IL-1 antagonism reduces hyperglycemia and tissue inflammation in the type 2 diabetic GK rat

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Abstract

Recent studies suggest an inflammatory process, characterized by local cytokine/chemokine production and immune cell infiltration, regulates islet dysfunction and insulin resistance in type 2 diabetes. However, the factor initiating this inflammatory response is not known. Here, we characterized tissue inflammation in the type 2 diabetic GK rat with a focus on the pancreatic islet and investigated a role for IL-1. GK rat islets, previously characterized by increased macrophage infiltration, displayed increased expression of several inflammatory markers including IL-1beta. In the periphery, increased expression of IL-1beta was observed primarily in the liver. Specific blockade of IL-1 activity by the IL-1 receptor antagonist (IL-1Ra) reduced the release of inflammatory cytokines/chemokines from GK islets in vitro and from mouse islets exposed to metabolic stress. Islets from mice deficient in IL-1beta or MyD88 challenged with glucose and palmitate in vitro also produced significantly less IL-6 and chemokines. In vivo, treatment of GK rats with IL-1Ra decreased hyperglycemia, reduced the proinsulin/insulin ratio, and improved insulin sensitivity. In addition, islet-derived proinflammatory cytokines/chemokines (IL-1beta, IL-6, TNFalpha, KC, MCP-1, and MIP-1alpha) and islet CD68(+), MHC II(+), and CD53(+) immune cell infiltration were reduced by IL-1Ra treatment. Treated GK rats also exhibited fewer markers of inflammation in the liver. We conclude that elevated islet IL-1beta activity in the GK rat promotes cytokine and chemokine expression, leading to the recruitment of innate immune cells. Rather than being directly cytotoxic, IL-1beta may drive tissue inflammation that impacts on both beta cell functional mass and insulin sensitivity in type 2 diabetes.
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Nonstandard abbreviations used: Interleukin-1 receptor antagonist (IL-1Ra), homeostatic model assessment for insulin resistance (HOMA-IR).

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ABSTRACT
Recent studies suggest an inflammatory process, characterized by local cytokine/chemokine production and immune cell infiltration, regulates islet dysfunction as well as in insulin resistance in type 2 diabetes. However, the factor initiating this inflammatory response is not known. Here, we characterized tissue inflammation in the type 2 diabetic GK rat with a focus on the pancreatic islet, and investigated a role for IL-1. GK rat islets, previously characterized by
increased macrophage infiltration, displayed increased expression of several inflammatory markers including IL-1β. In the periphery, increased expression of IL-1β was observed primarily in the liver. Specific blockade of IL-1 activity by the IL-1 receptor antagonist (IL-1Ra) reduced the release of inflammatory cytokines/chemokines from GK islets *in vitro*, and from mouse islets exposed to metabolic stress. Islets from mice deficient in IL-1β or MyD88 challenged with glucose and palmitate *in vitro* also produced significantly less IL-6 and chemokines. *In vivo*, treatment of GK rats with IL-1Ra decreased hyperglycemia, reduced the proinsulin/insulin ratio, and improved insulin sensitivity. In addition, islet-derived proinflammatory cytokines/chemokines (IL-1β, IL-6, TNFα, KC, MCP-1, MIP-1α) and islet CD68⁺, MHC II⁺, and CD53⁺ immune cell infiltration were reduced by IL-1Ra treatment. Treated GK rats also exhibited fewer markers of inflammation in the liver. We conclude that elevated islet IL-1β activity in the GK rat promotes cytokine and chemokine expression, leading to the recruitment of innate immune cells. Rather than being directly cytotoxic, IL-1β may drive tissue inflammation that impacts on both β-cell functional mass and insulin sensitivity in type 2 diabetes.

\section*{INTRODUCTION}

Obesity, insulin resistance, and type 2 diabetes are associated with chronic activation of the innate immune system (1-3). Indeed, data suggest the presence of an inflammatory phenotype in both pancreatic islets and insulin target tissues in animal models and human type 2 diabetes (1-3). With respect to
the pancreatic islet, laser-captured β-cells from patients with type 2 diabetes compared to non-diabetic controls have elevated levels of IL-1β (4) and various chemokines (5). Further, islets from patients with type 2 diabetes are infiltrated with macrophages, and human islets exposed to metabolic stress (elevated glucose and palmitate) release increased levels of cytokines and chemokines (6). Finally, treatment of type 2 diabetes patients with the IL-1 receptor antagonist (IL-1Ra (7, 8)) reduced hyperglycemia and improved β-cell function (9). Interleukin-1 mediated effects in both type 1 and type 2 diabetes have primarily focused on the direct cytotoxic effect of this cytokine (10, 11). However, whether IL-1 regulates other islet-derived cytokines/chemokines, and subsequent immune cell attraction in type 2 diabetes or animal models of this disease is unknown. Furthermore, the contribution of IL-1 to insulin resistance and peripheral tissue inflammation has been neglected. Thus, it remains to be determined whether elevated IL-1 activity contributes to the overall inflammatory tissue profile in type 2 diabetes.

With respect to animal models of type 2 diabetes, data suggest the presence of an islet inflammatory phenotype in the high-fat fed B6 mouse and in the GK rat (Paris colony) (6, 12). The GK rat is a spontaneous, non-obese model of type 2 diabetes originally established by inbreeding Wistar rats selected at the upper limit of normal glucose tolerance (13). GK animals have decreased β-cell mass during fetal development, however mild hyperglycemia only develops post-weaning at 4 weeks of age (14). Post-weaning, GK animals present with impaired β-cell glucose-stimulated insulin secretion and increased hepatic glucose production, while muscle and adipose tissue insulin resistance develops
at 2 months of age or later (15-17). Thus, similar to islets from human type 2 diabetes, GK rat islets are characterized by impaired insulin secretory function, increased macrophage infiltration, and fibrosis (12, 14, 18, 19).

In the present study, we characterized tissue inflammation in the type 2 diabetic GK rat with a focus on the pancreatic islet, and investigated a role for IL-1.

RESULTS

Characterization of sera from male Wistar control and type 2 diabetic GK rats at 2-months of age revealed increased fed levels of glucose, insulin, proinsulin, proinsulin/insulin ratio, leptin, and free fatty acids, in addition to increased alkaline phosphatase activity in the GK rat (SI Table 1). Circulating IL-6, chemokine KC, MCP-1, and MIP-1α levels were not significantly different between Wistar and GK rats at this age. Thus, as supported by previously published data (14) and both an increased proinsulin/insulin ratio and an increased HOMA-IR value (SI Table 1), 2-month-old GK rats display both β-cell dysfunction and insulin resistance compared to Wistar controls.

Characterization of islet and peripheral tissue inflammation in the GK rat.
We have previously published that 2-month-old GK rat islets are infiltrated with macrophages relative to Wistar controls (6, 12). Here, we went on to characterize GK rat islet inflammation in 2-month old males further with a focus on pro-inflammatory cytokines and chemokines. Indeed, caspase-1 and IL-1β mRNA were increased 5- and 16-fold respectively in isolated GK islets versus Wistar
control islets, suggesting the presence of active IL-1β (Figure 1A). Overall, there was a striking mRNA upregulation of numerous pro-inflammatory cytokines (IL-6, TNFα) and chemokines (KC, MCP-1, MIP-1α) in GK islets. In line with this, MyD88 and NFκB p65 (NFκB) expression were also increased approximately 2-fold, together with increased Toll-like receptor (TLR) 2 and 4 expression in GK islets (Figure 1A). Prohormone convertase 1 (PC1) and 2 (PC2), enzymes involved in insulin processing, were also slightly increased in GK rat islets (Figure 1A). To evaluate whether increased islet cytokine/chemokine mRNA expression translated to increased protein production, we measured the release of IL-1β, IL-6, TNFα, KC, MCP-1 and MIP-1α from GK islets ex vivo compared to age-matched Wistar control islets. Interleukin-1β and TNFα protein levels were below the limit of detection by the assay. However, the release IL-6, KC, MCP-1 and MIP-1α was higher in conditioned media from GK islets compared to Wistar islets (Figure 1B). Since we were unable to measure IL-1β protein, which is not surprising given the difficulty in measuring this cytokine in the presence of serum (20), the use of IL-1Ra in vitro was used to prove the presence of biologically active IL-1 in GK islets (Figure 2 and below). Overall, GK islets from 2-month-old animals are characterized by an inflammatory profile relative to age-matched Wistar control islets.

Interestingly, insulin sensitive tissues were not characterized by as strong inflammation in the 2-month-old male GK rat (Figure 1C). Only the liver displayed increased IL-1β and TNFα mRNA in the GK rat, while the macrophage marker CD68 was increased in liver and epididymal adipose tissue (Figure 1C). GK and
Wistar quadriceps skeletal muscle displayed no differences in the inflammatory markers assessed (Figure 1C).

**IL-1 regulates GK islet and metabolic stress-induced islet cytokine/chemokine release.** Interleukin-1β is known to regulate the expression of numerous cytokines and chemokines (9, 20). We reduced IL-1 activity in pancreatic islets *in vitro* with the IL-1 receptor antagonist (IL-1Ra). IL-1Ra partially blocked the release of IL-6, KC, MCP-1 and MIP-1α between 35-50 % from GK islets with no significant effect on Wistar islets (Figure 2). Thus, islet IL-1 activity contributes to the overall increased cytokine and chemokine release observed in the type 2 diabetic GK islets.

Similar to the GK rat, we have previously shown that chemokine KC and IL-6 are increasingly produced by islets from high fat diet (HFD) fed mice or islets exposed to metabolic stress (elevated glucose and palmitate) (6). Since IL-1β signaling is transduced via the IL-1 receptor in a MyD88-dependent manner (21, 22), we investigated the effect of metabolic stress on islet cytokine/chemokine release in MyD88 +/+, +/-, and -/- mouse islets. As seen in SI Figure 1A, elevated palmitate alone (0.5 mM), or in combination with high glucose (33 mM), stimulated islet chemokine KC and IL-6 release in a MyD88-dependent manner. Further, IL-1β stimulated release of these two factors was also MyD88-dependent (SI Figure 1B). To prove that IL-1 is involved in this inflammatory response to metabolic stress and that this is not just due to a developmental defect in the MyD88 -/- islets, we treated wild type islets with increased levels of glucose and palmitate alone or in combination, in the absence or presence of IL-
1Ra (SI Figure 1C). Indeed, IL-1Ra was able to inhibit both palmitate and palmitate plus glucose stimulated KC release, while also inhibiting IL-6 release under the latter conditions. Finally, to investigate whether these effects of IL-1Ra were due to inhibition of IL-1α or IL-1β, we performed experiments on IL-1β -/- mouse islets. While IL-1Ra was able to inhibit metabolic stress-induced KC and IL-6 release from wild type islets (B6) over 50%, IL-1Ra was ineffective in IL-1β -/- mouse islets (SI Figure 1D). Furthermore, IL-1β -/- mouse islets released significantly less chemokine KC and IL-6 in response to metabolic stress (SI Figure 1D). Therefore, similar to GK islets, metabolic stress induced islet chemokine KC and IL-6 release is partially IL-1-dependent; specifically, metabolic stress acts via Myd88 and IL-1β to induce islet cytokine and chemokine release. Thus, using two *in vitro* models, we have shown that IL-1 contributes to islet release of cytokines and chemokines.

**IL-1Ra treatment reduces GK rat hyperglycemia.** To investigate if IL-1Ra treatment could prevent islet inflammation *in vivo*, we treated 1-month-old GK rats with human recombinant IL-1Ra via both mini-osmotic pumps and by daily s.c. injections. GK animals present with mild hyperglycemia post- weaning (1-month of age) (23), and therefore pumps were implanted 2-3 days following weaning (SI Figure 2). We initially implanted mini-osmotic pumps to release IL-1Ra or saline (sham) continuously over time (on average 6.75 mg/kg/day of IL-1Ra) for 4 weeks. Basal fed plasma glucose (7.9 ± 0.1 mM sham (n=6), 7.9 ± 0.2 mM IL-1Ra (n=7)) and body weights were consistent between groups (42.5 ± 1.6 g sham (n=6), 39.9 ± 1.7 g IL-1Ra (n=7); at -5 days before implantation).
Elevated circulating human IL-1Ra was detected at the end of treatment in animals treated with IL-1Ra only (not shown). IL-1Ra treatment decreased fed hyperglycemia over the 4 weeks of treatment, with no effects on body weight (SI Figure 2AB). Fed plasma glucose values after 4 weeks of treatment were 7.9 ± 0.2 mM for the sham (n=6), and 7.3* ± 0.2 mM for the IL-1Ra group (n=7; *p<0.05). At the end of treatment, fed circulating insulin and proinsulin were both significantly reduced, with a trend towards an improved proinsulin/insulin ratio, and a decreased HOMA-IR in IL-1Ra treated animals (SI Figure 2C).

Animals were rapidly growing during the treatment period, therefore not allowing us to match IL-1Ra dose to body weight using the mini-osmotic pumps. Thus, we administered IL-1Ra s.c. twice daily at 10 and 50 mg/kg for 4 weeks. Before treatment, fed plasma glucose was lower in this set of animals compared to the pump experiment: 6.8 ± 0.2 mM, 6.4 ± 0.2 mM, and 7.1 ± 0.2 mM for twice daily injected GK saline controls (n=7), 10 mg/kg (n=5), and 50 mg/kg IL-1Ra-injected (n=8) groups respectively. Body weight between groups was 36.4 ± 2.0 g, 43.4 ± 3.0 g, and 42.6 ± 1.6 g, for groups in the same order as above at -5 days before treatment onset. Consistent with the mini-osmotic pump experiment, glycemia was reduced by high dose IL-1Ra treatment over the 4-week treatment period (Figure 3A, B). At the end of treatment, fed plasma glucose values were lower in both low and high dose IL-1Ra treated animals: 8.8 ± 0.3 mM GK saline control (n=7), 7.9 ± 0.1 mM* for 10 mg/kg IL-1Ra (n=5), and 7.9** ± 0.1 mM for 50 mg/kg IL-1Ra (n=8; *p<0.05, **p<0.01 vs. saline control). Further, at the end of treatment, both IL-1Ra treated groups had reduced fed circulating proinsulin,
and dramatically decreased proinsulin/insulin ratios compared to GK saline controls (Figure 3E-F). Interestingly, a lower dose IL-1Ra treatment significantly reduced fed insulin levels, while high dose IL-1Ra had little effect on circulating insulin (Figure 3D). Finally, calculated HOMA-IR indicated increased insulin sensitivity in both groups of IL-1Ra treated animals, while only low dose IL-1Ra treated animals showed consistent improvements during an insulin tolerance test (ITT; Figure 3GH).

In summary, as reported previously in humans and rodent models of type 2 diabetes (9, 24), IL-1Ra treatment of the type 2 diabetic GK rat reduced fed hyperglycemia. Based on both mini-osmotic pump experiments and s.c. injections, a lower dose of IL-1Ra improved both insulin sensitivity and β-cell insulin processing. In contrast, high dose IL-1Ra appeared to act more specifically on β-cell insulin processing with only minor effects on readouts of insulin sensitivity (HOMA-IR and ITT). Therefore, we focused mainly on characterization of islet inflammation in high dose IL-1Ra treated GK rats for the rest of the study.

**IL-1Ra has both islet and peripheral tissue anti-inflammatory actions.** We examined the islet inflammatory profile after 1-month of IL-1Ra treatment, either with pump implantation or daily injections. In the mini-osmotic pump experiment, IL-6, KC, MCP-1 and MIP-1α protein release were examined in conditioned media from isolated islets cultured for 48 h. Consistent with *in vitro* IL-1Ra effects, GK rat treatment with IL-1Ra *in vivo* decreased islet IL-6, KC, MCP-1 and MIP-1α protein release (SI figure 2D). After 4 weeks of twice daily s.c. injections
of IL-1Ra (50 mg/kg dose), GK islets were isolated and subjected to real-time mRNA analysis in order to examine a wider profile of inflammatory factors (Figure 4A). IL-1Ra treatment reduced the mRNA expression of islet caspase-1, IL-1β, IL-6, TNFα, chemokine KC, MCP-1, MIP-1α, MyD88, p65 NFκB, and TLR4. When comparing mRNA expression to GK untreated islets (Figure 1A), cytokine and chemokine expression was reduced at least 50% in all cases (Figure 4A). Finally, IL-1Ra treatment also enhanced expression of the insulin processing enzymes, proconvertase 1 and 2 (PC1, PC2), concomitant with increased insulin gene expression (INS1 and INS2) (Figure 4A).

Given the reduced expression of islet inflammatory markers, we performed immunohistochemistry for macrophages (CD68), mature granulocytes, myeloid precursors (CD53), and MHC class II protein expression in GK islets (Figure 4B-C). We found significantly reduced CD68+, MHC class II+, and CD53+ cells associated with IL-1Ra treated GK islets when quantifying this response (Figure 4C). Further, islet area among analyzed sections was not different between treatment groups (SI Figure 3). Thus, high dose IL-1Ra treatment reduced GK rat islet inflammation by reducing both islet cytokine/chemokine expression and islet immune cell infiltration.

Interleukin-1β is known to increase β-cell apoptosis in vitro (25). In order to assess whether IL-1Ra treatment was having effects on β-cell area in vivo we examined β-cell apoptosis and percent pancreatic β-cell area in IL-1Ra treated animals. In rats treated with mini-osmotic pumps, isolated islets from IL-1Ra treated animals showed reduced β-cell apoptosis (SI Figure 2E). Indeed, in vitro
treatment of isolated GK rat islets with IL-1Ra also reduced β-cell apoptosis to a similar degree (SI Figure 2E). Despite this reduction in ex vivo β-cell apoptosis, high dose IL-1Ra treated GK rats showed no difference in the percent pancreatic β-cell area compared to GK saline controls (Figure 4D). However, increased islet number per area was observed in IL-1Ra treated animals (SI Figure 4).

Because IL-1Ra treatment appeared to improve insulin sensitivity, we also analyzed inflammatory gene expression in liver, adipose, and skeletal muscle tissue after high dose s.c. IL-1Ra treatment (Figure 4E). IL-1Ra treatment most clearly reduced TNFα, MCP-1, and CD68 expression in the liver, with reduced TNFα expression also seen in adipose tissue (Figure 4E). In line with the reduced liver inflammation, elevated alkaline phosphatase activity in the GK rat was significantly reduced by s.c. IL-1Ra treatment: 416 ± 15 (n=4) for saline control and 372* ± 12 for IL-1Ra injected (n=5; *p<0.05). Furthermore, mRNA expression of liver PEPCK, the rate-limiting enzyme involved in gluconeogenesis, was reduced due to s.c. IL-1Ra treatment (relative mRNA level: 1.00 ± 0.03 and 0.81* ± 0.08 for GK saline control (n=4) and IL-1Ra treated groups, respectively; *p<0.05). Thus, while the GK rat is not characterized by strong peripheral tissue inflammation, IL-1Ra treatment primarily protected from increased expression of liver inflammatory markers.

Finally, we profiled circulating lipids in GK rats following treatment with IL-1Ra. There were no significant effects on lipids (FFA, triglycerides, Chol/HDL ratio, data not shown).
DISCUSSION

Recent data indicate that human type 2 diabetes is associated with islet inflammation (3), and that IL-1Ra treatment improves β-cell proinsulin/insulin processing and insulin secretion in human type 2 diabetes (9). In the present study we extend these studies using the GK rat as a model of human disease, with a focus on IL-1 as molecular link between islet inflammation and β-cell dysfunction. IL-1Ra treatment of the GK rat protected from increased islet proinflammatory cytokine expression, chemokine expression, islet immune cell infiltration, and improved insulin processing. These data show that IL-1 is a central regulator of a broad islet inflammatory signature characterized by immune cell infiltration, contributing to β-cell dysfunction in a type 2 diabetes model.

The GK rat colony is characterized by an early β-cell defect, followed by insulin resistance developing later in life (23). Published data and data presented here indicate that islet inflammation correlates with β-cell dysfunction in these animals (6, 12). In the present study, we extend on previous work and demonstrate that GK islets exhibit increased mRNA for numerous islet cytokines (IL-1β, IL-6, TNFα), chemokines (KC, MCP-1, and MIP-1α), and cytokine signaling intermediates (MyD88, NFκB), correlating with increased islet immune cell infiltration. Differences in experimental set up, or changes in mRNA stability and posttranslational processing may explain the discrepancy observed between islet cytokine/chemokine mRNA data and protein release. Regardless, the GK rat presents itself as a unique model to study the role of islet inflammation in a context of type 2 diabetes.
Interestingly, IL-1Ra treatment reduced islet caspase-1 mRNA 40% and IL-1β mRNA 50% compared to >90% reductions in TNFα and chemokine mRNAs. These data suggest that increased islet TNFα and chemokines are mainly IL-1 driven, while caspase-1, IL-1β, and IL-6 are partly increased in an IL-1 independent manner in the GK rat. These data support our in vitro data on isolated islets. Future analysis should elucidate the primary mechanism inducing islet IL-1β expression in the GK rat.

Similar to published data on humans and HFD mice (9, 24) IL-1Ra treatment via both mini-osmotic pumps and subcutaneous injections (twice daily 10 mg/kg and 50 mg/kg) reduced fed glycemia in the GK rat. While not completely preventing hyperglycemia in these animals, consistent reductions in blood glucose within the range of that previously reported with GLP-1, exendin-4, and gliclazide treatment in the GK colony were observed (26, 27). Our data extend these previous IL-1Ra studies to show that the effects of IL-1Ra treatment on glycemia can be via both improved β-cell insulin processing and effects on peripheral insulin sensitivity. The latter effect appears to be dose dependent. Improved insulin sensitivity due to a lower dose of IL-1Ra treatment was likely due to reductions in liver inflammation (reduced liver TNFα mRNA in treated animals: 1 ± 0.1 for saline control (n=4) vs. 0.67* ± 0.1 for IL-1Ra treated (n=5), fold of control; *p<0.05). Indeed, the effects of elevated TNFα on insulin resistance are well documented (1). Thus, we speculate that lower dose IL-1Ra in the GK rat is more effective at reducing peripheral tissue inflammation, while high doses of IL-1Ra are required to maximally inhibit islet inflammation. Of note,
the lower dose of IL-1Ra is around 20-fold higher than the dose administrated in a previous clinical study of IL-1 antagonism in patients with type 2 diabetes, which failed to uncover effects on insulin sensitivity (9). Therefore, the insulin sensitizing effect of IL-1Ra may be dose-dependent and U-shaped.

The effects of exogenous IL-1Ra treatment in the present study and those previously published in humans and mice (9, 24) contrast those elucidating the role of the IL-1 signaling system in glycemic and metabolic control using genetic knockout mouse models. Interleukin-1 receptor 1 knockout (KO) mice paradoxically develop maturity-onset obesity (28), while IL-1Ra KO mice are lean and resist HFD induced obesity (29, 30). We envisage two explanations for this apparent discrepancy. Appetite regulation occurs via modulation of the neuro-immune-endocrine axis (31). While the above mentioned genetic models display differences in body weight, treatment with IL-1Ra is not associated with alterations in body weights, neither in patients (9) nor in animal models (Figure 3C & (24)). Possibly, exogenous IL-1Ra does not impact the hypothalamus. An alternative explanation is that the genetic approach will completely block the IL-1 system, antagonizing a probable physiological role of very low concentrations of IL-1β (32).

Locally increased production of IL-1 in the islet and in insulin-sensitive tissues may (A) be directly cytotoxic to these tissues, (B) act directly to functionally affect insulin processing, secretion and action, and/or (C) induce recruitment of immune cells that subsequently contribute to impairment of tissue specific actions via production of additional cytokines and toxic substances. The
direct cytotoxic effects of IL-1β on the islet are well documented (33), however we did not detect any changes in percent pancreatic β-cell area due to IL-1Ra treatment, suggestive of no change in β-cell mass, which would be in agreement with data published on the HFD fed mouse (24). However, given the effects of IL-1Ra on reducing islet apoptosis ex vivo and increasing islet number, we cannot exclude the fact that IL-1Ra treatment may have altered the dynamics of β-cell apoptosis and/or proliferation/neogenesis over time. Our data do support a functional role of IL-1 inhibition with respect to insulin processing in the β-cell. Studies have documented that IL-1β down regulates PC1 and PC2 expression, impairing insulin processing, either alone or in combination with other cytokines such as IL-6 and TNFα (34-37). IL-1Ra reduced the local production of IL-1β, IL-6, and TNFα, likely explaining the improved insulin processing seen in our treated GK rats. Finally, IL-1 activity is known to increase the expression of chemokines in some tissues contributing to auto-inflammatory diseases (38, 39). Indeed, IL-1Ra treatment decreased islet chemokine expression both in vitro and in vivo, blocking subsequent islet immune cell infiltration. Indeed, the number of macrophages, MHC class II expressing cells, and granulocytes associated with GK islets were all markedly reduced by IL-1Ra, nearing levels observed in the control Wistar rat (6). Thus, IL-1Ra may have dual beneficial effects. First, IL-1Ra may protect from the direct effects of IL-1β on insulin processing or insulin signaling. Second, IL-1Ra may block IL-1β-induced chemokines and subsequently reduce immune cell infiltration and/or activation characteristic of GK islets. It remains to be determined to which degree the beneficial effects of
IL-1Ra are due to blocking the direct effects of IL-1 versus attenuating subsequent cytokine/chemokine release and immune cell infiltration.

The trigger of islet inflammation in the GK rat is unknown. Based on treatment of animals with phlorizin, and exacerbation of β-cell dysfunction by HFD feeding, it is hypothesized that glucolipotoxicity contributes to β-cell dysfunction in adult GK animals (40, 41). This is supported by hyperglycemia and increased circulating free fatty acids and triglycerides in the GK rat (SI Table 1). Indeed, we have previously found that a hyperglycemic and hyperlipidemic environment induces an islet inflammatory response \textit{in vitro} and in HFD fed mice (6), and our unpublished data show that lipid infusion into prediabetic BB rats exacerbates β-cell dysfunction while concomitantly increasing expression of islet cytokines and chemokines (J. Ehses, A. Giacca; manuscript in preparation). Therefore, we propose that the environment of metabolic stress in the GK rat may contribute to the islet inflammatory profile in these animals. In support of this, metabolic stress increased islet chemokine release in an IL-1 and MyD88-dependent manner. While the role of IL-1 in this response is supportive of our \textit{in vivo} data, the complete dependence of this response on the signaling intermediate, MyD88, is suggestive of signaling via other IL-1 family member receptors or Toll-like receptors being involved in this islet inflammatory response (42, 43). This warrants further investigation of these receptors as molecular links of metabolic stress induced islet inflammation.

The present study shows that IL-1Ra treatment improves GK hyperglycemia by improving both β-cell insulin processing (reducing the
proinsulin/insulin ratio) and insulin sensitivity. Reductions in hyperglycemia were paralleled by reductions in islet inflammation and anti-inflammatory effects on the liver. Thus, blocking IL-1 activity in type 2 diabetes may improve both \( \beta \)-cell function and insulin resistance by protecting cells from the direct toxic effects of IL-1 and/or by antagonizing the IL-1 induced inflammatory response.

**MATERIALS AND METHODS**

For detailed materials and methods please see the supporting information.

**Animals.** Experiments on the GK rat model (Paris colony) and Wistar controls were performed at the University Paris-Diderot, France. Animal experimentation was performed in accordance with accepted standards of animal care as established in the French National Center for Scientific Research guidelines and by Swiss veterinary law and institutional guidelines.

**Pancreatic islet isolation.** Rat islets and mouse islets were isolated as previously described (6, 18).

**RNA extraction and real-time PCR.** Total rat islet RNA was prepared as described (12). Liver, adipose, and muscle tissue RNA was extracted according to manufacturer’s instructions (Qiagen, Switzerland). Changes in mRNA expression were calculated using difference of \( C_T \) values as compared to a housekeeping gene (18S) and expressed relative to controls.

**Cytokines and chemokines.** Conditioned media from islets was assayed by Luminex™ (Millipore, Switzerland) as previously described (6).

**Free fatty acid preparation.** Palmitate was prepared as described (6) with
minimal endotoxin levels as tested (Cambrex, Charles City, IA, USA).

**In vivo IL-1Ra treatment.** IL-1Ra (kindly donated by Amgen, CA, USA) treatment of GK rats was performed by twice daily subcutaneous (s.c.) injections for 4 weeks. Prior to rat sacrifice an intraperitoneal (i.p.) insulin tolerance test (0.35 U/kg) was performed as previously described (44). At sacrifice organs were harvested for immunohistochemistry, islet isolations, and for total RNA isolation.

**Serum parameters.** Serum insulin, proinsulin, leptin, cytokines, FFA levels, Ketone, β-hydroxybutyrate levels, triglycerides and alkaline phosphatase activity were assayed according to the manufacturer's instructions (Mercodia, Sweden; Millipore; Wako Chemicals GmbH, Germany; Stanbio Laboratory, TX, USA; ABX Diagnostics, Montpellier, France). HOMA-IR was calculated as described (45).

**Immunohistochemistry.** GK rat pancreatic cryosections were incubated with anti-CD68, anti-MHC class II, anti-CD53, and anti–granulocyte antibodies as previously described (12), and immune cells quantified as previously (6).

**Statistics.** Data are expressed as means ± S.E. with the number of individual experiments presented in the figure and table legends. All data were analyzed using the nonlinear regression analysis program PRISM (GraphPad, CA, USA), and significance was tested using Student's t-test and analysis of variance (ANOVA) with Newman Keuls post-hoc test for multiple comparison analysis. Significance was set at p<0.05.

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Figure Legends

Figure 1: Pancreatic islet and peripheral tissue inflammation in the type 2 diabetic GK rat. (A) Total RNA was extracted from 2-month-old male Wistar and GK freshly isolated rat islets, and real-time PCR was performed for the indicated genes and normalized to a housekeeping gene (18S). Shown in parentheses are the fold increases in GK islets vs. Wistar controls. Casp-1= caspase-1. (B) Wistar and GK rat islets were isolated and cultured for 48h at 20 islets/dish, and conditioned media were assayed for the indicated cytokine/chemokine. Data were normalized for total islet protein. Data represent 3 different (A) and 4 different (B) islet isolations with islets pooled from 2-3 animals each time, and
experiments performed in triplicate. (C) Real-time PCR was performed on cDNA from liver, adipose, and muscle tissue from 2-month old male Wistar and GK rats. Data are representative of 4 animals per strain and are shown as fold increases vs. Wistar controls. Where * represents p<0.05 as determined by Student’s t-test.

**Figure 2: IL-1Ra inhibits GK islet cytokine and chemokine release *in vitro*.** Isolated Wistar and GK islets were plated at 20 islets/well and treated *in vitro* without (-) or with 1000 ng/mL IL-1Ra (+) for 48h. Thereafter, conditioned media were removed and assayed for (A) IL-6, (B) chemokine KC, (C) MCP-1, and (D) MIP-1α, and data were normalized for total islet protein. Data are presented relative to GK untreated. Data represent 3 different islet isolations with islets pooled from 2-3 animals each time, and experiments performed in quadruplicate. Where * represents p<0.05 as determined by ANOVA with Newman Keuls post-hoc analysis.

**Figure 3: IL-1Ra treatment reduces hyperglycemia, reduces the circulating proinsulin/insulin ratio, and improves insulin sensitivity in the GK rat.** Four-week-old male GK rats were injected s.c. twice daily with saline (n=7; GK saline), 10 mg/kg/injection (n=5), or 50 mg/kg/injection IL-1Ra (n=8; GK IL-1Ra) for 4 weeks as shown in scheme. Animal groups had similar starting blood glucose values (see text). (A) Delta (Δ) fed blood glucose, (B) area under the curve (AUC) for Δ fed blood glucose values over 4 weeks of treatment, and (C) Δ body weight
during treatment are shown. (D) At the end of treatment circulating fed insulin, (E) proinsulin, and (F) proinsulin/insulin ratio were determined. (G) HOMA-IR was calculated, and (H) an insulin tolerance test (0.35 U/kg) was performed at the end of treatment with saline or IL-1Ra. Where n represents the total number of animals treated in 2 separately conducted experiments, and * represents p<0.05, ** p<0.01, *** p<0.001 compared to saline control and # p<0.05, ## p<0.01 compared to low dose IL-1Ra as determined by ANOVA with Newman Keuls post-hoc analysis.

Figure 4: IL-1Ra treatment reduces tissue inflammation in the GK rat. (A) Pancreatic islets were isolated from GK rats following treatment with IL-1Ra by twice daily s.c. injections (GK saline n=6, GK IL-1Ra (50 mg/kg/injection) n=5). Total RNA was extracted from isolated islets and real-time PCR was performed for the indicated genes and normalized to 18S and expressed relative to GK saline controls. (B, C) Immunohistochemistry was performed for CD68, MHC class II, CD53, and granulocytes associated with islets and quantified (n=3 for both treatment groups). (D) β-cell area/total pancreatic area was quantified by immunohistochemistry (n=3 for both treatment groups). (E) Real-time PCR was performed on cDNA samples from liver, adipose, and muscle tissue from GK saline (n=4) and IL-1Ra treated animals (n=5) (E). Where n represents the number of animals analyzed, and * represents p<0.05 as determined by Student’s t-test.
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