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PSCs and GLP-1R: occurrence in normal pancreas, acute/chronic pancreatitis and effect of their activation by a GLP-1R agonist

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There is increasing concern about the development of pancreatitis in patients with diabetes mellitus who received long-term glucagon-like peptide-1 (GLP-1) analog treatment. Its pathogenesis is unknown. The effects of GLP-1 agonists on pancreatic endocrine cells are well studied; however, there is little information on effects on other pancreatic tissues that might be involved in inflammatory processes. Pancreatic stellate cells (PSCs) can have an important role in pancreatitis, secreting various inflammatory cytokines/chemokines, as well as collagen. In this study, we investigated GLP-1R occurrence in normal pancreas, acute pancreatitis (AP)/chronic pancreatitis (CP), and the effects of GLP-1 analog on normal PSCs, their ability to stimulate inflammatory mediator secretion or proliferation. GLP-1 receptor (GLP-1R) expression/localization in normal pancreas and pancreatitis (AP/CP) tissues were evaluated with histological/immunohistochemical analysis. PSCs were isolated from male Wistar rats. GLP-1R expression and effects of GLP-1 analog on activated PSCs was examined with real-time PCR, MTS assays and western blotting. In normal pancreas, pancreatic β cells expressed GLP-1R, with only low expression in acinar cells, whereas in AP or CP, acinar cells, ductal cells and activated PSCs expressed GLP-1R. With activation of normal PSCs, GLP-1R is markedly increased, as is multiple other incretin-related receptors. The GLP-1 analog, liraglutide, did not induce inflammatory genes expression in activated PSCs, but induced proliferation. Liraglutide activated multiple signaling cascades in PSCs, and the extracellular signal-regulated kinase pathway mediated the PSCs proliferation. GLP-1Rs are expressed in normal pancreas and there is marked enhanced expression in AP/CP. GLP-1-agonist induced cell proliferation of activated PSCs without increasing release of inflammatory mediators. These results suggest chronic treatment with GLP-1R agonists could lead to proliferation/chronic activation of PSCs, which may lead to important effects in the pancreas.
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Glucagon-like peptide-1 (GLP-1) analogs and DPPIV inhibitors are increasingly used long term in patients with diabetes mellitus worldwide.[1–4] These drugs appear to be generally safe, however, a concern receiving increasing attention is the development of pancreatitis.[5–15] At present, the pathogenesis of this effect is unknown.

GLP-1 is released postprandially by intestinal L cells and its activation of GLP-1 receptors (GLP-1Rs) on β cells stimulates insulin biosynthesis/secretion.[16,17] GLP-1Rs are expressed not only in pancreatic β cells, but also in other organs, such as brain, heart, stomach, muscle and liver.[18,19] Although the effects of GLP-1 on pancreatic endocrine cells have been well studied, there is little information on the effects of GLP-1R in other pancreatic exocrine tissues that might be involved in inflammatory conditions of the pancreas.

Recent studies establish the important role of various resident pancreas cells (particularly acinar cells and pancreatic stellate cells (PSCs)) in various aspects of pancreatitis (acute pancreatitis (AP)/chronic pancreatitis (CP)), especially leukocyte attraction via secretion of chemokines and cytokines and expression of adhesion molecules.[20–28] PSCs, not only can secrete various inflammatory cytokines/

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chemokines,[29–31] which can participate in inflammatory processes, they can also proliferate and secrete collagen.[32,33] Although numerous stimulants can affect PSCs including activation of some G-protein-coupled receptors (PGE2, angiotensin, CCK-A, CCK-B),[34–39] it is unknown if GLP-1R occurs on PSC either in normal pancreas or pancreatic disease states or, whether PSCs are activated in AP or CP, or if it does occur, whether the GLP-1R agonists affect the PSC signaling cascade, growth or ability to release inflammatory mediators. To address these questions, in this study we have examined the occurrence of the GLP-1R in PSCs in normal pancreas, in AP/CP models, as well as the ability of the GLP-1R agonist, liraglutide, to alter function of PSCs and effect PSCs behavior.

MATERIALS AND METHODS

Materials

Liraglutide was from Shoyaku (Fukuoka, Japan), U0126 and cerulein from Sigma-Aldrich (St Louis, MO, USA), Recombinant rat PDGF-BB from R&D Systems (Minneapolis, MN, USA) and rabbit anti-phosphorylated extracellular signal-regulated kinase (ERK) antibody, rabbit anti-phosphorylated JNK antibody, rabbit anti-phosphorylated p38 antibody, rabbit anti-phosphorylated Akt antibody, mouse anti-total IκB antibody anti-rabbit IgG-HRP-conjugated antibody and anti-mouse IgG-HRP-conjugated antibody from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-GLP-1R antibody, mouse anti-glial fibrillary acidic protein (GFAP) and mouse anti-alpha smooth muscle actin (α-SMA) were from Abcam PLC (Cambridge, UK), Goat anti-type I collagen antibody from Southern Biotechnology (Birmingham, AL, USA), rabbit antibody against glyceraldehydes-3-phosphate dehydrogenase (GAPDH) from Trevigen (Gaithersburg, MD, USA) and anti-rabbit IgG Alexa488-conjugated antibody, anti-mouse-IgG-Alexa555-conjugated antibody, anti-goat-IgG-Alexa350-conjugated antibody and Hoechst33342 were from Invitrogen (Carlsbad, CA, USA).

Animals

Fifteen-week-old male Wistar rats and Wistar/Bonn–Kobori (KOB) rats (KBT Oriental, Saga, Japan) were used. All animal procedures were performed in accordance with the guidelines of the Committee on Animal Care (Kyushu University). L-arginine-induced AP was performed by administrating a single intraperitoneal injection of 4.0 g/kg body weight L-arginine monohydrochloride in 0.9% sodium chloride (pH7.0) as described previously.[40] Rats were fed *ad libitum* after the treatment and killed 72 h later. Cerulein-induced AP was also performed by injecting intraperitoneally into the right lower quadrant with a 50 μg/kg/body weight of cerulein dissolved in 0.9% saline in a volume of 100 μl. Injections were at hourly intervals up to seven injections. Animals were killed 8 h after the first injection of cerulein. To study results in CP, a male Wistar/Bonn–KOB rats[41,42] were used as previously described.[31]

Isolation of PSCs and Cell Culture

PSCs were isolated from the pancreas of male Wistar rats and were maintained as previously described.[43] Cell purity was assessed initially by a typical star-like configuration and by detecting vitamin A autofluorescence and was >95%. To further assess the purity, in the third passage (activated PSCs) using immunohistochemistry to assess the co-expression of α-SMA (marker of activated stellate cells) and the presence of a cell (Hoechst33342) using ImageJ (NIH). We found the purity of PSCs was >99%. Furthermore, trypsin mRNA expression as a marker for acinar cell contamination was assessed using PCR (primers: Table 1, PCR conditions: Tm = 60 °C, cycles 40). In the third passage of PSCs and was >1 million times lower than in acinar cells, indicating contamination by acinar cells was negligible. Unless otherwise specified, PSCs were incubated in serum-free medium for 24 h before the addition of experimental reagents. The ERK-specific inhibitor (U0126) was added 30 min before the addition of liraglutide.

Expressional Changes of Incretin-Related and Pancreatitis-Related mRNAs in PSCs: Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from PSCs using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as previously described.[44] Total RNA was reverse transcribed into first-strand complementary DNA and RT-PCR was performed using a LightCycler Real-Time PCR system (Roche, Switzerland) as previously described.[44] To control for variations in the reactions, all PCR data were normalized against GAPDH expression. The PCR products were separated in a 2% agarose gel and visualized under UV illumination. Sequences of primers were listed on Table 1.

Histochemical Analysis

Specimens from the body of each pancreas were used for histopathological analysis. The pancreas specimens were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Tissue sections were stained with hematoxylin/eosin (HE) or Masson-Trichrome (MT), which preferentially labels collagen fibrils.

Immunofluorescent Staining

Immunofluorescent staining was performed as previously described.[43] Briefly, double-immunofluorescent staining for GLP-1R and GFAP (staining quiescent PSCs), type I collagen/α-SMA (staining activated PSCs) was performed as previously described.[31] Hoechst33342 was used for nuclear counter staining. The degree of colocalization of GLP-1R and α-SMA was calculated using ImageJ (NIH).

Western Blotting

Western blot analysis was performed as previously described.[43] Briefly, cells were lysed in RIPA buffer and cellular

Table 1 Sequences of primers used in this study

Gene	Sequence
Rat GLP-1R	
Sense	5′-CTGAGGAACAGCTCCTGTCG-3′
Anti-sense	5′-AGCTGACAAGGATGGCTGAA-3′
Rat GLP-2R	
Sense	5′-GGGGAAGTGTTCCAAGAAGC-3′
Anti-sense	5′-GTGTGAGCTGCATGTGGAGA-3′
Rat GIPR	
Sense	5′-AGATCTTCCCACCTCCCAGA-3′
Anti-sense	5′-TCTGTGTGTCCATCCATCCA-3′
Rat GlucagonR	
Sense	5′-CTCTGCGAAGACCTCATTGG-3′
Anti-sense	5′-GAGGCCCTTCTGAATCCAAC-3′
Rat proglucagon	
Sense	5′-CGCCATAGCTGAGGAACTTG-3′
Anti-sense	5′-CTCTGGTGGCAAGGTTATCG-3′
Rat GIP	
Sense	5′-AGACCTGCTCTCTGCTGCTG-3′
Anti-sense	5′-ATGGGATCGGAACTCAACCT-3′
Rat DPPIV	
Sense	5′-CCCAGGTCCAAGCATACAAA-3′
Anti-sense	5′-CTCAGAAAACGGTGCCAGTC-3′
Rat α-SMA	
Sense	5′-CCTCAGGGTGCTCGTGGAT-3′
Anti-sense	5′-CAGGACTGCCAGGCTCTCC-3′
Rat TNF-α	
Sense	5′-CTGGTGGTACCAGCAGATGG -3′
Anti-sense	5′- GGAGGCTGACTTTCTCCTGG -3′
Rat IL-6	
Sense	5′-CCACCAGGAACGAAAGTCAA-3′
Anti-sense	5′-CAGTCCCAAGAAGGCAACTG-3′
Rat IL-1β	
Sense	5′-GCACAGTTCCCCAACTGGTA-3′
Anti-sense	5′-CCGACCATTGCTGTTTCCTA-3′
Rat MCP-1	
Sense	5′-ACGTGCTGTCTCAGCCAGAT-3′
Anti-sense	5′-GTTCTCCAGCCGACTCATTG-3′
Rat CINC-1	
Sense	5′-CCACACTCAAGAATGGTCGCG-3′
Anti-sense	5′-AGACGCCATCGGTGCAATC-3′
Rat CX3CL1	
Sense	5′-CACAAGATGACCTCGCCAAT-3′
Anti-sense	5′-GCTGTCTCGTCTCCAGGATG-3′
Rat type I collagen	
Sense	5′-AGTTGGTGATGATGCCGTGTT-3′
Anti-sense	5′-ATGGGCCAAAAGGACAGCTAT-3′
Rat trypsin	
Sense	5′-AGATTCCTGCCAGGGTGACT-3′
Anti-sense	5′-CCATAGCCCCAGGAGACAAT-3′
Rat GAPDH	
Sense	5′-GCTCTCTGCTCCTCCCTGTT-3′
Anti-sense	5′-CACACCGACCTTCACCATCT-3′

proteins were fractionated by electrophoresis on a polyacrylamide gel (Bio-Rad, Hercules, CA, USA). The proteins were transferred onto a nitrocellulose membrane (Bio-Rad), and it was incubated for 2–6 h with primary antibodies (at 1 : 1000–10 000 dilutions). After incubating with HRP-conjugated secondary antibody (at 1 : 10 000 dilution), the proteins were visualized by using an ECL kit and ImageQuant LAS 4000mini (GE Healthcare). Levels of phosphorylated ERK, JNK, p38, Akt, total IκB and GAPDH were determined by General-Purpose Analysis Software Multi Gauge.

Cell Proliferation Assay: MTS Assay

Cell proliferation was assessed by the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Madison, WI, USA) as previously described.[44]

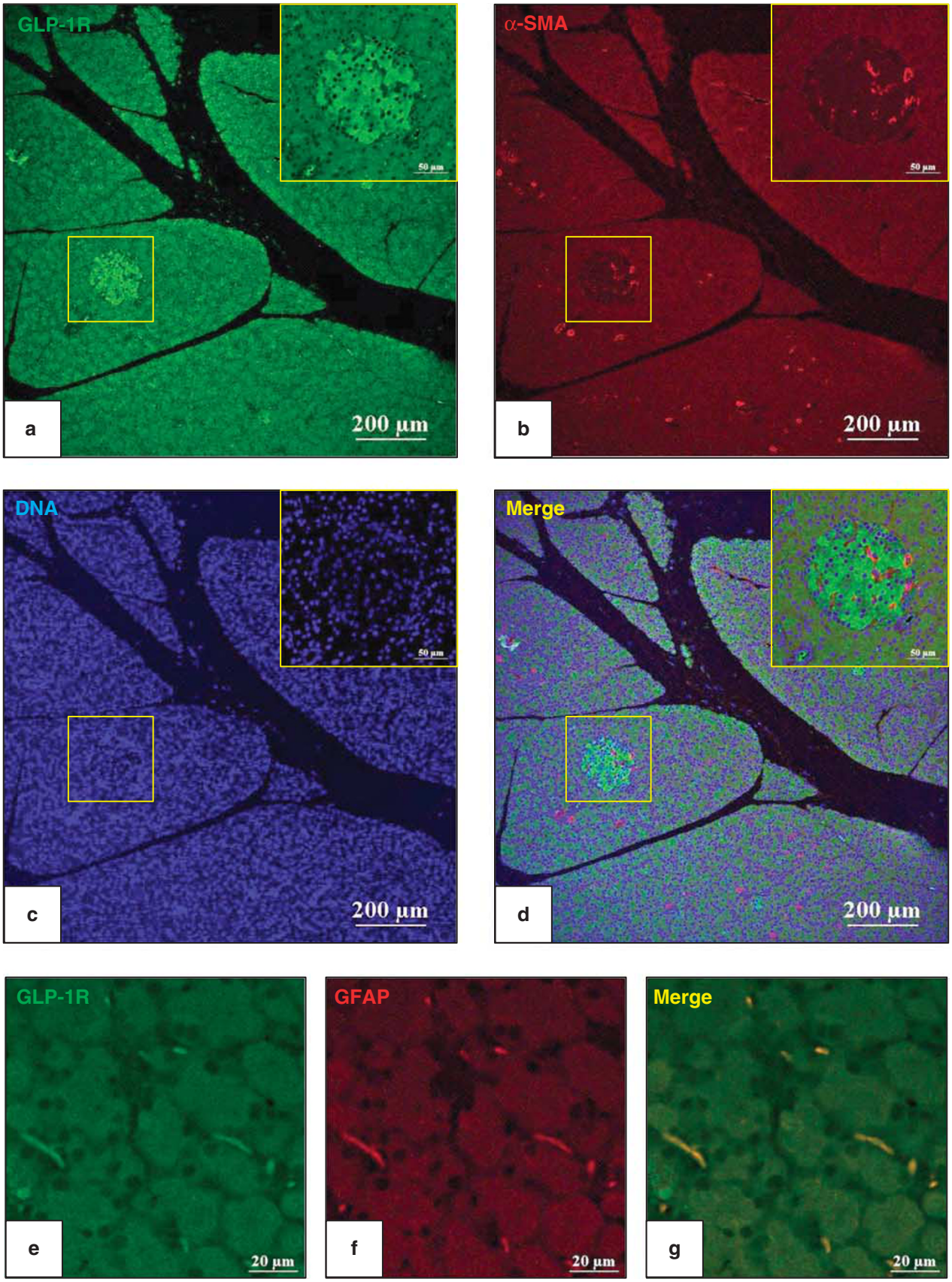
Statistical Analysis

Results are expressed as the means (s.e.m.) of 3–4 separate cell preparations. The two-tailed unpaired Student's *t*-test was used for the statistical analyses. *P*-values of <0.05 were considered statistically significant.

RESULTS

Expression of the GLP-1R in Normal Pancreas

Initially, we performed immunofluorescent staining to evaluate the expression of the GLP-1R in normal pancreas from male Wistar rats (Figures 1 and 2). As previously reported,[45] high expression of the GLP-1R occurred in pancreatic islets (Figure 1a). α-SMA-positive capillary vessel cells in the islets did not show GLP-1R stain (Figures 1b and d). Only weak staining for GLP-1R was seen in acinar cells. In the normal pancreas specimen, no α-SMA-positive PSCs (activated myofibroblast-like cells) were seen, however, GFAP-positive quiescent PSCs showed low expression of the GLP-1R (Figures 1 e–g). To investigate further expression of GLP-1R in normal islets, the GLP-1R distribution was examined in β cells (central islet area) and α cells (islet-periphery for GLP-1R; Figures 2a–f). GLP-1R was only present in β cells, a finding similar to some reports,[45,46] however, different from others, which reported the GLP-1R also in α cells.[47–49]


GLP-1R
50 μm
200 μm
a
α-SMA
50 μm
200 μm
b
DNA
50 μm
200 μm
c
Merge
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GLP-1R
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e
GFAP
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f
Merge
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g

Expressions of GLP-1R in Activated PSCs in the Pancreas of Rats with AP

We next investigated whether GLP-1R expression was present in a model of AP (L-arginine pancreatitis-model (72 h) and cerulein-induced pancreatitis-model (8 h)). Double-staining for α-SMA and GLP-1R (Figure 3) showed a marked increase in GLP-1R expression throughout the inflamed pancreas (Figures 3a–h) and also α-SMA-positive cells (activated PSCs) were seen throughout the pancreas, especially in the interstitial area (Figures 3b and f). Double-positive staining cells (yellow) were observed supporting the conclusion that GLP-1R was present on α-SMA-positive cells (activated PSCs; Figures 3d and h). In general, the changes were milder in the cerulein-induced AP model (Figures 3e–h) than in the model of L-arginine-induced AP (Figures 3a–d). To determine the degree that GLP-1R colocalized in activated PSCs, we performed quantitative immunohistochemistry of GLP-1R and α-SMA-positive cells (activated PSCs; Figure 3). GLP-1R was found in 83% of the activated PSCs. We also performed the reverse analysis by determining the percentage of GLP-1R-positive cells that were activated PSCs and found that (Figure 3a) it was 24%. Dilated pancreatic duct cells (DCs) also expressed the GLP-1R (Figure 3d).

GLP-1R Expression in Pancreatic Sections from Model of CP

We next performed similar studies in a model of CP (Wistar/Bonn–KOB rat; Figure 4). HE and MT (for collagen fiber) staining showed destruction of the normal pancreatic structure, as well as fibrosis in peri-islet-areas (P-IAs) and stromal fibrotic areas (FAs; Figures 4a–e). GLP-1R was markedly increased in both islets and acinar tissue. GLP-1R was also expressed in a type I collagen abundant lesion of the P-IA and FA, especially in spindle-shaped fibroblasts (Figures 4k–o).

Expression of GLP-1R in Activated PSCs in the Pancreas of Rats with CP

The possible expression of GLP-1R in activated PSCs in the CP model was investigated in more detail (Figure 5). To determine whether the spindle-shaped fibroblasts seen in Figure 4 were activated PSCs, we performed double-staining of α-SMA and GLP-1R in an area showing fibrosis (Figures 5a–d). A marked increase in α-SMA-positive cells was seen throughout the pancreas, especially near areas of pancreatic islet cell destruction (Figure 5b). In this area, double-positive stained cells (yellow) were observed for GLP-1R and α-SMA, demonstrating that GLP-1R was expressed on the most activated PSCs (Figure 5d). Similar to Figure 3a, we determined the degree that GLP-1R is colocalized with activated PSCs. GLP-1R is colocalized in 99% of the activated PSCs (shown by positive α-SMA stain). These data (Figures 3a and 5) demonstrate that by far the majority of activated PSCs in this chronic model of pancreatitis have GLP-1R. We also performed the reverse analysis by determining the percentage of GLP-1R-positive cells that were activated PSCs, and which was 71% in CP models (Figure 5a). These data support the conclusion that GLP-1R expression in these inflammatory conditions (AP/CP), can also occur in non-stellate cells (acinar cells, islet cells and inflammatory cells). The proportion of GLP-1R occurring in non-stellate cells is higher when the inflammation is mild (76% AP, Figure 3a; 29% CP, Figure 5). DCs also expressed GLP-1R (Figures 5a and d).

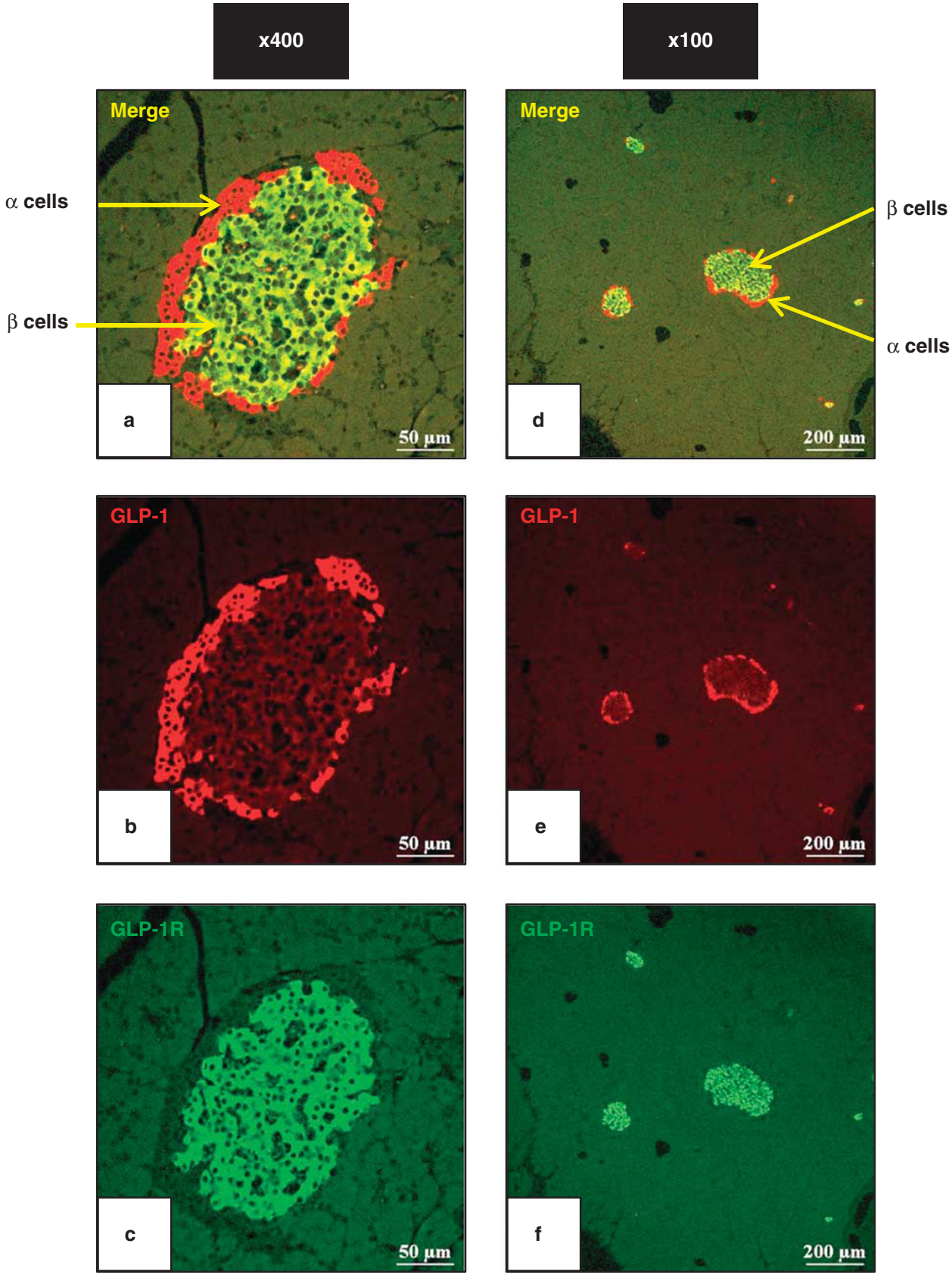
Both Quiescent and Activated PSCs from Normal Rat Expressed the GLP-1R *In Vitro* and the Expression is Increased in Activated PSCs

To further investigate the expression of GLP-1R in normal pancreas and the result of its activation, we studied PSCs isolated from normal pancreas in both a quiescent phase (first passage: 1 day; Figure 6a) and an activated phase (third passage) *in vitro* (Figure 6b). Activation of the PSCs during the third passage was confirmed by demonstrating the presence of immunofluorescent staining for α-SMA (Supplementary Figure S1). GLP-1R expression by immunohistochemical staining suggested that there was a marked increase in the expression in the PSCs, with activation (compare Figures 6a *vs* 6b). We confirmed that all of the PSCs in both quiescent and activated state expressed GLP-1R with nuclear staining (Supplementary Figure S1). Not only GLP-1R, but also other incretin-related mRNAs (GLP-2R, GIPR, GlucagonR, proglucagon, GIP and DPPIV) were expressed in PSCs in sequential 1–4 passages (Figure 6c). Quantitative real-time PCR revealed greater expression of GLP-1R, GLP-2R and GIP-R mRNA in activated PSCs from the third passage (activated) compared with quiescent PSCs from the first passage (quiescent) (Figure 6d).

The GLP-1 Agonist, Liraglutide, did not Stimulate Inflammatory mRNA Expression, but Stimulated Cell Proliferation of PSCs

We next examined whether the GLP-1R agonist, liraglutide, stimulated inflammatory responses in PSCs (Figure 7a). Liraglutide (1 μM) did not increase the mRNA expression of inflammatory cytokines (TNF-α, IL-6 and IL-1β) or

Figure 1 Expression of glucagon-like peptide-1 receptor (GLP-1R) in normal pancreas of Wistar rats. Expression of GLP-1R, alpha-smooth muscle actin (α-SMA) (activated pancreatic stellate cells (PSCs)) and glial fibrillary acidic protein (GFAP) (quiescent PSCs) in the pancreas of 15-week-old Wistar rats was examined by immunofluorescence staining. In the top four panels **a**–**d**, figures are × 100 and the area in the square is shown at × 200 in the insert. α-SMA (red) and GLP-1R expression (green) are shown. In the bottom panels **e**–**g**, figures are × 1000 magnification of an area showing two PSCs. GFAP (red) and GLP-1R expression (green) are shown. This figure demonstrates that acinar cells stained faintly for GLP-1R, whereas islets stained strongly, and that quiescent normal PSCs also have tGLP-1R expression. These pictures are representative of four experiments.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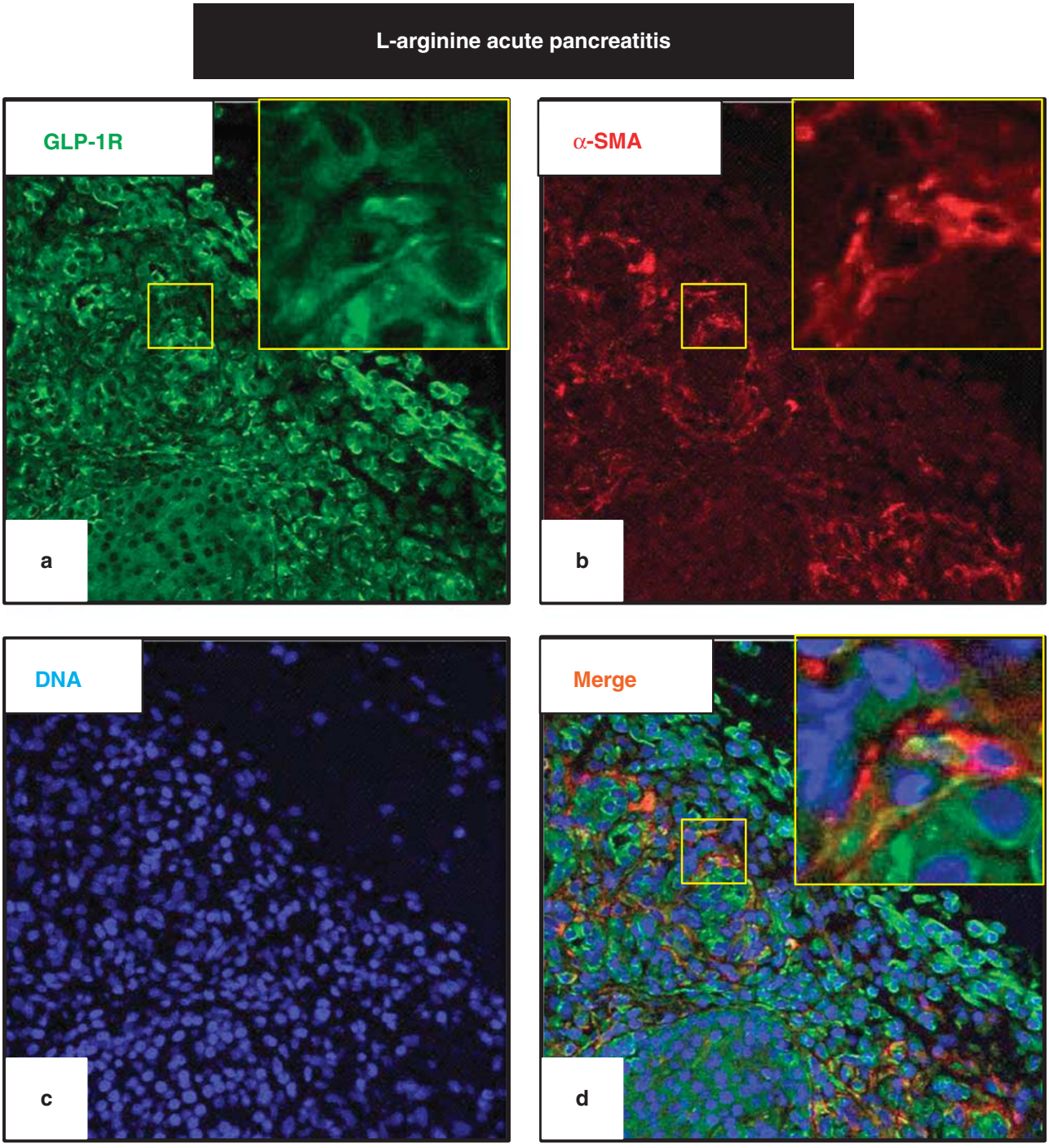


Figure 3 Glucagon-like peptide-1 receptor (GLP-1R) expression in activated pancreatic stellate cells (PSCs; alpha-smooth muscle actin (α-SMA) positive) in an area of acute inflammation of L-arginine-induced acute pancreatitis (AP). Expression of GLP-1R and α-SMA (to identify activated PSCs) in the pancreas of L-arginine-induced AP model rats and cerulein-induced AP model rats were examined by immunofluorescence staining. Panels **a**–**d** are × 400 magnification and the area in the square is shown in the insert at × 2000. Panels **e**–**h** are × 1000 magnification. Shown are GLP-1R expression (in green: **a**, **e**), α-SMA (in red: **b**, **f**) and nuclei (in blue: **c**, **g**). A merged image is shown in panels **d** and **h**. This figure shows AP. There is marked GLP-1R expression in activated PSCs (α-SMA-positive cells) and dilated pancreatic duct cells (DCs). These pictures are representative images from four experiments.

Figure 2 Expression of glucagon-like peptide-1 (GLP-1) in pancreatic islet cells of normal pancreas of Wistar rats. Expression of the GLP-1R in the pancreas of 15-week-old Wistar rats was examined by immunofluorescence staining. In the left panels **a**–**c**, figures are × 400 magnification of an area showing a pancreatic islet. GLP-1 (red) (α cells) and GLP-1R expression (green) are shown. In the right panels **d**–**f**, figures are × 100 magnification of an area showing expression in other islets (α and β cells). GLP-1 (red) and GLP-1R expression (green) are shown. This slide shows GLP-1R is present in β cells and not α cells. These pictures are representative of four experiments.

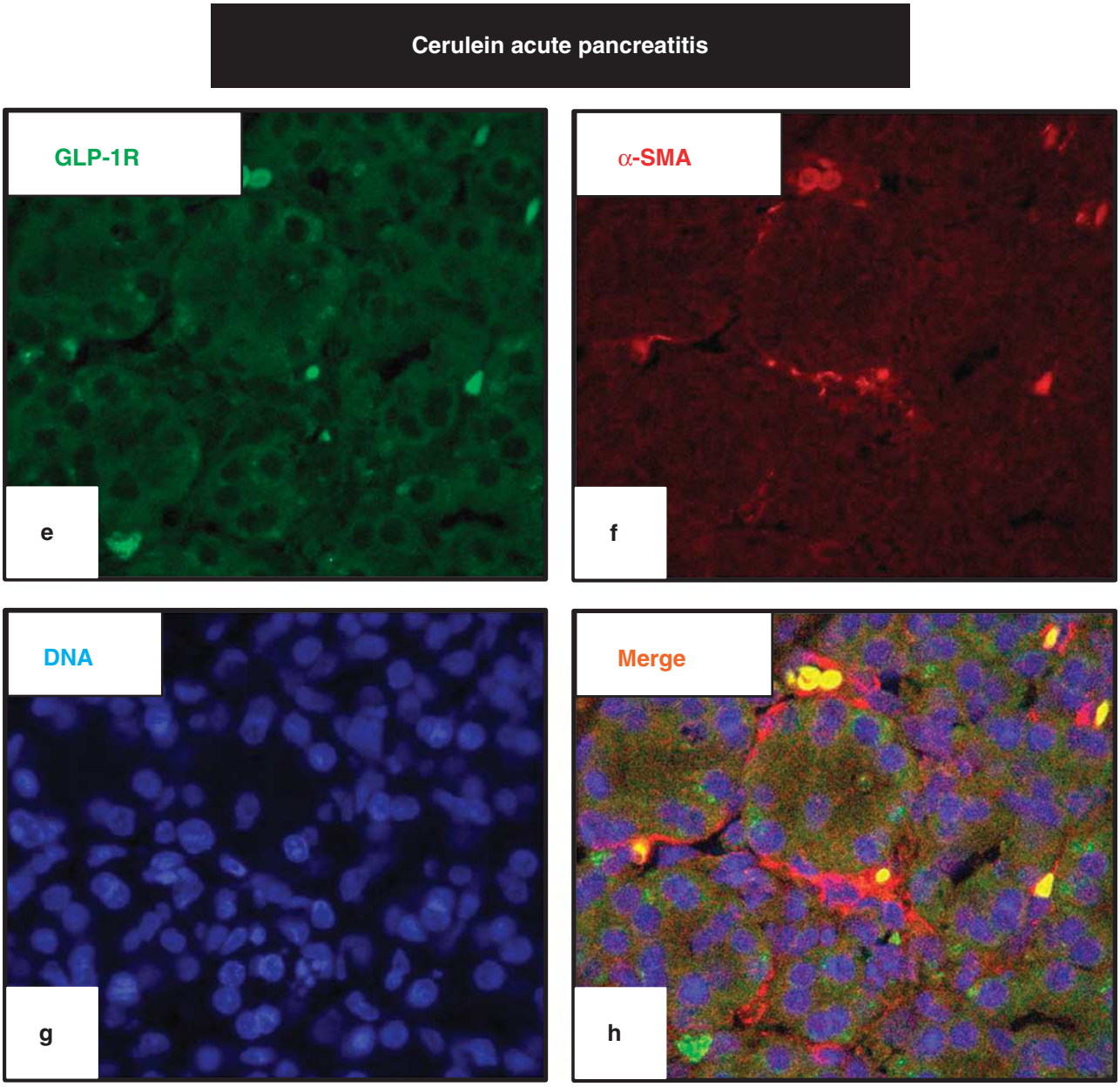


Figure 3 Continued.

chemokines (MCP-1, CINC-1 and CX3CL1; Figure 7a). α-SMA and type I collagen mRNA, which are cell activation markers, were also not increased by liraglutide (Figure 7a). However, liraglutide stimulated PSCs proliferation at 24 and 48 h after its addition (Figure 7b).

The GLP-1 Agonist, Liraglutide, Activated Various Cell Signaling Cascades in PSCs

Phosphorylation of MAPKs (ERK, JNK and p38) and Akt was rapidly stimulated by liraglutide (by 5 min) with a maximal response between 5 and 15 min (lanes 2 and 3; Figure 7c). Degradation of total IκB also occurred rapidly with liraglutide treatment indicating NF-κB activation. PDGF-BB was used as a positive control and activated each of the cell signaling pathways (lane 6).

ERK Activation Has an Essential Role in Liraglutide-Induced Cell Proliferation of PSCs

ERK activation in PSCs is important in stimulating the growth effects of numerous stimuli.[43,50] To assess this, U0126, an ERK inhibitor, at the indicated concentrations (1–10 μM) was added 30 min before liraglutide (10 μM). Liraglutide-induced cell proliferation was completely abolished by U0126, demonstrating ERK activation is important in mediating liraglutide-induced proliferation of PSCs (Figure 7d).

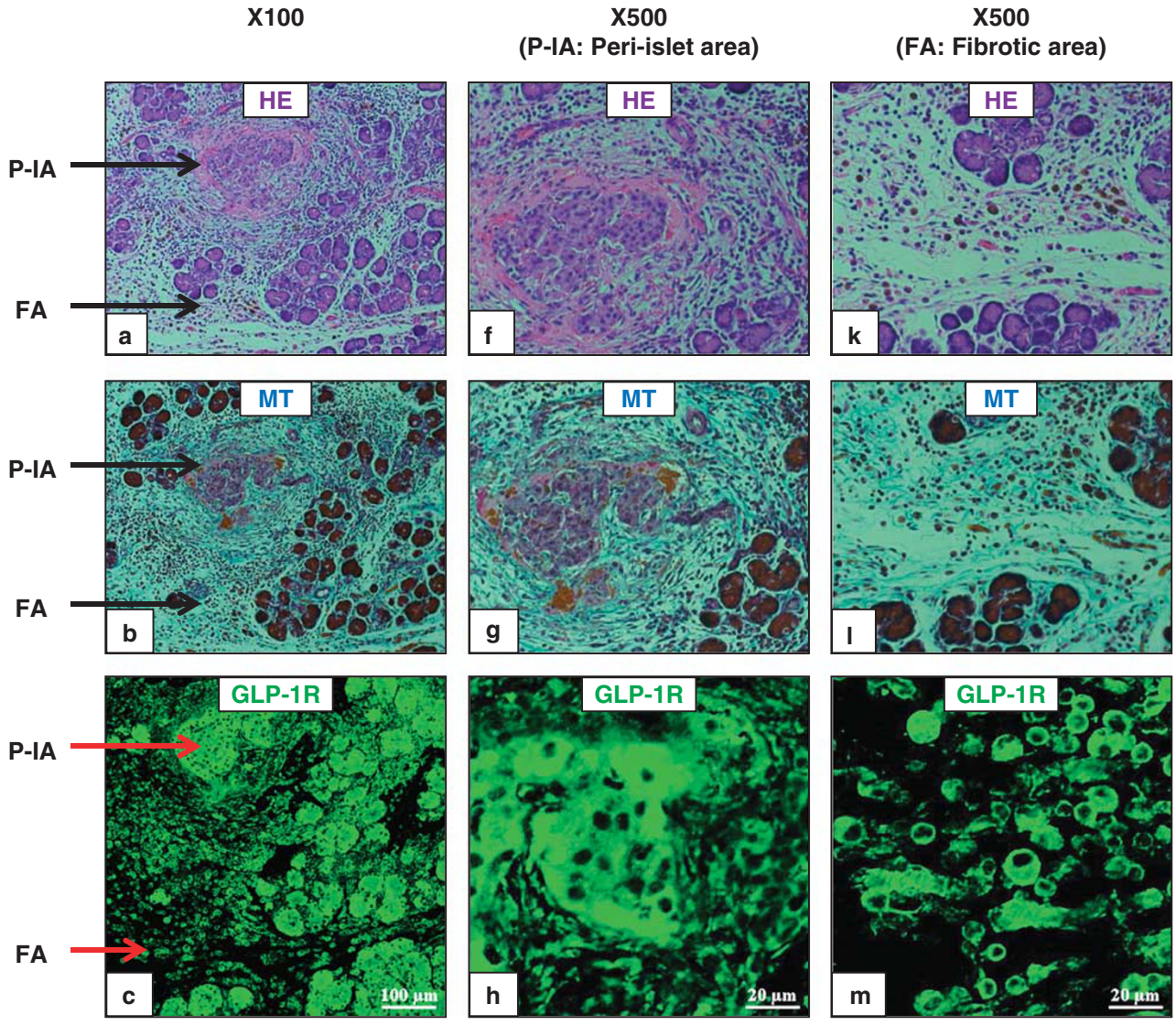


Figure 4 Glucagon-like peptide-1 receptor (GLP-1R) and type I collagen expression in pancreas of Wistar/Bonn–Kobori (WBN/Kob) rats (a chronic pancreatitis (CP) model). Shown are representative sections showing peri-islet area (P-IA) and a fibrotic area (FA) at the indicated magnifications (× 100, × 500). Expression of GLP-1R and type I collagen in the pancreas of 15-week-old WBN/Kob rats was examined by immunofluorescence staining. Panels **a**, **f** and **k** show hematoxylin-eosin (HE) staining, panels **b**, **g** and **l** Masson Trichrome (MT) staining for collagen fibrils, panels **c**, **h** and **m** GLP-1R staining (green), panels **d**, **i** and **n** type I collagen staining (blue) and panels **e**, **j** and **o** a merged image of type I collagen and GLP-1R. These pictures are from sequential sections showing the same area. This figure demonstrates in this CP model increased collagen deposition (MT staining) and increased GLP-1R expression in areas where increased collagen is present (merged picture). These pictures are representative images from four experiments.

DISCUSSION

GLP-1 analogs and DPPIV inhibitors are now one of the main drugs used for treatment in patients with diabetes mellitus.[1–3] The use of these drugs has generally been associated with few side-effects, however, a concern receiving increasing attention is the possible development of pancreatitis.[7,8] A number of studies report an association of GLP-1-agonist use and pancreatitis,[10–15] however, others either do not show this association or dispute it.[51–53] At present, the pathogenesis of this effect is unknown. PSCs are increasingly recognized as having an important role in various aspects of pancreatitis, especially CP.[26,27] PSCs secrete various inflammatory cytokines/chemokines,[29,30] which can participate in inflammatory processes and can also secrete collagen and have an essential role in fibrosis.[32,33] Numerous stimulants activate PSCs, including activation of some G-protein-coupled receptors (PGE2, angiotensin, CCK-A, CCK-B).[34–39] However, it is unknown if GLP-1R occurs on PSCs in normal pancreas, on activated PSCs in AP or CP or if this does occur, whether the GLP-1R agonists affect the PSC signaling cascade, PSC growth or ability to release inflammatory mediators. To address these questions, in this study we examined the occurrence of the GLP-1R in PSCs in normal pancreas, in AP/CP models, as well as the ability of

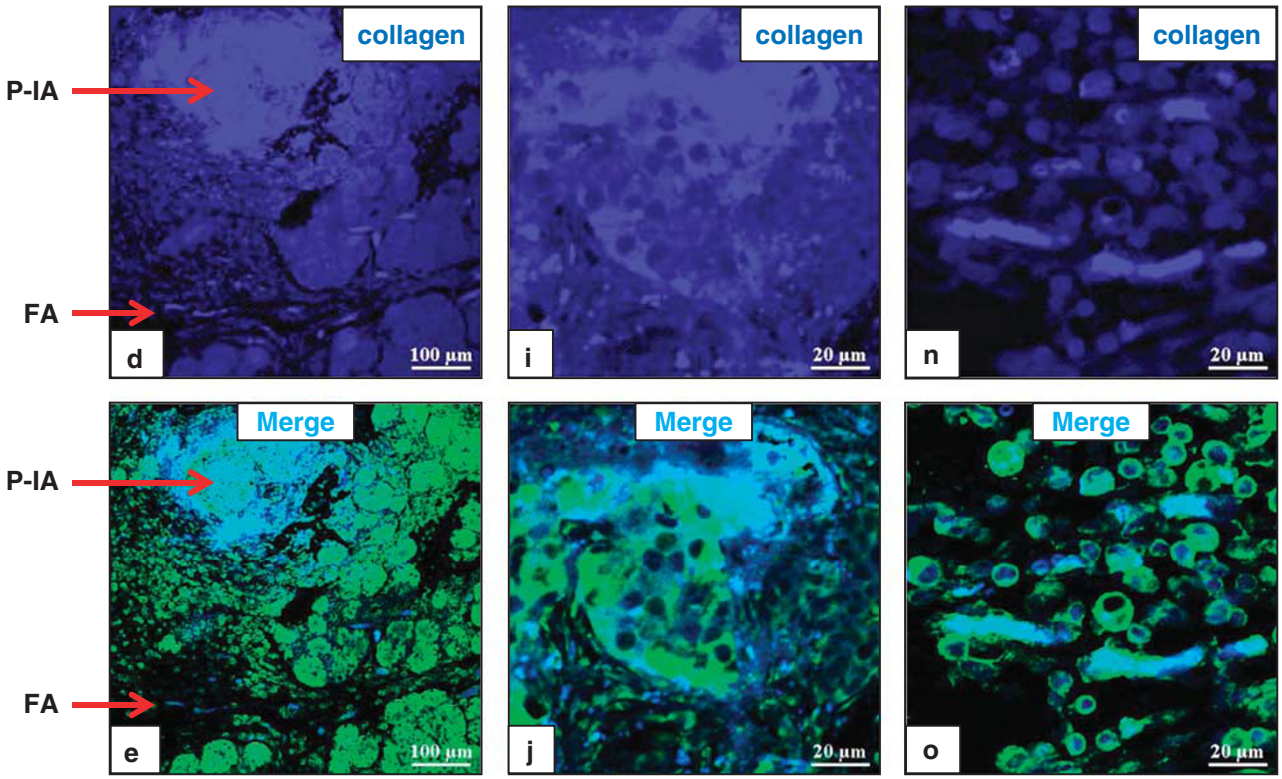


Figure 4 Continued.

the GLP-1R agonist, liraglutide, to alter function of PSCs and effect PSCs behavior.

We first examined GLP-1R expression in normal pancreas, followed by its expression in PSCs in the pancreas of various pancreatitis models (AP/CP). Our results show GLP-1R is present predominantly in islets of normal tissue, although it is also weakly present in acinar cells. In the normal islets, we found it present in β cells, not α cells, which is consistent with findings in some studies,[45,54] but differ from others.[47] GLP-1R has been identified by various methods on islets of numerous species (rat, human and hamsters)[55–57] and GLP-1R activation has also been shown to alter islet cell function (insulin release).[56] Hörsch *et al.*[45] reported that the GLP-1R had a high density in the β-cell zone and were not detected in the α-cell zone, similar to our results. This distribution pattern is in accordance with the numerous studies reporting GLP-1-binding sites on β-cell lines.[58,59] In contrast, the possibility of GLP-1R expression in α cells is controversial, with some reporting it is present,[47,48] whereas others report no GLP-1R in α cells, similar to our results.[45,54]

Our findings that normal pancreatic acinar cells have a low level of expression of GLP-1R is similar to those from some reports[60,61] but differ from others, which report no GLP-1R in normal pancreatic acinar cells by assessing GLP-1R mRNA levels.[45,57] Furthermore, the pancreatic acinar cell line, AR42J cells responds to GLP-1R agonist (increased cAMP)[60] raising the possibility that acinar cells might potentially contribute to liraglutide-induced pancreatitis. Further investigation focusing on acinar cell responses to liraglutide treatment would be required in the future. We did not find GLP-1R on DCs in normal pancreas. This result is similar to the findings in two other studies.[45,57] Finally, in normal pancreas, we did not find GLP-1R in normal pancreatic arteries. This result is consistent with another study.[62]

A number of our results, in both AP and CP models, are consistent with the conclusion that GLP-1Rs are expressed in activated PSCs and expression is increased over normal pancreas. First, we observed an increased number of activated PSCs in L-arginine-induced AP, which is consistent with findings from a previous report,[63,64] which demonstrated increased α-SMA expression (a marker for activated PSCs) coincided with TGF-β1 activation in this model. Second, in the CP model (WBN/KOB-rat), we found increased numbers of PSCs, which is consistent with findings in other studies.[65,66] Third, we found in both the pancreatitis models a marked overexpression of GLP-1R, which generally colocalized with activated PSCs or with areas of marked increased in PSC activity in the CP model with enhanced fibrosis. However, in our study, the localization of PSCs (peri-acinar and peri-islet) in pancreatitis differed in some aspects from other reports. In some studies, activated PSCs are reported in vascular walls,[37,66] whereas we could not find this. We did, however, find that dilated vascular structures in our AP model

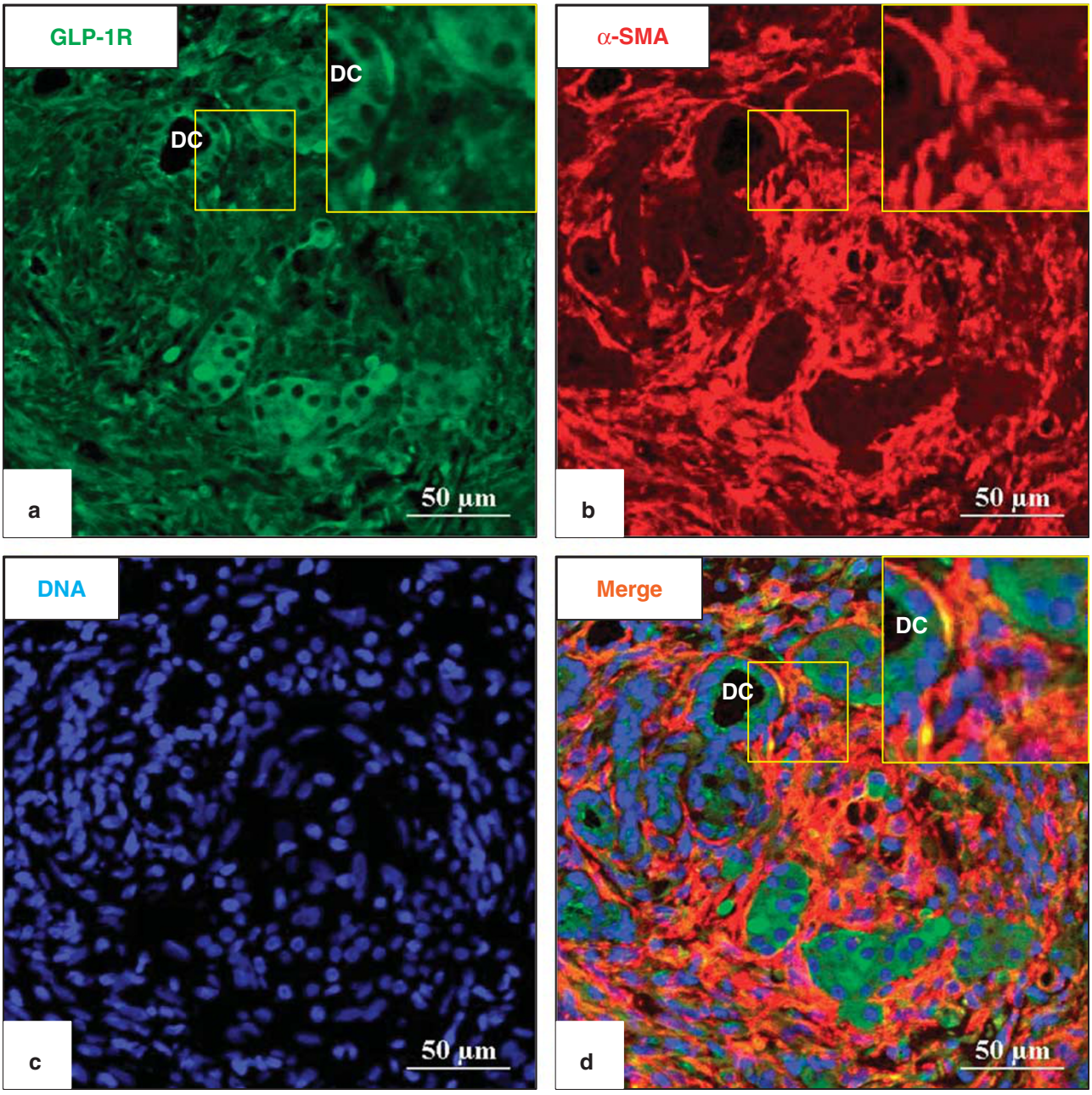
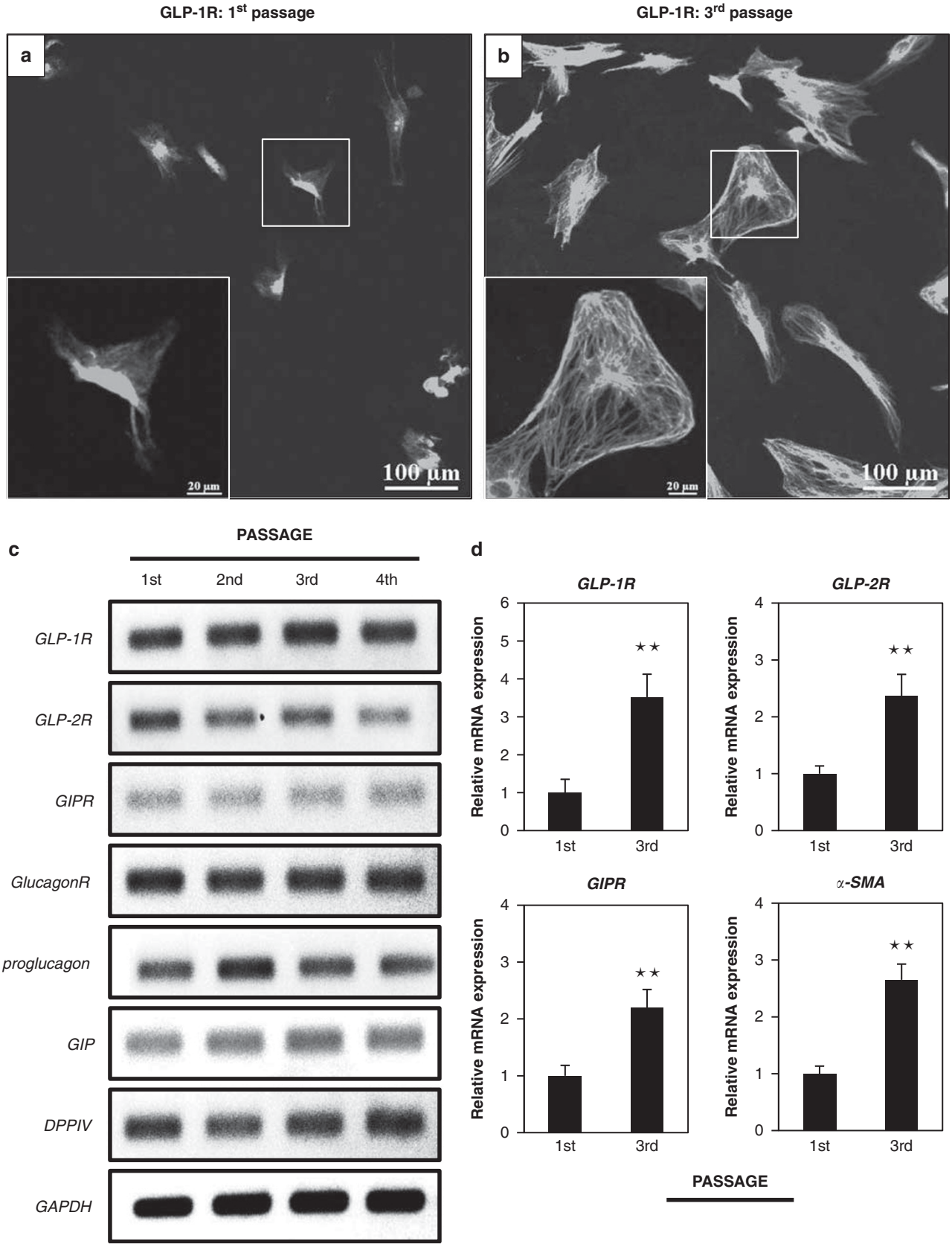


Figure 5 Glucagon-like peptide-1 receptor (GLP-1R) expression in activated pancreatic stellate cells (alpha-smooth muscle actin (α-SMA) positive) and surrounding areas in the pancreas of Wistar/Bonn–Kobori (WBN/Kob) rats (a chronic pancreatitis (CP) rat model). Expression of GLP-1R and α-SMA (markers for activated PSCs) in the pancreas of 15-week-old WBN/Kob CP model rats was examined by immunofluorescence staining. Panels **a**–**d** are × 500 magnification and the area in the square is shown in the insert at × 1000. Shown are GLP-1R expression (in green: **a**), α-SMA (in red: **b**) and nuclei (in blue: **c**). A merged image is shown in panel **d**. This figure shows in a CP model, the marked GLP-1R expression colocalizes with activated PSCs (α-SMA positive). These pictures are representative images from four experiments. DC, ductal cell.

stained positive for α-SMA. We found activated PSCs not only in peri-acinar lesions, but also in peri-islet lesions in the CP model that differs from that reported in another pancreatitis study, which showed only a peri-acinar location of PSCs.[65,66]

In quiescent PSCs, we found the expression of GLP-1R, as well as numerous other incretin-related receptors including the receptors for glucagon, gastric inhibitory peptide (GIP) receptor and GLP-2R. This result is similar to others in pancreatic β cells, which are reported to express glucagon receptors and GIP receptors as well as GLP-1R, whereas α cells express glucagon and GIP receptors, but no GLP-1Rs.[54] In culture-activated PSCs from normal pancreas, our results show there is a marked increase in the expression of GLP-1R


GLP-1R: 1st passage
GLP-1R: 3rd passage
a
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PASSAGE
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with activation, as well as an increase in numerous other incretin-related receptors. Similar to our results with GLP-1R, an increased expression in activated PSCs of the AT1 receptor,[36] ET receptor[67] and PAR2[68] have also been reported. These results suggest the possibility that these GPCRs, including GLP-1R may have an important role in conditions where PSCs are activated such as AP/CP, and may contribute to the various changes seen with these diseases.

With liraglutide treatment, we did not find an increased expression of mRNA's of inflammatory cytokines (TNF-α and IL-1β) or chemokines (MCP-1 and CX3CL1) in activated PSCs. These results are consistent with numerous findings in various studies in other tissues. Specifically, liraglutide did not induce ROS or increase PTX3 (an inflammatory signal) expression in endothelial cells in one study,[69] and in fact, it attenuated the increases in both ROS and PTX3 induced by TNF-α, suggesting it was not increasing inflammatory activity. Exendin-4, a GLP-1R agonist, also suppressed basal expression of several inflammatory mediators and in combination with phosphodiesterase inhibitors, decreased CXCL10 expression by IFN-γ (a type II-interferon) in human islets and in MIN6 cells (a mouse β-cell line).[70] Finally, GLP-1 attenuated IL-1β-mRNA expression induced by lipopolysaccharide (a component of Gram-negative bacteria) in rat astrocytes.[71] These results are consistent with the conclusion that in PSCs and other cells with GLP-1Rs, GLP-1R agonists do not stimulate inflammatory responses, and may, in fact, attenuate their development by other inflammatory stimulants. From our data, we cannot establish whether the prolonged GLP-1R agonist treatment affects quiescent PSCs and induces them to proliferate and stay activated. Therefore, at present we cannot address the events of induction of the pancreatitis.

GLP-1 has not only insulinotropic effects in β cells, but also has cell-proliferative and anti-apoptotic effects.[72,73] A proliferative effect by GLP-1 agonists has also been reported in mouse skin cells and the pancreatic acinar cell line, AR42J.[74,75] Our results show that the GLP-1R agonist, liraglutide, stimulated proliferation of PSCs. These results are similar to the effects of activation of two other GPCRs on PSCs, the AT1R and PAR2-R, which also result in PSC cell proliferation.[35,36,68] In contrast, our results differ from activation of ET-1 receptors on PSCs, which does not stimulate proliferation.[67,76] The role of the proliferative effect on PSCs of liraglutide and the other GPCRs in AP or CP at present is unknown, as is the possibility that prolonged treatment with GLP-1R agonists for diabetes or other diseases, may lead to increased fibrosis due to enhanced PSC growth and activation.

In other cells, various signaling cascades, including activation of MAPK, phospho-inositol-3-phosphate kinase (PI3K), adenylate cyclase and PKCζ by GLP-1 agonists, mediate the effects of GLP-1R activation on various cellular functions including insulin secretion and biosynthesis, β-cell proliferation and neogenesis.[16,77,78] We found several signaling cascades are activated by liraglutide in PSCs including ERK, JNK, p38, Akt and IκBα. Furthermore, the ERK activation was essential for GLP-1R-mediated PSC proliferation. These results have similarities and differences from GLP-1R-mediated growth reported in other cells. GLP-1-induced cell proliferation of β cells is inhibited by a ERK inhibitor, suggesting the involvement of ERK.[73] Furthermore, ERK activation by GLP-1 in mouse skin cells[74] mediates its proliferative effect. In contrast, in other studies,[72,73] protein kinase A, PI3K and PKC-zeta activation are needed for GLP-1-induced cell proliferation of β cells. Our finding of high basal ERK level and a rapid increase in ERK activation with liraglutide that return to normal by 30 min is similar to reports by others in PSCs with other stimuli and in other cells with ERK activation.[79,80] Our results with GLP-1R-mediated PSC proliferation also have similarities and differences with the ability of other GPCRs to stimulate growth in PSCs. Similar to our findings, PSC cell proliferation induced by activation of ERK signaling pathway is needed for growth mediated by PAR2-receptor or angiotensin-receptor activation, however, the latter effect requires EGFR transactivation.[35,68] These results show that ERK is one of the important signal cascades mediating cell proliferation of PSCs.

In conclusion, we demonstrated the GLP-1R expression in normal pancreas occurs primarily in β cells and only weakly in acinar cells or quiescent PSCs. However, in AP/CP there is marked increase in GLP-1R, primarily in PSCs. Furthermore, with activation of normal PSCs, there is marked increase in the GLP-1R level. However, in activated PSCs, liraglutide, a GLP-1R agonist, did not stimulate release of inflammatory cytokines/chemokines, however, it stimulated PSC proliferation, which was dependent on ERK activation. Chronic GLP-1 use in humans has recently been reported to cause expansion of both exocrine and endocrine pancreas and led to concerns about its unattended proliferative effects in these pancreatic tissues.[81] Our study

Figure 6 PSCs express incretin-related genes as well as GLP-1R *in vitro* and the expression increases with PSC activation. In the top panels **a**–**b**, expression of GLP-1R protein in both freshly isolated PSCs (first) and culture-activated PSCs (third passage) are shown. Magnification is × 200 and the area in the box is shown in the insert at × 500. Panel **c** shows a representative RT-PCR result for different incretin-related mRNAs at passages 1–4. Panel **d** shows the quantitation of RT-PCR result from four experiments for passages 1–3. $^{**}P<0.01$, comparing passages 1–3. These results were representative of four experiments. DPPIV, dipeptidyl peptidase IV; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLP-1R, glucagon-like peptide receptor-1; GLP-2R, glucagon-like peptide receptor-2; GIPR, gastric inhibitory peptide receptor; GlucagonR, glucagon receptor; GIP, gastric inhibitory peptide; PSC, pancreatic stellate cell.

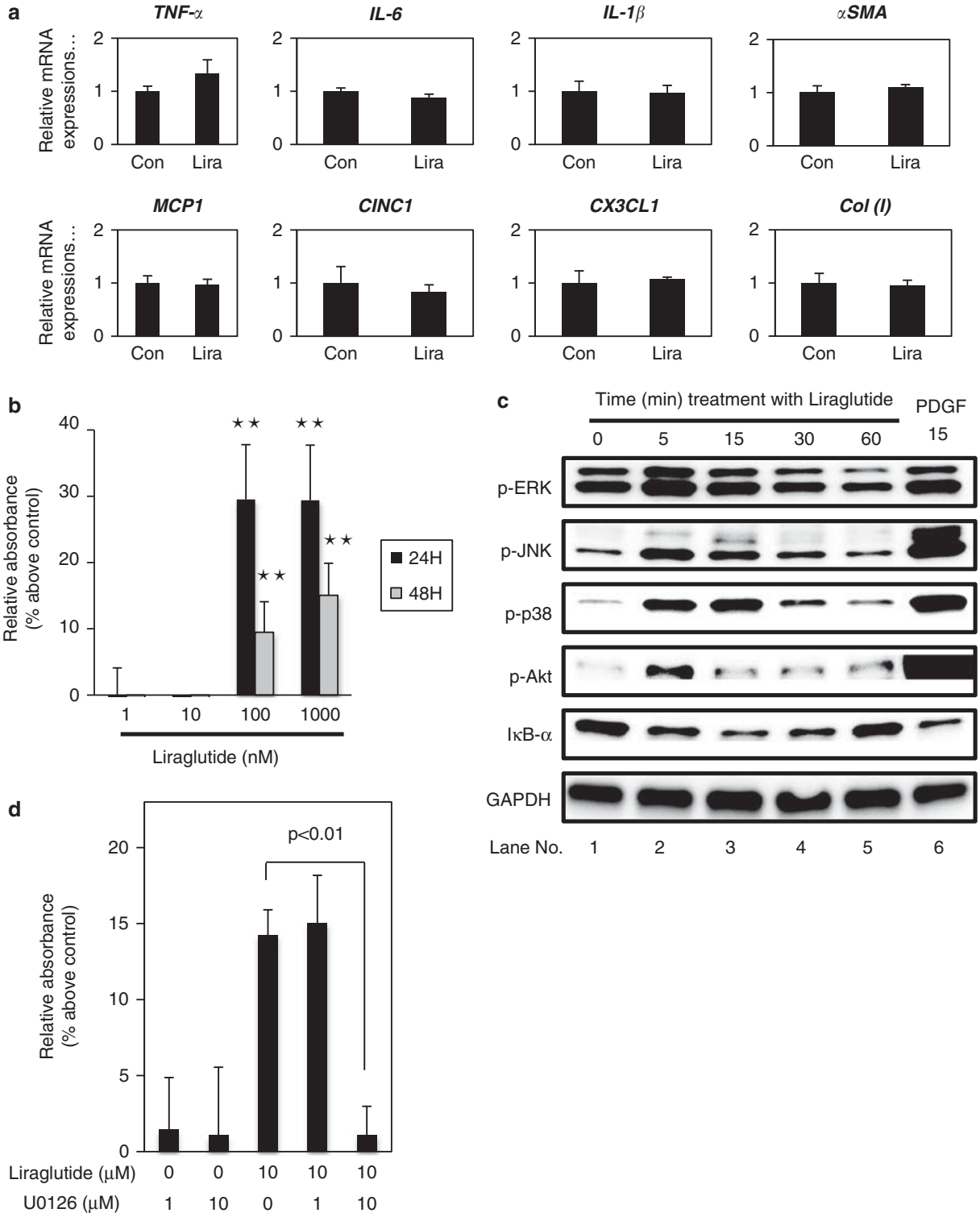

Figure 7 Effects of the glucagon-like peptide-1 (GLP-1) agonist, liraglutide, on inflammatory mediators, proliferation of PSCs and activation of signaling cascades, and effects of the ERK inhibitor, U0126, on basal or liraglutide stimulated growth. Panel **a** shows the RT-PCR results from four experiments for passages 3–5 of PSCs, comparing non-treatment (Con) with treatment with liraglutide (1000 nM) for 6 h. Panel **b** shows the concentration-dependent effect of liraglutide on cell proliferation after 24 and 48 h. Panel **c** shows the liraglutide-induced signaling cascade activation. Results for phospho-ERK, phospho-JNK, phospho-p38 and phospho-Akt were determined using specific phospho-antibodies. Results with IκB-α were determined using a specific IκB-α antibody. The results were determined at the indicated times with liraglutide and after a 15 min incubation with PDGF-BB. Panel **d** shows effects of U0126, a specific inhibitor of ERK. PSCs (passages 3–5; 2000 cells per well) were pre-incubated for 24 h in serum-free medium and then incubated for 24 h with indicated concentrations of liraglutide alone or with U0126. U0126 was added 30 min before liraglutide. Growth was determined by MTS assay using coloriometric microplate reader. Results are the means ± s.e.m. from four experiments. $^{**}P < 0.01$, comparing untreated and treated with liraglutide.

raises a similar concern for its long-term proliferative effect on PSCs. At present, it remains unclear whether these changes in PSCs contribute to the development of pancreatitis seen in patients with chronic use of GLP-1R agonists, or whether they could lead to complications of the increased PSCs proliferation due to long-term use of GLP-1 agonists.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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