm$ SEM 340/380 fluorescence ratios (a, c, f, g) or as the peak amplitude response (b); $n=34$ –37 cells, three experiments. Quantification of *Gpr75* mRNA (d) and protein (e) after exposure of MIN6 beta cells to non-interfering RNAs (control) or siRNAs, with (e) β -actin expression in the same samples. Data for mRNA quantification (d) are mean $\pm$ SEM, $n=3$, $*p<0.05$. The blot (e) is representative of three separate experiments

perfusing mouse and human islets in a temperature-controlled apparatus. Figure 4a demonstrates that recombinant mouse CCL5 (20 nmol/l) induced a small, sustained increase in insulin secretion at 2 mmol/l glucose (2.1 ± 0.6 -fold peak stimulation, $p<0.05$). These islets were then able to mount a significant ($p<0.01$) increase in insulin secretion in response to 20 mmol/l glucose, indicating that the islets were metabolically active and exposure to CCL5 had not compromised their insulin secretory function. In parallel experiments, increasing the glucose concentration from 2 to 20 mmol/l initiated a biphasic secretory response from mouse islets (Fig. 4b), while administration of mouse CCL5 in the continued presence of 20 mmol/l glucose induced a further increase in secretion over the stable second phase of glucose-stimulated

insulin secretion. This potentiation of insulin secretion by CCL5 was rapid in onset, but not readily reversible, as the response was maintained for 20 min after removal of CCL5.

In similar experiments with isolated human islets, recombinant human CCL5 (10 nmol/l) caused a transient, yet pronounced 5.4 ± 0.7 -fold increase in insulin secretion at 2 mmol/l glucose, with islets responding to a subsequent exposure to 20 mmol/l glucose with a typical biphasic secretory response (Fig. 5a). Figure 5b shows that CCL5 also potentiated glucose-induced insulin release from human islets, but in contrast to the secretory profile obtained with mouse islets (Fig. 4b), the potentiation of insulin secretion from human islets by CCL5 was transient and insulin secretion returned to

Fig. 4 CCL5 stimulates insulin secretion from mouse islets. Isolated mouse islets were perfused with buffers supplemented with 20 nmol/l recombinant mouse CCL5 (black circles) for the periods shown at 2 mmol/l (a) and 20 mmol/l (b) glucose. In all experiments islets were initially perfused with a buffer containing 2 mmol/l glucose. (b) Islets were also exposed to 20 mmol/l glucose alone for 60 min (white circles). Data, representative of three separate experiments, are percentages of insulin secretion at 2 mmol/l glucose and are expressed as means $\pm$ SEM, $n=4$

that seen in the 20 mmol/l glucose plateau during the period of exposure to CCL5.

CCL5 improves glucose tolerance in vivo Lean ICR mice and insulin-resistant *ob/ob* mice were fasted overnight before

being subjected to glucose tolerance tests following a single intraperitoneal administration of glucose (2 g/kg) in the absence or presence of 65 pmol mouse CCL5. This dose of CCL5 was selected to replicate a circulating CCL5 concentration of approximately 30 to 40 nmol/l, assuming a mouse blood volume of 60 μ l per g body weight. We chose a slightly higher concentration for in vivo studies as the N-terminal dipeptide of CCL5 is cleaved by dipeptidyl peptidase 4 (DPP4) [28, 29], potentially reducing levels of bio-available CCL5 in vivo. There were no significant differences ($p>0.2$) in the weights of the control mice or those treated with CCL5 (Fig. 6a), or in the fasting glucose concentrations (Fig. 6b), although, as expected, the *ob/ob* mice were significantly heavier and had higher fasting plasma glucose concentrations than the lean mice ($p<0.001$). CCL5 improved glucose tolerance in lean (Fig. 6c) and obese (Fig. 6d) mice, with reductions in average blood glucose concentrations seen as early as 15 min after administration ($p<0.05$) and enhanced glucose clearance being sustained for at least 60 min after CCL5 administration. Calculation of the AUCs for the glucose tolerance tests confirmed that CCL5 reduced blood glucose levels in lean and obese mice ($p<0.05$ and $p=0.08$ respectively). In some glucose tolerance tests with lean mice, blood was retrieved 30 min after CCL5 administration for quantification of plasma insulin levels. From Fig. 6g it can be seen that the glucose-induced elevation of insulin was enhanced by CCL5, although this was not statistically significant. Insulin tolerance tests indicated that administration of 65 pmol CCL5 had no significant effect on insulin sensitivity (Fig. 6h).

Discussion

CCL5 was originally characterised as a T cell-specific protein [30] so most earlier studies focused on its pro-inflammatory

Fig. 5 CCL5 stimulates insulin secretion from human islets. Isolated human islets were perfused with buffers supplemented with 10 nmol/l recombinant human CCL5 at 2 mmol/l (a) and 20 mmol/l (b) glucose. In all experiments islets were initially perfused with a buffer containing

2 mmol/l glucose. Data, representative of three separate experiments, are percentages of insulin secretion at 2 mmol/l glucose and are expressed as means $\pm$ SEM, $n=4$

Fig. 6 CCL5 improves glucose tolerance in vivo. Body weight (**a**) and fasting blood glucose concentrations (**b**) in lean and *ob/ob* mice administered 2 g/kg glucose alone (black bars) or 65 pmol CCL5 and 2 g/kg glucose (white bars). Blood glucose concentrations in lean (**c**) and *ob/ob* (**d**) mice after an i.p. glucose challenge in the absence (circles) or presence (diamonds) of CCL5. AUCs were calculated from the glucose tolerance data for lean (**e**) and *ob/ob* (**f**) mice. (**g**) Increase in plasma

insulin in response to i.p. delivery of glucose alone (control) or glucose in the presence of CCL5. (**h**) Blood glucose concentrations in lean mice after an i.p. insulin challenge in the absence (circles) or presence (diamonds) of CCL5. Data are means $\pm$ SEM of repeated measures, $n=4-6$, * $p<0.05$ vs average blood glucose concentrations in mice after an i.p. glucose challenge in the absence of CCL5 and *** $p<0.001$ vs corresponding conditions in lean mice

role. However, here we present evidence of an alternative role for CCL5, namely to influence normal beta cell function via GPR75 activation. *Ccl5* mRNA and protein have been identified in islets from non-diabetic BALB/c [6] and C57BL/6 mice [31], and we have confirmed expression of this chemokine in islets isolated from ICR mice and from healthy human donors. We found that CCL5 was constitutively expressed by mouse and human islet cells, although cellular localisation varied between the two species, with CCL5 being expressed primarily by alpha cells in mouse islets, while being detected in alpha and in beta cells in human islets.

The expression of CCL5 receptors by healthy islets has not been investigated previously. In the current study, the quantification of *CCR1*, *CCR3* and *CCR5* mRNA in non-diabetic human and mouse islets indicates that they were either undetectable or present at levels $<10\%$ of that of the novel CCL5 receptor, GPR75. These low levels of CCR chemokine receptors suggest that they are unlikely to have a major function in normal islet physiology, but do support the notion that endogenous or circulating CCL5 regulates islet function in a non-immune environment through activation of GPR75.

The identification of GPR75 as the most abundant CCL5 receptor in islets, its localisation to beta cells and previous reports that this receptor couples via Gq to elevate $[Ca^{2+}]_i$ [10]

led us to investigate whether similar signal transduction occurred in beta cells. Determining the optimal exogenous CCL5 concentration needed to identify whether this chemokine acts via GPR75 to regulate beta cell signalling is challenging. In studies investigating the effects of CCL5 in GPR75-transfected Human embryonic kidney cells and Chinese hamster ovary cells, the EC_{50} for effects on IP_3 generation and Ca^{2+} mobilisation ranged from 0.1 to 0.3 nmol/l [10], while 1 nmol/l CCL5 has been reported to significantly elevate $[Ca^{2+}]_i$ in spinal cord synaptosomes [32]. In addition, the circulating CCL5 concentration in healthy humans is 5.8 to 7.8 nmol/l [33, 34], yet as CCL5 is expressed by islet cells, its local concentration may be higher than that reaching islets via the circulation. We therefore examined the effects of CCL5 on changes in $[Ca^{2+}]_i$ in MIN6 beta cells over the range 0.25 to 25 nmol/l. In these experiments, CCL5 at concentrations as low as 0.25 nmol/l caused rapid and reversible increases in $[Ca^{2+}]_i$, with similar stimulatory effects being observed in the presence of 2.5 and 25 nmol/l CCL5. We therefore used lower concentrations of CCL5 and found that concentration-dependent elevations of $[Ca^{2+}]_i$ were obtained using 0.025 to 25 pmol/l, suggesting a high sensitivity of beta cells to exogenous CCL5. The PLC inhibitor U73122 reduced the CCL5-induced elevation of $[Ca^{2+}]_i$, suggesting signalling via Gq-

coupled GPR75. A calcium influx component was also identified, since the stimulatory effects of CCL5 on $[Ca^{2+}]_i$ were abolished in the absence of extracellular Ca^{2+} . The involvement of GPR75 in CCL5-stimulated increases of $[Ca^{2+}]_i$ was confirmed by the loss of stimulatory effects in beta cells in which GPR75 had been downregulated by exposure to *Gpr75* siRNAs. These observations, together with the low abundance of CCRs in islets, are consistent with the notion that CCL5 activation of beta cell GPR75 receptors stimulates Gq-mediated IP_3 production and Ca^{2+} mobilisation, as occurs in GPR75-transfected cells [10].

Elevations of $[Ca^{2+}]_i$ play key roles in beta cell stimulus–secretion coupling [35], so perfusions were carried out to identify whether CCL5 stimulated insulin secretion. These dynamic secretion experiments revealed that stimulatory effects of CCL5 were observed at sub-stimulatory (2 mmol/l) and maximal stimulatory (20 mmol/l) glucose concentrations in mouse and human islets, but secretory profiles were species-specific. Thus, CCL5 induced sustained elevations of insulin release from mouse islets that were reversible at 2 mmol/l glucose, but still evident upon CCL5 withdrawal at 20 mmol/l glucose. In contrast, in human islets the rapid responses to CCL5 were not sustained for the duration of exposure to the agonist, with only short-term elevations of insulin release, indicating that CCL5-stimulated secretory profiles differ between species.

Agents that stimulate insulin secretion in vivo can reduce hyperglycaemia by promoting glucose uptake and storage, so the effects of in vivo administration of CCL5 on glucose and insulin tolerance were examined in mice. CCL5 is a substrate for DPP4 [28, 29]. This protease is known to rapidly inactivate the insulin secretagogue glucagon-like peptide 1, and indeed, DPP4 inhibitors are used clinically in the treatment of type 2 diabetes to maintain glucagon-like peptide 1 levels in vivo [36, 37]. It has been reported that DPP4-mediated cleavage of the N-terminal serine and proline in CCL5 affects its binding affinity to CCR1, -3 and -5 [28, 29], but it is not known whether cleavage of CCL5 reduces its biological activity or affects its interaction with GPR75. Our in vivo experiments were carried out by administering glucose solutions supplemented with CCL5, rather than pre-administering CCL5; this was done to limit CCL5 degradation and possible changes in its receptor-binding characteristics. We also used CCL5 at a dose of 65 pmol per mouse to achieve higher concentrations in vivo than those used in vitro; our aim was to counteract the reduced availability caused by DPP4-induced cleavage in vivo. Our results indicate that CCL5 improved glucose tolerance in lean and insulin-resistant *ob/ob* mice, and that it also increased insulin secretion in vivo, without having any effects on insulin sensitivity.

In conclusion, we have shown here that islet cells express the chemokine CCL5 and its recently identified receptor GPR75, while mRNAs encoding the conventional chemokine

receptors CCR1, -3 and -5 are undetectable or expressed at very low levels in normal islets. Our results also indicate that exogenous administration of CCL5 increases beta cell $[Ca^{2+}]_i$ via GPR75 and stimulates insulin secretion in vitro and in vivo; it also improves glucose tolerance in vivo.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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