Extracellular acidification stimulates GPR68 mediated IL-8 production in human pancreatic β cells

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Acute or chronic metabolic complications such as diabetic ketoacidosis are often associated with extracellular acidification and pancreatic β-cell dysfunction. However, the mechanisms by which human β-cells sense and respond to acidic pH remain elusive. In this study, using the recently developed human β-cell line EndoC-βH2, we demonstrate that β-cells respond to extracellular acidification through GPR68, which is the predominant proton sensing receptor of human β-cells. Using gain- and loss-of-function studies, we provide evidence that the β-cell enriched transcription factor RFX6 is a major regulator of GPR68. Further, we show that acidic pH stimulates the production and secretion of the chemokine IL-8 by β-cells through NF-κB activation. Blocking of GPR68 or NF-κB activity severely attenuated acidification induced IL-8 production. Thus, we provide mechanistic insights into GPR68 mediated β-cell response to acidic microenvironment, which could be a new target to protect β-cell against acidosis induced inflammation.

In biological systems, cells actively partake in maintaining homeostasis of their environmental milieu within a precise range of physiological parameters. Cellular systems also foster the unique ability to respond and adapt to physiological stress, preserving survival and function. Signal transduction across cell membrane, through surface receptors is fundamental to detect and respond to changes in the local milieu1. Protons (H+) represent an important component of the extracellular milieu2. The extracellular fluids and blood pH are tightly regulated and maintained judiciously at ~7.4 but under many patho-physiological circumstances such as inflammation, ischemia and tumor formation, acidosis occurs in the localized microenvironment3.

Cells sense extracellular protons concentration by a number of mechanisms4,5. Ion channels such as transient receptor potential V1 and acid-sensing ion channels (ASICs) represent one sensing mechanism. Such channels are predominantly expressed on sensory neurons and act as proton sensors for pain and nociception signals6,7. A sub-family of G protein-coupled receptors (GPCR) represents a second type of proton sensing mechanism. This includes four members: GPR4, GPR68 (or Ovarian cancer G protein-couple receptor 1, OGR1), GPR65 (or T-cell death-associated gene 8, TDAG8) and GPR132 (or G2A). These receptors sense moderate extracellular pH within a narrow range (pH 6.0 to 7.6) and signal via a variety of intracellular pathways. For example, GPR68 is coupled to the Gq11-phospholipase-C/Ca2+ pathway, whereas GPR4 and GPR65 are coupled to the Gs-adenyl-cyclase/cAMP pathway8,9.

Insulin-producing pancreatic β-cells are highly differentiated cells that play a critical role in maintaining glucose homeostasis. They are factories dedicated to produce and secrete insulin in a tightly regulated fashion10. β-cells sense a myriad of circulating factors such as glucose, neurotransmitters and hormones that regulate their function under physiological conditions11. They are also sensitive to inflammatory cytokines that are implicated in their destruction in type 1 diabetes (T1D)12,13. A recurring complication of T1D is diabetic ketoacidosis (DKA) resulting in ketonemia and metabolic acidosis14 with extracellular acidification of the pancreatic microenvironment15,16. However, the mechanism by which human β-cells sense proton concentration and transmit their signal remains largely unknown. It is likely that moderate acidosis in the pancreatic microenvironment is...
primarily sensed through the proton sensing GPCR because i) ASICs ion channels are not reported to be present in islets; ii) TRPV1 channels, even though reported to be expressed in some β-cell lines, sense acidic pH (pH 4–5)17–21. Information is limited on the expression and function of proton sensing GPCRs in pancreatic β-cells. Impaired glucose-stimulated insulin secretion has been described in GPR68 knockout mice, however the role of proton sensing GPCRs in human β-cells remains to be explored22.

Here, we provide evidence that GPR68 is the predominant proton sensor receptor expressed by human β-cells. Its expression is tightly regulated by RFX6, a β-cell enriched transcription factor23. We also show using the human β-cell line Endo-CβH224 that extracellular acidification activates GPR68, inducing the production and secretion of the chemokine IL-8 through NF-κB activation. In conclusion, proton sensing via GPR68 is a novel mechanism for the induction of inflammatory response in human pancreatic β-cell.

**Results**

The proton-sensing receptor GPR68, a target of RFX6, is expressed in EndoC-βH2 cells and human islets. Our previously published transcriptomic analyses (GEO No: GSE48101) indicated that EndoC-βH2 cells express mRNA coding for the proton-sensing receptor GPR6824. We validated these data by Real-Time-quantitative PCR (RT-qPCR) that indicated that GPR68 mRNA expression was enriched in EndoC-βH2 cells compared to the duct cell line SKPC (Fig. 1a). Transient transfection of EGFP tagged human GPR68 construct in EndoC-βH2 cells showed its predominant localization on the plasma membrane (Supplementary Fig. 1). GPR68 was almost the sole proton sensing GPCR expressed in EndoC-βH2 cells, the other ones (GPR4, GPR65, GPR132) being expressed at nearly undetectable levels (Fig. 1a). Similar data were obtained using human islet preparations that expressed GPR68, but not GPR65 and GPR132 (Fig. 1b). Of note, GPR4 was detected in human islets and not in EndoC-βH2 cells (Fig. 1a), which could be due to its expression by non-β-cells present in human islet preparations like endothelial cells25,26.

RFX6 is a key transcription factor highly expressed in β-cells and required for their function. Our previous transcriptomic analyses indicated that siRNA-mediated RFX6 knock-down decreased GPR68 expression in EndoC-βH2 cells [FC, −3.85; p = 7.78.10^{-4}] (GEO No: GSE9049) without effecting the expression of other proton sensing receptors25. Further validation by RT-qPCR showed that decreased expression level of RFX6 mRNA (63.92 ± 10.5%) was consistently accompanied by decrease in the level of GPR68 mRNA (59.73 ± 15%) in EndoC-βH2 cells (Fig. 2a). Similar results were obtained in human islets where decreased RFX6 expression (79.34 ± 13%) resulted in a 42.15 ± 10% decrease of GPR68 transcripts (Fig. 2b). Additionally, overexpression of wtRFX6 but not p.V506G mutant RFX625,26 increased the expression of GPR68 transcripts (Fig. 2c). GPR68 expression was also enhanced following transfection of EndoC-βH2 cells with a trans-activation domain VP16 conjugated RFX6 (Fig. 2d), while transfection of a trans-repression domain conjugated KRAB-RFX6 in EndoC-βH2 cells resulted in a decreased expression of GPR68 (Fig. 2e). GPR68 is thus the major proton sensor expressed in β-cells, its expression being tightly controlled by RFX6.

**GPR68 is involved in proton-induced inositol phosphate (IP) production in Human β-cells.**

GPR68 is a proton-sensing Gq/11 coupled receptor that stimulates IP formation to elicit pH dependent responses8. To examine if acidification of the extracellular medium activates Gq/11 pathway in EndoC-βH2 cells, we incubated cells at either physiological pH 7.4 or acidic pH 6.4. Buffered pH media did not alter cell morphology, viability (Supplementary Fig. 2a–c) or insulin secretion in EndoC-βH2 cells (Supplementary Fig. 3). Acidic pH 6.4 induced a significant increase in IP formation (Fig. 3a). This effect was blocked by YM-254890, a selective Gq/11 inhibitor27, demonstrating the selective role of Gq/11 pathway in pH dependent responses in EndoC-βH2 (Fig. 3a). On the other hand, proton did not modulate cAMP production measured at pH 7.4 or 6.4 (Fig. 3b),...
further indicating that Gs-coupled receptors such as GPR4 and GPR132 are not involved in proton sensing in EndoC-βH2 cells. We next show that acidic pH-stimulated IP production was GPR68-dependent. Indeed control (siNT) treated β-cells sensed normally the extracellular acidic pH (6.4) and responded by increasing IP formation. In contrast siRNA-mediated GPR68 depletion significantly decreased this induction (Fig. 3c). Accordingly, siRFX6 treatment lead to decreased GPR68 expression also inhibited proton induced IP formation (Fig. 3c). Thus, GPR68 is involved in proton-induced IP production in EndoC-βH2 cells.

EndoC-βH2 cells express and secrete the pro-inflammatory cytokine IL-8 upon exposure to acidic pH. Extracellular acidic microenvironment has been reported to induce the expression of pro- and anti-inflammatory cytokines in a variety of cell types. We examined the expression of selected cytokines by EndoC-βH2 cells exposed to acidic pH. RT-qPCR analysis of cells incubated at pH 7.4 or pH 6.4 for 24 h showed the induction of IL-8 transcripts in cells exposed to acidic pH (Fig. 4a). At all intermediate pH tested between 7.4 and 6.4, IL-8 mRNA expression increased while pH decreased (Fig. 4b). Low pH-induced IL-8 mRNA expression was detected as early as 8 h following low pH exposition and increased at later time points (24 and 48 h) (Fig. 4c). Following incubation at pH 6.4, IL-8 protein was detected in the conditioned medium of EndoC-βH2 cells. PMA, a strong inducer of IL-8 in human EndoC-βH2 cell model, was used as positive inducer.
**IL-8 induction in β-cells by extracellular acidification is NF-κB-dependent.** NF-κB is a central mediator of inflammatory response29 and RELA, a major subunit of NF-κB complex, is a mediator of IL-8 transcription30. As EndoC-βH2 cells secrete inflammatory cytokine IL-8 in response to acidification of their medium, we investigated whether IL-8 production requires NF-κB activation. We showed nuclear translocation of RELA, a subunit of NF-κB complex upon acidic pH treatment in EndoC-βH2 cells (Fig. 6a). EMSA performed using cellular extracts from EndoC-βH2 showed that DNA-binding activity of NF-κB increased in a time-dependent manner when cells were exposed to acidic pH 6.4 (Fig. 6b). Consistent with these results, cell treatment with JSH-23, a potent NF-κB activation inhibitor II, significantly decreased acidic pH induced IL-8 mRNA expression (Fig. 6c). Moreover, a siRNA that efficiently targeted RELA (Fig. 6d, left), decreased the acidic pH-mediated IL-8 mRNA induction (Fig. 6d, right). Thus, in EndoC-βH2 cells low pH induces the up-regulation of IL-8 mRNA through the activation of NF-κB complex.

**IL-8 secreted by human β-cells in acidic conditions attracts neutrophils.** IL-8 is a chemotactic pro-inflammatory cytokine that mediates the recruitment and activation of neutrophils during inflammation31. We examined if IL-8 secreted by EndoC-βH2 cells exposed to acidic pH induces neutrophil chemotaxis. We performed in-vitro migration assay using CD16-positive human blood neutrophils and EndoC-βH2 cell conditioned media as chemo-attractant (Fig. 7a). When compared to pH 7.4 condition medium, acidic conditioned medium significantly attracted blood neutrophils. This chemotactic migration was abrogated by anti-IL-8 antibody indicating that neutrophil migration to acidic conditioned medium is dependent on the presence of IL-8 (Fig. 7b).

**Discussion**

We and others recently showed that in adult human and mouse pancreatic β-cells, the transcription factor RFX6 controls insulin secretion by modulating calcium homeostasis21,32. In the present study, we demonstrate that RFX6 plays a pivotal role in extracellular proton sensing by regulating the expression of the G-protein coupled receptor GPR68 in human β-cells. We next demonstrate that extracellular signal acidification activates GPR68 which induces the production of inflammatory chemokine IL-8 through activation of the NF-κB complex.
GPR68 is one of the 4 known proton sensing GPCRs together with GPR4, 65 and 132. Our results indicate that GPR68 is the predominant GPCR of this family in the human β-cell-line EndoC-βH2 cells. This appears to be also the case in primary human β-cells. Indeed, human islet preparations that contain β-cells, but also some endocrine and non endocrine pancreatic cells, express GPR68 but no GPR65 and GPR132 (our present data and ref. 25). GPR4 expression is also detected in human islet preparations. However, while GPR68 remains expressed in islet β-cell enriched fractions, GPR4 expression levels collapse in such fraction25. Such data suggest that GPR68 is expressed in primary human β-cells, while GPR4 is expressed in non-β-cells in islet preparations.

Signaling via GPCR plays major role in response to neurotransmitters, hormones and environmental stimuli33,34. This is also the case in pancreatic β-cells, where signaling mediated by a number of GPCRs regulate β-cell expansion and function35–37. However, limited information is available on the regulation of GPCR expression38,39. Here, using gain- and loss-of-function approaches, we provide strong evidence that RFX6 is a major positive regulator of GPR68 expression. RFX6 knockdown down-regulates GPR68 in EndoC-βH2 cells and in human islets. Likewise, overexpression of wtRFX6 enhances GPR68 expression. Finally, converting RFX6 into a
**Methods**

**Culture of human cell lines and islets.** EndoC-βH2 cells were cultured in low-glucose (1g/L) Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing L-glutamine and sodium pyruvate, supplemented with 2% BSA fraction V (Roche Diagnostics), 50μM 2-mercaptoethanol, 10 mM nicotinamide (Calbiochem), 5.5μg/ml transferrin (Sigma-Aldrich), 6.7 ng/ml selenite (Sigma-Aldrich), 100 μ/ml penicillin and 100 μg/ml streptomycin. Cells were seeded on Matrigel (1%)/fibronectin (2μg/ml) (Sigma-Aldrich) coated plates and cultured at 37°C and 5% CO2. The human duct cell line SKPC58 was cultured in high glucose DMEM (4.5 g/L) supplemented with 10% fetal calf serum (Biowest), 100 μg/ml penicillin and 100 μg/ml streptomycin. Human islets were isolated and maintained as described. 

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![Image of a and b diagrams](https://www.nature.com/scientificreports/)
Preparation of buffered culture media. Culture media at different pH were prepared as described. Briefly, low glucose DMEM without sodium bicarbonate (Sigma, D2902) was buffered with HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid; Sigma, H0887), EPPS.
(4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid; Sigma, E9502) and MES (2-(N-Morpholino)ethanesulfonic acid; Sigma, M3671) (8mM each) and pH was adjusted with HCl/NaOH. They were kept iso-osmotic by adding NaCl and NaHCO3. The pH stability was monitored at the initiation and completion of the experiments.

RNA isolation, reverse transcription and RT-qPCR. Total RNA was extracted from EndoC-β2H2 cells using RNeasy Plus Micro kit (Qiagen). First strand cDNA was prepared using Maxima First Strand cDNA synthesis kit (ThermoFisher). RT-qPCR was performed using Power SYBR Green mix (Applied Biosystems) with ABI Prism 7300 sequence detector (Applied Biosystems). Cyclophilin A transcript levels were used for normalization of each target gene. The custom primers were designed with IDT Primer-Quest online software and the amplification efficiency for each primer was determined with serial dilution of total cDNA from EndoC-β2H2/human islets cDNA. Primer sequences are listed in Supplementary Table.

siRNA Transfection. EndoC-β2H2 cells were transfected using Lipofectamin RNAiMAX (Life Technologies) and ON-TARGETplus siRNA SMARTpool for human RFX6/GPR68/RELA gene (40 nM) or ON-TARGETplus Non-targeting pool (siNT) (Dharmacon, Thermo Scientific) as described23. Human islet samples were partially dissociated with Accutase (PAA Laboratories) and siRNA transfections were performed as described23.

DNA Transfection. Human RFX6 constructs (pRIG-RFX6, pRIG-Mut506-RFX6, pRIG-KRAB-RFX6 and pRIG-VP16-RFX6)23 were used in this study. The MGC Human GPR68 cDNAclone (Clone ID: 6971805) was purchased (Open Biosystems; Thermo Scientific) and sub-cloned into pEGFP-N1 (Clontech). EndoC-β2H2 cells were transiently transfected with DNA using Lipofectamin2000 (Invitrogen) following manufacturer’s instructions in Opti-MEM. GFP-positive cells were FACS sorted 24–48 h post transfections and RNA expression was analyzed by RT-qPCR.

Electrophoretic mobility shift assay for NF-κB. EndoC-β2H2 cells were cultured at pH 7.4 (8h) or 6.4 (1, 2 and 8h) or with PMA (100 ng/ml at pH 7.4 for 8h). Cellular extracts were prepared and NF-κB activation was analyzed by electrophoretic mobility shift assay (EMSA) using the human immunodeficiency virus long terminal repeat tandem κB oligonucleotide as κB probe61.

IP and cAMP formation assay. IP formation was quantified with HTRF (Homogeneous Time-Resolved Fluorescence) based “Cisbio IP-One Tb” (Cisbio, Bagnoles-sur-Cêze, France) assay kit, following manufacturer’s instructions. EndoC-β2H2 cell suspensions (5 × 10^6 cells) were treated in 384-well plate (16 μl volume) with modified stimulation buffer (10 mM Hepes, 10 mM MES, 1 mM CaCl2, 0.5 mM MgCl2, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl) at pH 7.4 or 6.4 for 60 min at 37°C. Where indicated, cells were pretreated with YM-254890, a selective Goαs inhibitor17 for 30 min prior to incubation with the IP stimulation buffer and maintained throughout the IP determination. IP measurements were performed in triplicates and experiments were repeated at least three times. Samples were read on a TECAN Infinite F500 (Tecan Group, Ltd., Männedorf, Switzerland) with excitation at 320 nm and emission at both 620 nm and 665 nm.

Figure 7. IL-8 secreted by EndoC-β2H2 cells in acidic conditions attracts neutrophils. (a) Neutrophils were isolated from whole blood using MACSxpress kit, analyzed for the expression of CD16 and used for in-vitro transwell migration assay. (b) Neutrophil chemotaxis was tested using conditioned media from EndoC-β2H2 cells cultured at pH 7.4 or pH 6.4 for 72 h. Acidic pH 6.4 conditioned medium pre-treated for 10 min with Anti-human IL-8 (1 μg/ml) was also used as well as pH 7.4 conditioned medium supplemented with recombinant human IL-8 (50 ng/ml). Data are represented as migration index, calculated by assigning a value of 1 to the number of migrating neutrophils towards pH 7.4-conditioned medium. Data are mean ± SEM of 4 blood donors. *p < 0.05; **p < 0.01 (one-way ANOVA, followed by a Tukey’s multiple comparisons post-test).
cAMP activity was measured using a cAMP-HTRF assay kit (Cisbio) following manufacturer's instructions. EndoC-βH2 cells (5 × 10^5) were treated in 384-well plate (12 μl final volume) with stimulation buffer (PBS containing 10 mM of each HEPES, MES, and 0.5 mM IBMX, at pH 7.4 or 6.4) for 30 min at room temperature. Cells were lysed using kit lysis buffer and cAMP was then measured in 384 well plates (HTRF) with TECAN Infinite F500.

**Immunocytochemistry and immunoblotting.** EndoC-βH2 cells were cultured on Matrigel/fibronectin coated 4-well chambers slide (Nunc Lab-Tek) and processed for IL-8 and RELA immunostaining as described using anti-IL-8 (1:1,000; BD554717; BD Biosciences) or anti-RELA (1:200; sc8008; Santa Cruz Biotechnology) antibodies. Images were acquired with a Leica Leitz fluorescent microscope equipped with cooled 3-chip charge coupled device camera (Hamamatsu C5810; Hamamatsu) and processed using ImageJ software.

For immunoblot assays, total cellular proteins were prepared as described. Proteins (25 μg) were resolved by SDS PAGE, immunoblotted with antibodies against RELA (sc8008, 1/250 dilution) and Actin (1/1000, Sigma-Aldrich). Membranes were incubated with species-specific HRP-linked secondary antibodies (1:5000) and visualization was performed following ECL exposure.

**IL-8 Elisa.** Secreted IL-8 protein levels were determined using commercially available Human IL-8 ELISA MAX Deluxe kit (BioLegend #431504) as per manufacturer's instructions. EndoC-βH2 cells were treated with pH 7.4 or 6.4 or with 100 ng/ml PMA (at pH 7.4) and culture supernatants were collected and stored for ELISA.

**Neutrophil migration assay.** Blood samples were obtained from the pediatric endocrinology and diabetes center at Necker Enfants-Malades hospital, Paris, France in accordance with the approved guidelines. All the experimental protocols were approved by the local ethic committee (CPP - Paris Ile de France, France). Informed consent was obtained from all subjects. Neutrophils were isolated using a MACSxpress human neutrophil isolation kit (Miltenyi Biotec). Red Blood Cell lysis buffer was used to remove residual erythrocytes. The purity of isolated neutrophils was consistently between 98–99% based on CD16 staining. Chemotaxis assay was performed in 24-well micro chemotaxis chamber using 6.5 mm Transwell with 3 μm PVP-free polycarbonate filter membrane (Costar). Neutrophils (2 × 10^5 cells in 200 μl PBS) in upper chamber were allowed to migrate towards 500 μl of conditioned medium produced during 72 h by EndoC-βH2 cultured at pH 7.4 or 6.4. In some experiments, conditioned medium was supplemented with Anti-human IL-8 (1 μg/ml for 10 min; BD554717; BD Bioscience) or with recombinant human IL-8 (50 ng/ml; BioLegend). After 2 h at 37°C, migrating cells were recovered with Accutase (Sigma) in the lower chamber and numbered by flow cytometry. Results are expressed as migration index: number of migrating neutrophils in a defined condition divided by number of migrating neutrophils towards pH 7.4 conditioned medium.

**Statistics.** Graphs were constructed by using PRISM software (version 5.02 GraphPad). Quantitative data are presented as the mean ± SEM from at least three independent experiments, unless indicated. For comparison between two mean values, statistical significances were estimated using two-tailed Student’s t-test. For comparison between three or more variables, one-way ANOVA was used with Tukey’s multiple comparisons post-test. Statistical significance was set at p < 0.05.

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Author Contributions
V.C. designed research, performed experiments, analyzed data and wrote the manuscript. A.K. performed IP and cAMP assays. P.R. performed experiments and participates in manuscript preparation. F.C. performed EMSA experiments and contributed to the manuscript writing. C.R. participates in FACS based experiments. R.J. and M.A. analyzed data and participates in manuscript writing. O.A. designed experiments, analyzed data and participates in manuscript writing. R.S. designed research, analyzed data and wrote the manuscript.

Additional Information
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