Glucagon-Like Peptide-1 Receptor Activation Modulates Pancreatitis-Associated Gene Expression But Does Not Modify the Susceptibility to Experimental Pancreatitis in Mice

Jacqueline A. Koehler, Laurie L. Baggio, Benjamin J. Lamont, Safina Ali, and Daniel J. Drucker

OBJECTIVE—Clinical reports link use of the glucagon-like peptide-1 receptor (GLP-1R) agonists exenatide and liraglutide to pancreatitis. However, whether these agents act on the exocrine pancreas is poorly understood.

RESEARCH DESIGN AND METHODS—We assessed whether the antidiabetic agents exendin (Ex)-4, liraglutide, the dipeptidyl peptidase-4 inhibitor sitagliptin, or the biguanide metformin were associated with changes in expression of genes associated with the development of experimental pancreatitis. The effects of Ex-4 when administered before or after the initiation of caerulein-induced experimental pancreatitis were determined. The importance of endogenous GLP-1R signaling for gene expression in the exocrine pancreas and the severity of pancreatitis was assessed in *Glp1r*$^{-/-}$ mice.

RESULTS—Acute administration of Ex-4 increased expression of egr-1 and c-fos in the exocrine pancreas. Administration of Ex-4 or liraglutide for 1 week increased pancreas weight and induced expression of mRNA transcripts encoding the anti-inflammatory proteins pancreatitis-associated protein (PAP) (RegIIIβ) and RegIIIα. Chronic Ex-4 treatment of high-fat–fed mice increased expression of PAP and reduced pancreatic expression of mRNA transcripts encoding for the proinflammatory monocyte chemotactic protein-1, tumor necrosis factor-α, and signal transducer and activator of transcription-3. Sitagliptin and metformin did not significantly change pancreatic gene expression profiles. Ex-4 administered before or after caerulein did not modify the severity of experimental pancreatitis, and levels of pancreatic edema and serum amylase were comparable in caerulein-treated *Glp1r*$^{-/-}$ versus *Glp1r*$^{+/+}$ mice.

CONCLUSIONS—These findings demonstrate that GLP-1 receptor activation increases pancreatic mass and selectively modulates the expression of genes associated with pancreatitis. However, activation or genetic elimination of GLP-1R signaling does not modify the severity of experimental pancreatitis in mice. ***Diabetes*** **58:2148–2161, 2009**

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Glucagon-like peptide (GLP)-1, a peptide hormone secreted by enteroendocrine cells in the distal small bowel and colon, exerts a diverse set of complementary actions on islet β-cells, resulting in glucose-dependent augmentation of insulin biosynthesis and secretion (1). GLP-1 also restores glucose sensitivity to diabetic β-cells and promotes expansion of β-cell mass via stimulation of β-cell proliferation and enhancement of β-cell survival (2). Moreover, exogenous GLP-1 administration inhibits glucagon secretion and gastric emptying and induces satiety, leading to weight loss after prolonged GLP-1 receptor (GLP-1R) activation (3). Taken together, these actions of GLP-1 lead to significant improvement in glucose homeostasis and have fostered the development of GLP-1R agonists, exemplified by synthetic exendin (Ex)-4, for the treatment of type 2 diabetes (3,4).

The majority of studies examining GLP-1 biology in the pancreas has focused on α- and β-cells within the endocrine pancreas; however, GLP-1 exerts a number of actions in the exocrine pancreas. GLP-1R agonists induce transdifferentiation of pancreatic exocrine cells to an endocrine cell phenotype in vitro (5), and GLP-1 inhibits hypoglycemia-induced pancreatic bicarbonate and protein secretion in the isolated perfused pig pancreas (6). Moreover, exogenous GLP-1 induces neural transmission converging on the pancreas via depolarization of neurons within the dorsal motor nucleus of the vagus that project to the exocrine pancreas (7).

Although the biology of GLP-1 action in the exocrine pancreas remains poorly understood, the clinical use of the first approved GLP-1R agonist, exenatide, has been associated with case reports of pancreatitis, and some have speculated this may be related to the venomous origin of the Ex-4 peptide from the lizard (8). Although there is limited scientific information linking GLP-1 receptor activation to the pathogenesis of pancreatic inflammation, pancreatitis has also been reported in clinical trials of the human GLP-1R agonist liraglutide (9). In contrast, analysis of a health care claims database did not reveal an increased incidence of pancreatitis in hospitalized patients previously treated with exenatide versus other antidiabetic agents (10).

As GLP-1 inhibits pancreatic exocrine secretion (6), a putative mechanism associated with the development of pancreatitis (11), it seems possible that sustained GLP-1 receptor activation will increase the susceptibility for development of pancreatic inflammation. Ex-4 regulates the pancreatic expression of the Reg gene family (12), and

changes in expression of RegIIIβ (also known as pancreatitis-associated protein [PAP]) have been associated with divergent effects on pancreatitis susceptibility and pancreatic necrosis in vivo (13,14).

Accordingly, we examined whether GLP-1 receptor activation modulates expression of genes known to be associated with the development of inflammation or acute pancreatitis in mice. We employed two structurally distinct GLP-1R agonists, Ex-4 and liraglutide, as well as a dipeptidyl peptidase (DPP)-4 inhibitor, sitagliptin, and the biguanide metformin to assess pancreatitis-associated gene expression in mice. The effect of Ex-4 administration before or after the administration of low-dose caerulein, a chemical cholecystokinin (CCK) mimic that produces secreatogue-induced pancreatitis (15), was assessed in wild-type mice, and changes in pancreatic gene expression and the susceptibility to pancreatitis were analyzed in *Glp1r*$^{-/-}$ mice.

Experimental procedures. Experiments were carried out according to protocols approved by the animal care committees of the University Health Network and Mount Sinai Hospital. Mice were housed under a 12-h light/dark cycle with access to standard or high-fat rodent diet (45% kcal from fat; Research Diets, New Brunswick, NJ). Male C57Bl/6J mice (8–10 weeks old) were obtained from Taconic Laboratories (Hudson, NY) and were allowed to acclimatize to the animal facility for 7 days before each experiment. *Glp1r*$^{-/-}$ mice in the C57Bl/6J background were generated as described (16).

Reagents. Ex-4 was from Chi Scientific (Maynard, MA) and dissolved in PBS. Forskolin and caerulein were from Sigma Chemical (St. Louis, MO). Caerulein was dissolved in saline (0.9% NaCl) and forskolin in DMSO. Liraglutide was from Novo Nordisk (Bagsværd, Denmark). Sitagliptin was from Merck (Rahway, NJ).

Induction of experimental pancreatitis. Pancreatitis was induced in 9- to 11-week-old male C57Bl/6J mice or 3-month-old male *Glp1r*$^{-/-}$ and littermate control *Glp1r*$^{+/+}$ mice using the CCK receptor agonist caerulein (17). Caerulein dose-response experiments were performed in male 9- to 11-week-old C57Bl/6J mice to identify a submaximal dose of caerulein that would produce a low level of pancreatic inflammation. Secretagogue-induced pancreatitis was elicited by administration of five sequential hourly intraperitoneal injections of caerulein, at doses of 6, 12, 24, or 50 μg/kg body wt as described (11,17,18); control animals received an equal volume of saline. Mice were killed by CO_2 inhalation 1 h after the final caerulein injection.

Pretreatment with Ex-4. To assess whether prior GLP-1R activation exacerbates the subsequent development of pancreatitis, male C57Bl/6J mice were injected (intraperitoneally) twice daily with either 10 nmol/kg Ex-4 or PBS for 7 days. Sixteen hours after the last Ex-4 injection, mice were administered 5 hourly intraperitoneal injections of caerulein at 3 or 6 μg/kg body wt, as indicated, and killed by CO_2 inhalation 1 h after the final caerulein injection.

Posttreatment with Ex-4. To assess whether a low level of pancreatitis was worsened by subsequent GLP-1R activation, male C57Bl/6J mice were administered 5 hourly intraperitoneal injections of caerulein (6 μg/kg) followed by twice daily intraperitoneal injections with either 10 nmol/kg Ex-4 or PBS for up to 6 days. Mice were killed 1, 24, 72, or 144 h (day 0, 1, 3, and 6, respectively) after the final caerulein injection such that mice killed on day 1 received two injections of PBS or Ex-4, one in the evening (~3 h after the last caerulein injection) and one in the morning (~6 h before being killed). Similarly, the final injection with Ex-4 or PBS was 6 h before being killed for

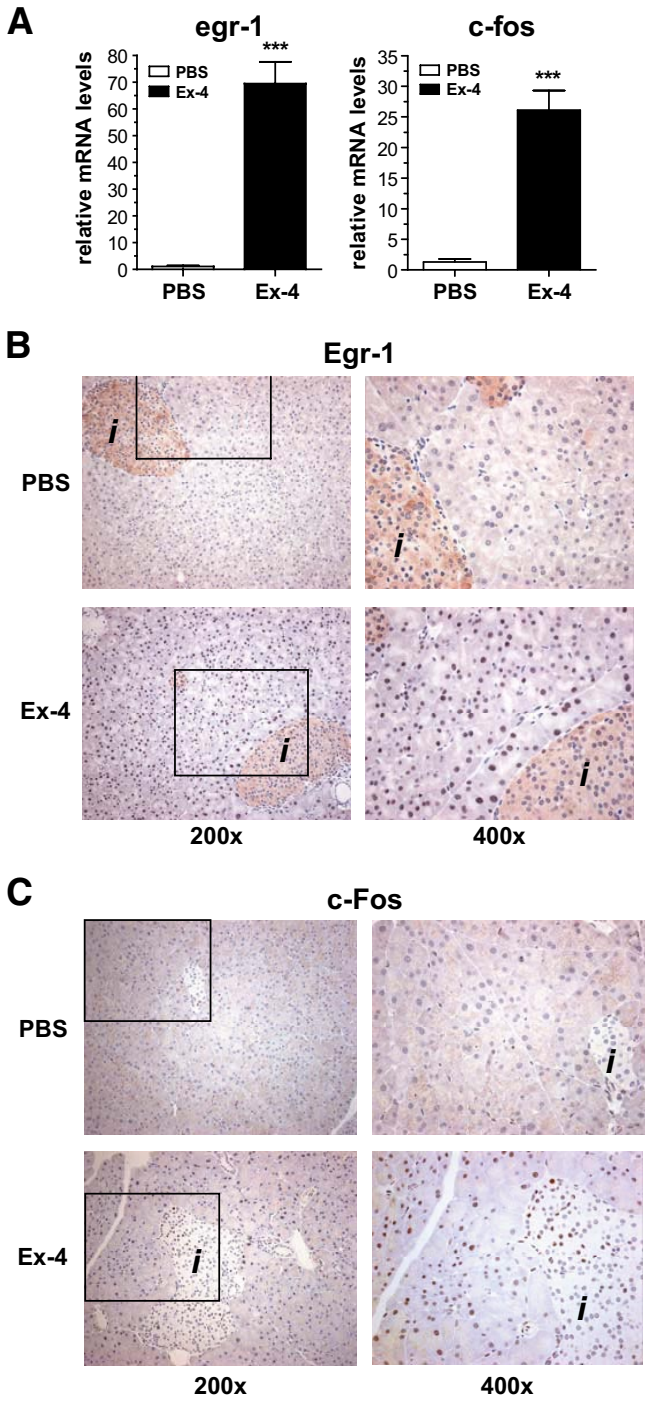
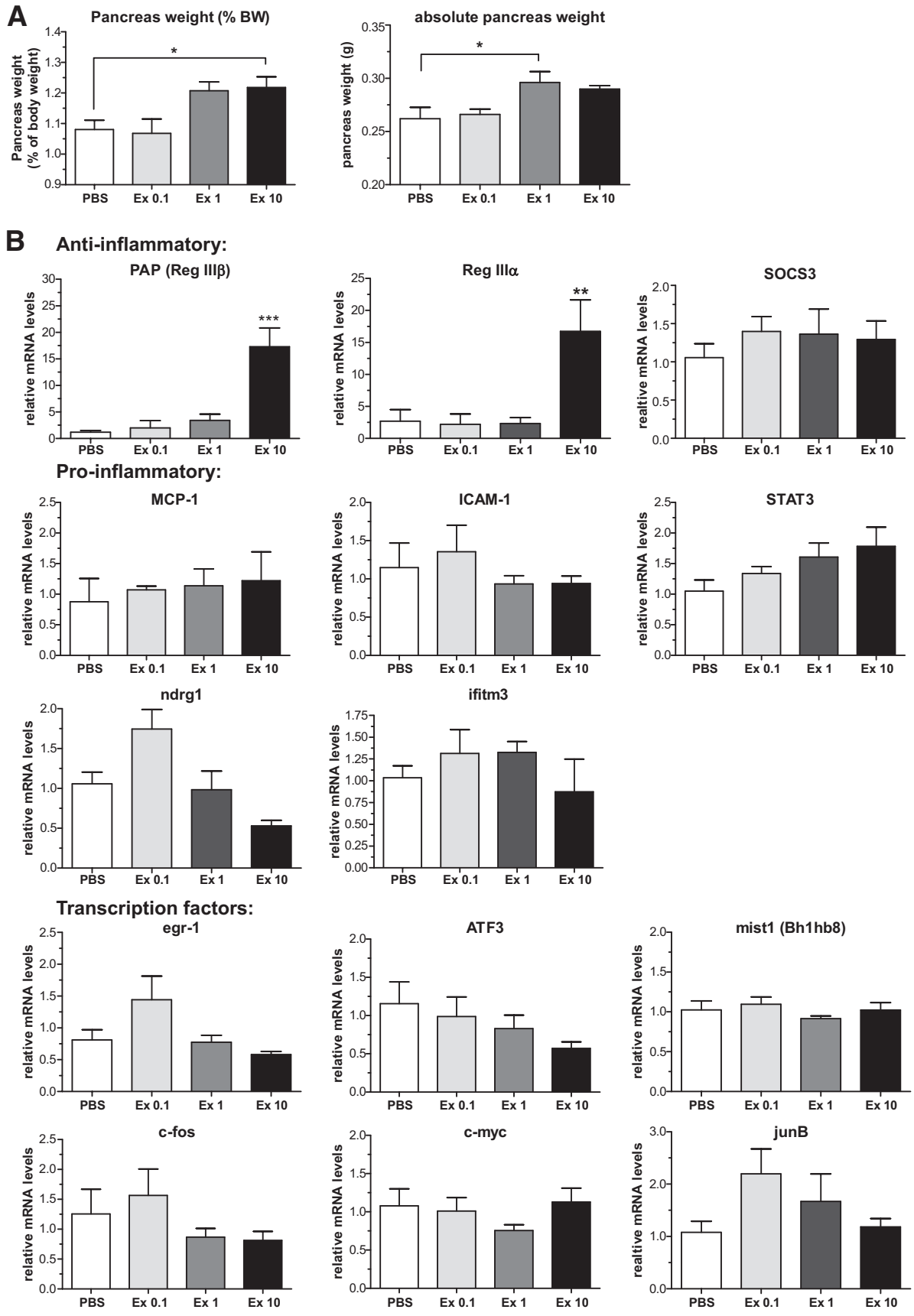


FIG. 1. Acute induction of egr-1 and c-fos transcripts by Ex-4. Male C57Bl/6J mice were subcutaneously injected with a single dose of Ex-4 (1 μg) or vehicle alone (PBS). *A*: Total RNA was isolated from pancreas 45 min after injections and reverse transcribed, and the levels of the indicated transcripts were determined by real-time PCR, normalized to 18S rRNA content, and shown relative to the control (PBS)-treated group. Results are expressed as means ± SE. ***$P < 0.001$ PBS- versus Ex-4–treated mice, $n = 4$ in each group. Immunohistochemical localization of egr-1 (*B*) and c-fos (*C*) in the pancreas of mice treated for 45 min with vehicle (PBS) or Ex-4. Photomicrographs are representative of four mice per group. Islets are represented with an *i*. Magnification ×200 or ×400. (A high-quality digital representation of this figure is available in the online issue.)


A
Pancreas weight (% BW)
Pancreas weight (% of body weight)
absolute pancreas weight
pancreas weight (g)
PBS
Ex 0.1
Ex 1
Ex 10
B
Anti-inflammatory:
PAP (Reg IIIβ)
Reg IIIα
SOCS3
relative mRNA levels
realtive mRNA levels
Pro-inflammatory:
MCP-1
ICAM-1
STAT3
ndrg1
ifitm3
Transcription factors:
egr-1
ATF3
mist1 (Bh1hb8)
c-fos
c-myc
junB

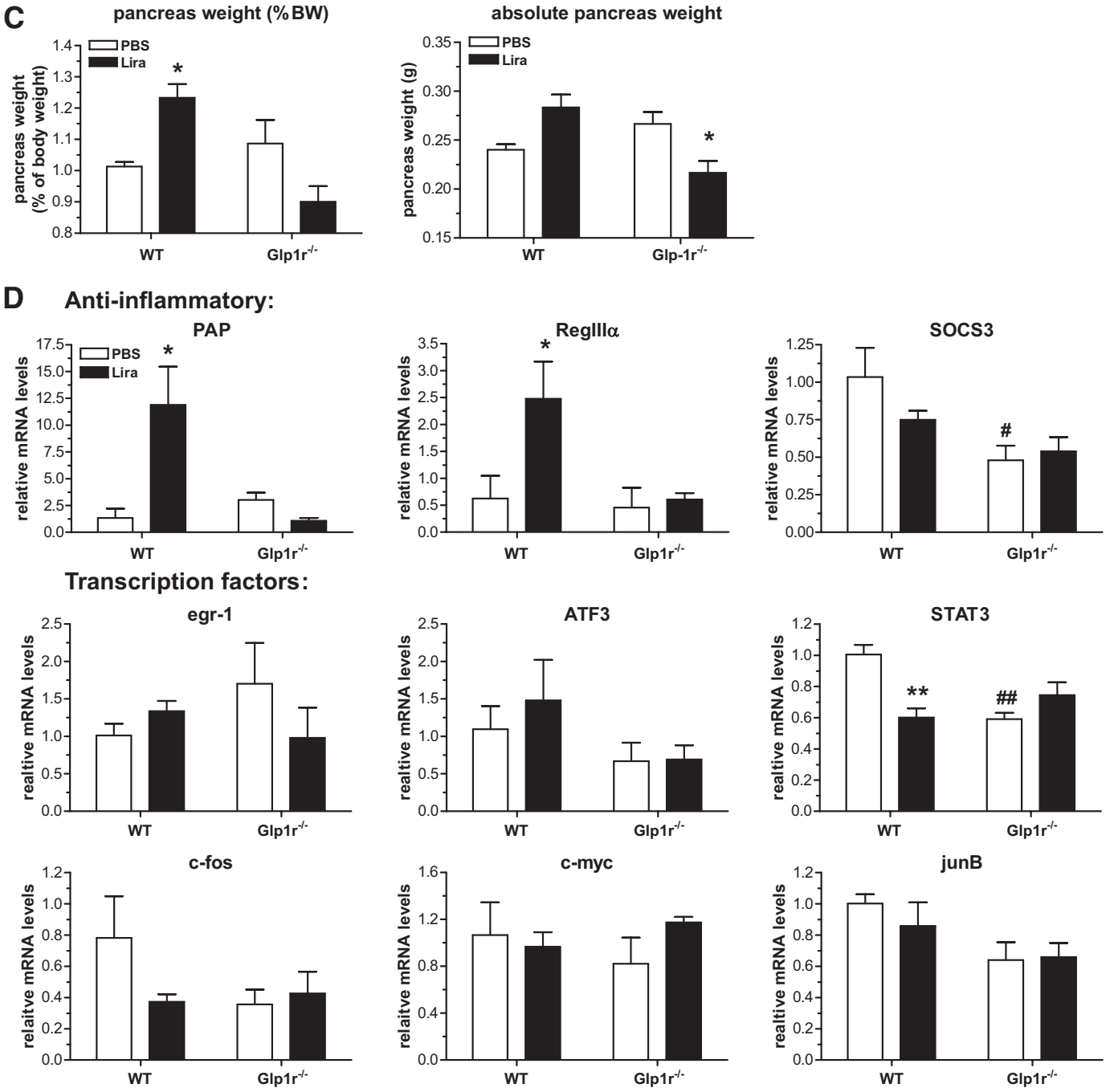


FIG. 2. GLP-1R agonists increase pancreatic weight and regulate gene expression in a GLP-1R–dependent manner. *A*: Pancreas weight of mice treated twice daily with the indicated doses of Ex-4 for 1 week is shown as a percentage of the final body weight (*left panel*) or absolute pancreas weight (*right panel*). Results are expressed as means ± SE. *$P < 0.05$ PBS- versus Ex-4–treated mice, $n = 5$ in each group. *B*: Expression profiles of genes associated with pancreatitis in the pancreas of mice treated twice daily for 1 week with the indicated doses of Ex-4 (0.1–10 nmol/kg). Total RNA was isolated from pancreas samples and reverse transcribed, and the levels of the indicated transcripts were determined by real-time quantitative PCR, normalized to levels of 18S or cyclophillin mRNA transcripts (for SOCS3, STAT3, junB, ndrg1, and ifitm3), and shown relative to the control (PBS)-treated group. Results are expressed as means ± SE. **$P < 0.01$, ***$P < 0.001$ PBS- versus Ex-4–treated mice, $n = 5$ in each group. Pancreas weight shown as a percentage of the final body weight (*left panel*) or absolute pancreas weight (*right panel*) (*C*), and expression profiles of genes associated with pancreatitis (*D*) in the pancreas of wild-type *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice treated twice daily for 1 week with 75 μg/kg liraglutide. Levels of mRNA transcripts were determined by real-time quantitative PCR, normalized to the 18S rRNA content, and shown relative to control (PBS)-treated wild-type mice. Results are expressed as means ± SE. *$P < 0.05$, **$P < 0.01$ PBS- versus liraglutide-treated mice, #$P < 0.05$, ##$P < 0.01$ wild-type versus *Glp1r*$^{-/-}$ mice, $n = 3$ in each group.

all remaining mice (day 3 and 6). To determine whether loss of GLP-1R signaling protected mice from development of experimental pancreatic inflammation, pancreatitis was induced in 3-month-old male *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice by administration of 5 hourly intraperitoneal injections of caerulein (6 μg/kg). Mice were killed by CO_2 inhalation 1 h after the final caerulein injection.

Acute peptide administration and high-fat feeding studies. To assess whether GLP-1 receptor activation regulates gene expression in the exocrine pancreas of

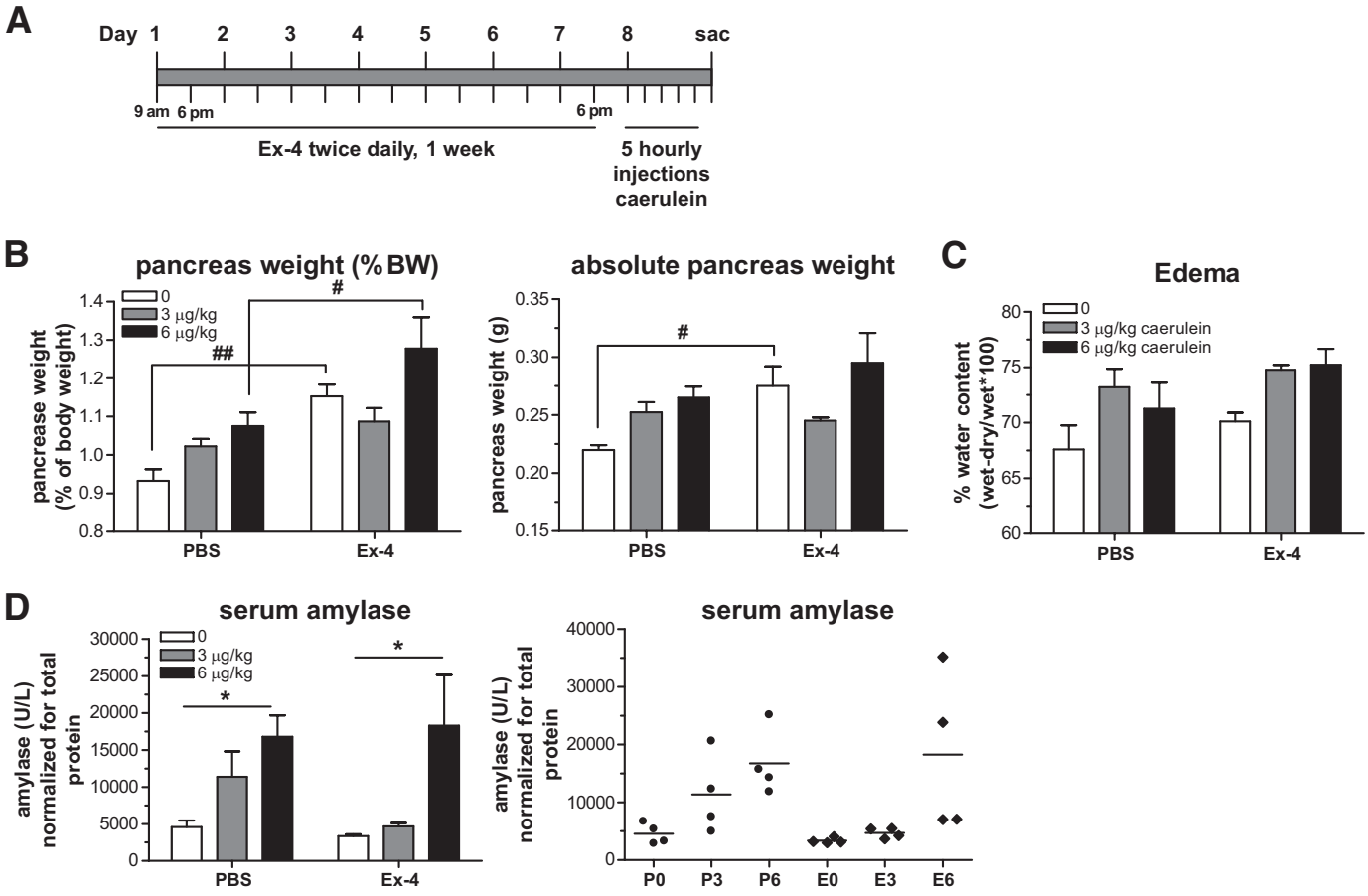
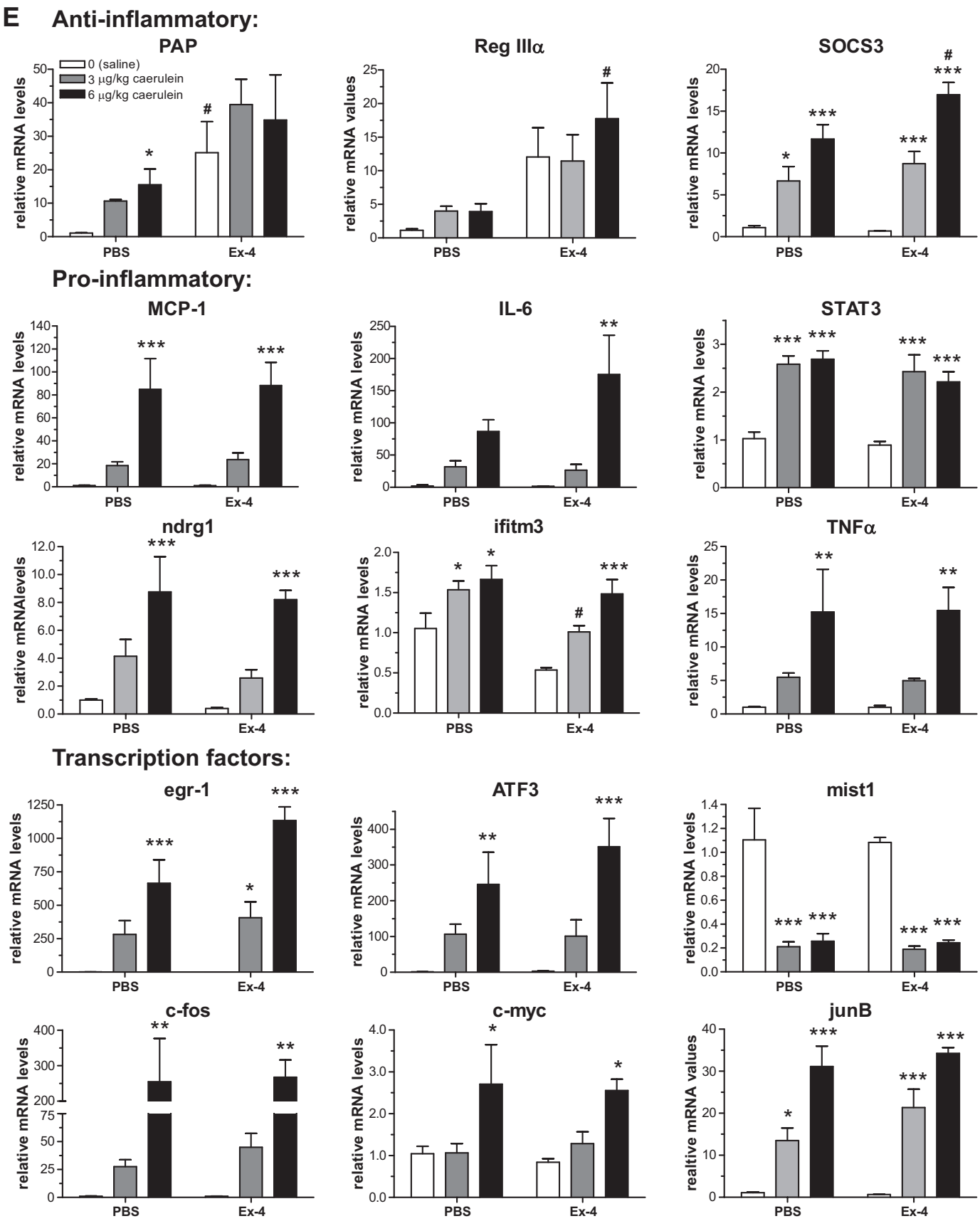


FIG. 3. Effect of prior exposure to Ex-4 on the severity of caerulein-induced acute pancreatitis. Mice were treated twice daily with PBS or 10 nmol/kg Ex-4 for 1 week followed by 5 hourly injections with the indicated concentrations of caerulein. *A*: Schematic representation of experimental design. *B*: Pancreas weight shown as a percentage of the final body weight (*left panel*) or absolute pancreas weight (*right panel*), and pancreatic water content (edema) (*C*) and serum amylase (*D*) were assessed in mice 6 h after the initial caerulein injection. Shown are means ± SE, (*C* and *D*) means ± SE (*left panel*), and individual serum amylase levels (*right panel*). #$P < 0.05$, ##$P < 0.01$ PBS versus Ex-4 treatment, *$P < 0.05$ saline versus caerulein treatment, $n = 4$ in each group. *E*: Expression profiles of pancreatitis-associated genes in the pancreas of mice treated with PBS or 10 nmol/kg Ex-4 for 1 week before caerulein administration as indicated above. Levels of the indicated transcripts are plotted relative to levels from pancreata of control (PBS 1 week + saline treatment) group as determined by real-time quantitative PCR normalized to the 18S rRNA content or cyclophillin mRNA levels (for STAT3, SOCS3, ndrg1, and ifitm3). Results are expressed as means ± SE. #$P < 0.05$ PBS versus Ex-4 treatment, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, saline versus caerulein treatment, $n = 4$ mice in each group.

normal mice, male C57Bl/6J mice were injected subcutaneously with a single dose of Ex-4 (1 μg), Ex-4 + 15% glucose (G + X), or PBS, and mice were killed 30, 45, 60, or 90 min later by CO_2 inhalation. To determine whether high-fat feeding and induction of insulin resistance (19) modifies the effects of Ex-4 on the exocrine pancreas, 4-week-old wild-type male mice were fed either standard rodent diet or high-fat diet (45% kcal from fat) for 8 weeks; during the last 4 weeks mice on high-fat diet were given twice daily intraperitoneal injections of PBS or Ex-4 (24 nmol/kg). Separately, to assess the impact of diabetes on the GLP-1R–dependent regulation of exocrine pancreatic gene expression, 13-week-old male C57BL/6 mice were maintained on a high-fat diet for 8 weeks, given one dose of streptozotocin (STZ, 100 mg/kg), and after 4 more weeks were allocated to four treatment groups for an additional 8 weeks of *1)* high-fat diet and twice daily intraperitoneal injections of PBS, *2)* high-fat diet and metformin ($500\ mg \cdot kg^{-1} \cdot day^{-1}$) provided in the drinking water, *3)* high-fat diet and twice daily intraperitoneal injections of Ex-4 (3 nmol/kg), or *4)* high-fat diet and sitagliptin ($\sim 370\ mg \cdot kg^{-1} \cdot day^{-1}$) provided in the diet.

Preparation of serum and tissue samples. Animals were killed by CO_2 inhalation 1, 24, 72, or 144 h after the final caerulein injection. Blood samples were collected by cardiac puncture, placed on ice for 30 min, and centrifuged at 4°C for 10 min at 14,000 rpm, and serum was stored at −80°C. The pancreas was removed, weighed, placed on ice, and cut into sections for RNA, protein, histology, and edema analyses. For RNA isolation, a section of pancreas was homogenized in TRIzol reagent (Invitrogen Life Technologies, San Diego, CA), frozen on dry ice, and stored at −80°C until further analysis. Tissue samples for histology were fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin. Remaining tissue samples were snap frozen in liquid nitrogen and stored at −80°C until further analysis.

Evaluation of the severity of pancreatitis. Quantification of serum amylase was performed using the Phadebas amylase test (Magle Life Sciences, Cambridge, MA) using 10 μl serum (20). Pancreatic edema (21) was quantified by measuring tissue water content after desiccation to a constant weight at room temperature for 72 h (dry weight). The results were calculated and



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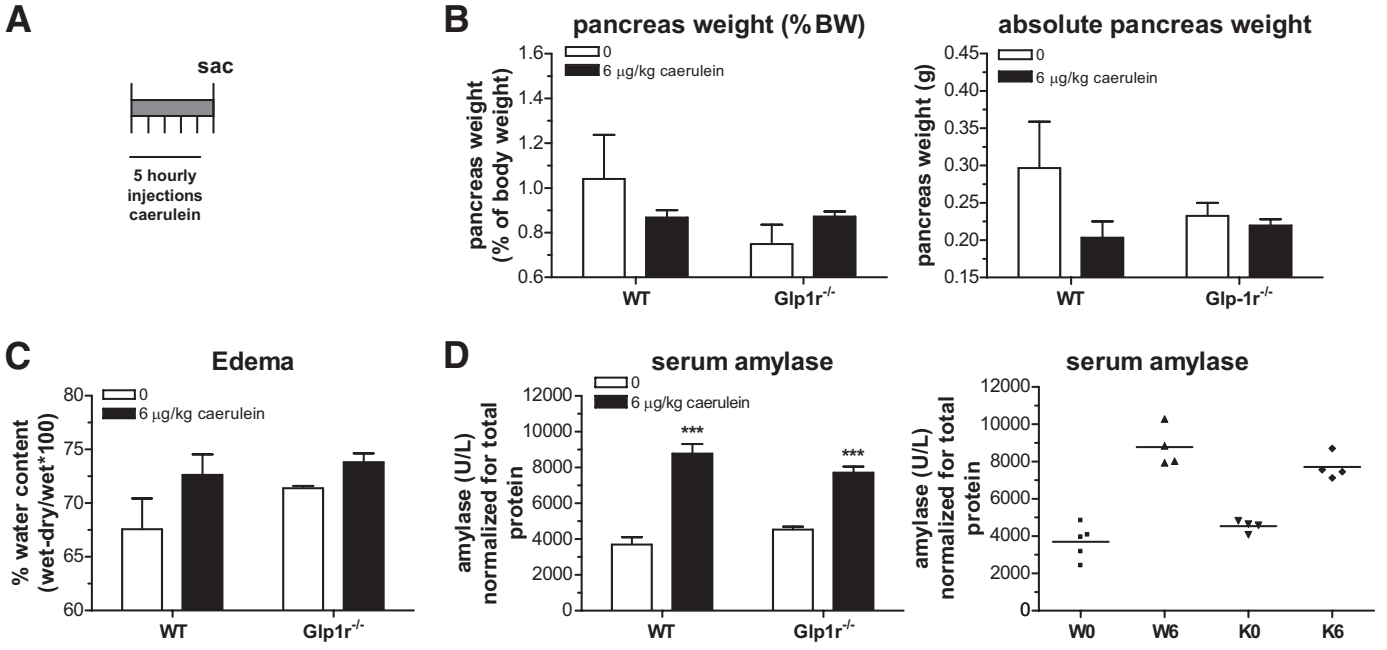


FIG. 4. Susceptibility of *Glp1r*$^{-/-}$ mice to caerulein-induced pancreatitis. *Glp1r*$^{+/+}$ (wild-type) and *Glp1r*$^{-/-}$ mice were administered 5 hourly injections of 6 μg/kg caerulein. *A*: Schematic representation of experimental design. Pancreas weight (*B*) shown as a percentage of the final body weight (*left panel*) or absolute pancreas weight (*right panel*), and pancreatic water content (edema) (*C*) and serum amylase (*D*) were assessed in mice 6 h after the initial caerulein injection. Shown are means ± SE (*D*) (*left panel*) and individual serum amylase levels (*right panel*). ***P < 0.001 saline versus caerulein treatment, n = 5 (saline) or n = 4 (caerulein) mice for each genotype. *E*: Expression profiles of pancreatitis-associated genes in the pancreas of wild-type and *Glp1r*$^{-/-}$ mice after caerulein administration. Levels of mRNA transcripts relative to the wild-type control (saline)-treated group are shown normalized to 18S rRNA content. Results are expressed as means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 saline versus caerulein treatment. ##P < 0.01 Glp1r$^{+/+}$ versus *Glp1r*$^{-/-}$ mice, n = 5 (saline) or n = 4 (caerulein) mice for each genotype.

expressed as a percentage of wet weight (wet weight−dry weight/wet weight × 100).

RNA isolation and quantitative real-time PCR. RNA from pancreatic tissue was extracted using TRIzol reagent; after first-strand cDNA synthesis, real-time quantitative PCR was carried out as described (22). Quantification of transcript levels was performed with the ABI PRISM SDS 2.1 software. Cyclophilin mRNA or 18S rRNA was used for normalization as expression of both remained unaltered regardless of treatment.

Histology. Paraffin-embedded tissues were sectioned (5 μm) and stained with hematoxylin-eosin. Immunohistochemistry was carried out using indirect immunoperoxidase detection with NovaRED substrate (Vector Laboratories, Burlington, ON, Canada) followed by hematoxylin counterstaining. Primary rabbit polyclonal antibodies were used at dilutions recommended by the manufacturer and included: rabbit anti–c-fos (Sigma-Aldrich), rabbit anti–egr-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and rat anti-mouse neutrophils (MCA771GA; AbD Serotec, Kidlington, Oxford, U.K.).

Ex vivo pancreas preparation and cAMP assay. Pancreatic fragments were prepared from C57Bl/J6 mice. Briefly, the pancreas was rapidly cut into small pieces, cultured in DMEM + 1% BSA + 10% trasylol, washed three times with DMEM + 1% BSA + 5% trasylol, resuspended in DMEM + 1% BSA + 10% trasylol, and aliquoted such that each tube contained ~4% of the entire pancreas. Preparations were then stimulated with 100 nmol/l Ex-4 or 20 μmol/l forskolin for 15 or 30 min at 37°C and frozen on dry ice. Samples were thawed, sonicated with ice-cold ethanol (65% final concentration), and cellular debris removed by centrifugation at 13,000 rpm. cAMP was measured from dried aliquots of ethanol extracts using a cAMP radioimmunoassay kit (Biomedical Technologies, Stoughton, MA).

Statistical analysis. Statistical significance was assessed by one-way or two-way ANOVA using Bonferroni's multiple comparison post hoc test and, where appropriate, by unpaired Student's t test using GraphPad Prism 4 (GraphPad Software, San Diego, CA). A P value of <0.05 was considered to be statistically significant.

RESULTS

Ex-4 acutely increases egr-1 and c-fos expression in the exocrine pancreas. Mice treated with GLP-1R agonists exhibit a significant increase in pancreas weight (23) that cannot be attributed solely to increased β-cell mass. To determine whether GLP-1R activation induces a program of gene expression in the exocrine pancreas associated with development of pancreatic growth or inflammation, we examined whether Ex-4 enhanced the expression of immediate early genes known to play important roles in regulating cell proliferation. Ex-4 rapidly and robustly increased pancreatic levels of egr-1 and c-fos (Fig. 1*A*), c-myc, and junB mRNA transcripts (supplementary Fig. 1, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0626/DC1). Moreover, the egr-1 protein was not upregulated in islets but was localized exclusively to nuclei of cells within the exocrine pancreas (Fig. 1*B*), whereas nuclear c-fos expression was observed in both islets and exocrine tissue (Fig. 1*C*). Hence, GLP-1R activation induces a robust induction of gene and protein expression in the exocrine pancreas.

Treatment with Ex-4 induces expression of PAP. Egr-1 is an early response gene that encodes a transcription factor that regulates cell proliferation, growth, and

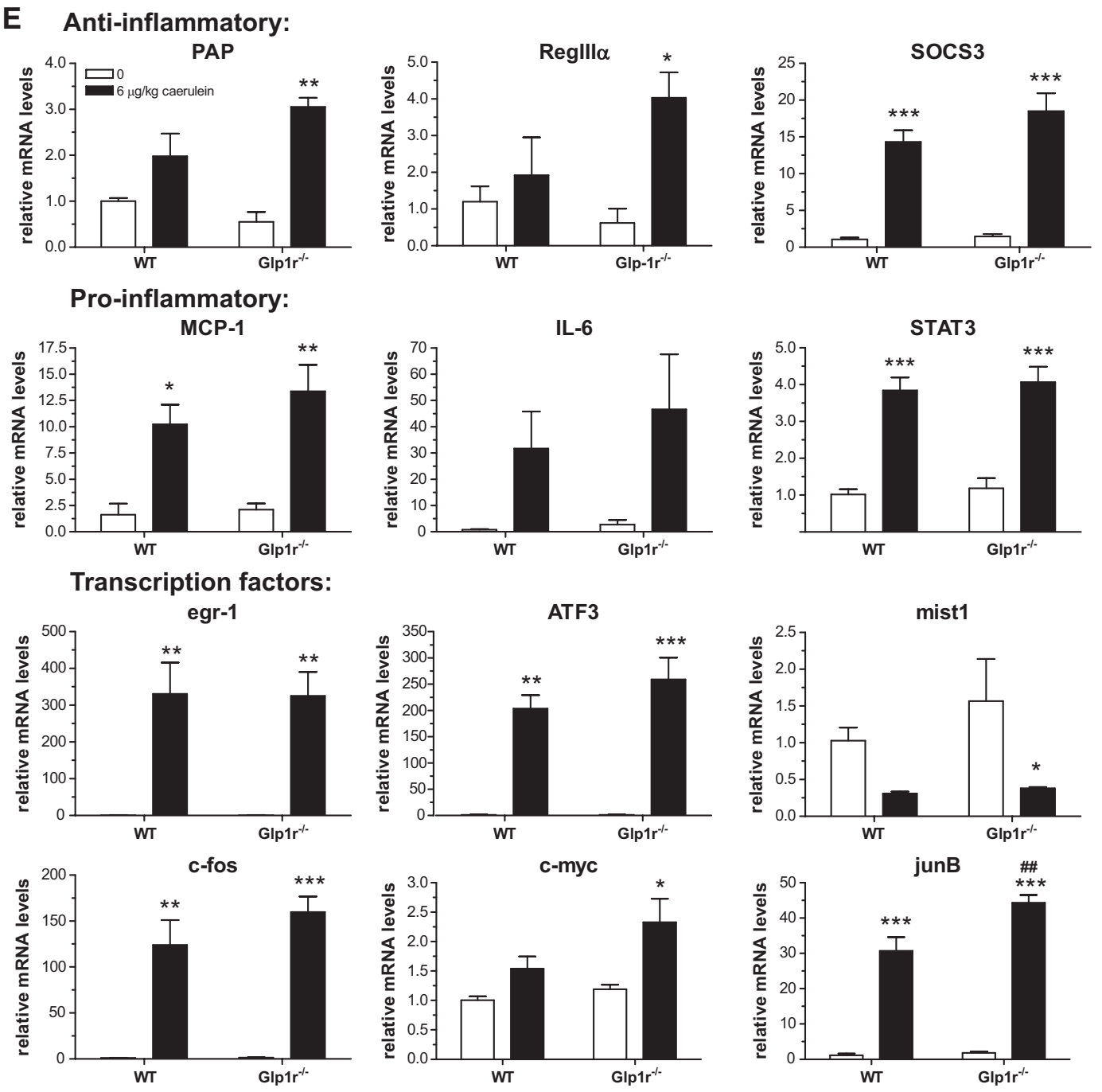


FIG. 4. Continued.

apoptosis (24). Induction of egr-1 expression occurs early in the course of caerulein-induced pancreatitis, and inflammation-related gene expression is attenuated in *egr-1*$^{-/-}$ mice with experimental pancreatitis (25). Accordingly, we examined whether agents that activate the GLP-1Rs upregulate expression of genes associated with pancreatitis. Mice were treated with Ex-4 for 1 week (supplementary Fig. 2), and the expression of pancreatitis-associated genes was assessed by real-time quantitative PCR. Ex-4 (1 or 10 nmol/kg twice daily for 1 week) increased pancreas weight (Fig. 2*A*) but had no effect on levels of mRNA transcripts encoding the proinflammatory mediators (monocyte chemotactic protein [Mcp]-1, intracellular adhesion molecule-1, signal transducer and activator of transcription [STAT]-3, ndrg1, or ifitm3) or transcription factors (egr-1, activating transcription factor (ATF)-3, mist1, c-fos, c-myc, and junB) associated with acute pancreatitis (Fig. 2*B* and supplementary Table 1). In contrast, Ex-4 markedly increased pancreatic expression of the anti-inflammatory gene PAP (Reg IIIβ) and Reg IIIα (Fig. 2*B*). The increase in pancreatic weight and induction of PAP and RegIIIα gene expression was not specific to Ex-4 but was also observed in mice treated with the human GLP-1R agonist liraglutide (Fig. 2*C* and *D*). These actions required a functional GLP-1R as liraglutide had no effect on pancreatic weight or induction of gene expression in *Glp1r*$^{-/-}$ mice (Fig. 2*C* and *D*). Moreover, liraglutide reduced the expression of the proinflammatory transcription factor, STAT3, in wild-

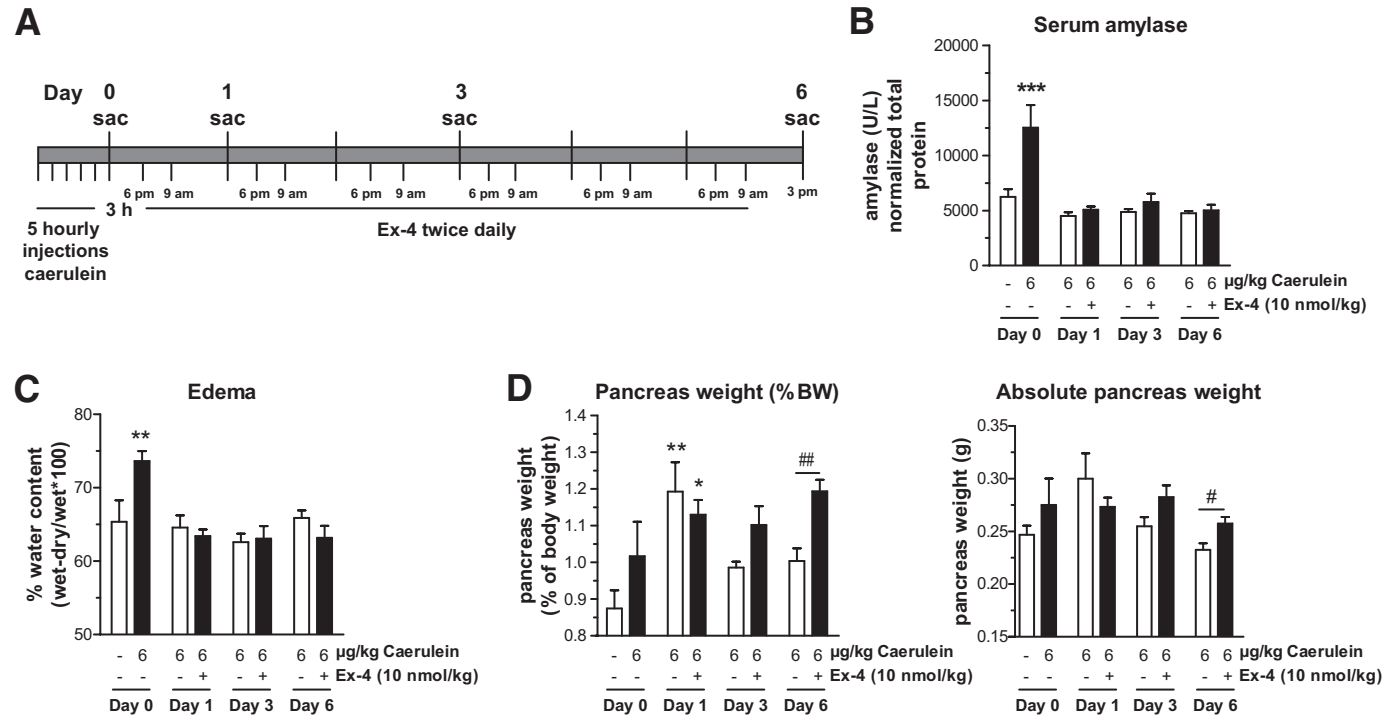


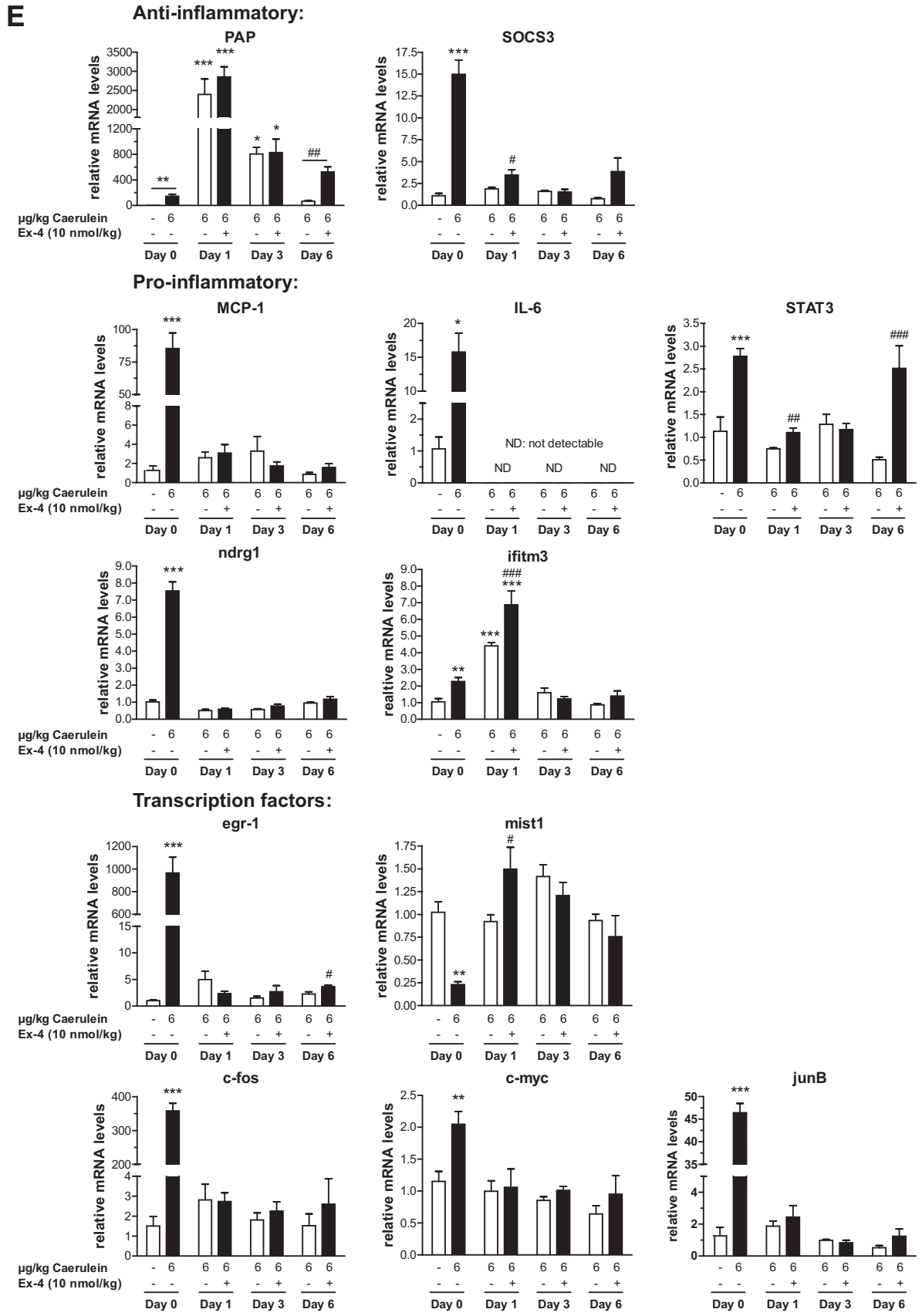
FIG. 5. Ex-4 does not modify severity of or recovery from caerulein-induced pancreatitis. Mice were administered 5 hourly injections with 6 μg/kg caerulein followed by twice daily injections with PBS or 10 nmol/kg Ex-4. *A*: Schematic representation of experimental design. Serum amylase (*B*), pancreatic water content (edema) (*C*), and pancreas weight (*D*), shown as a percentage of the final body weight (*left panel*) or absolute pancreas weight (*right panel*), were assessed in mice 6 h after the initial caerulein injection (day 0) or 24 h (day 1), 72 h (day 3), or 144 h (day 6) after the final caerulein injection. Day 1 mice received two treatments with either PBS or Ex-4. Shown are means ± SE. #$P < 0.05$, ##$P < 0.01$ PBS versus Ex-4 treatment, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ saline versus caerulein treatment, $n = 4$ mice in each treatment group. *E*: Expression profiles of genes associated with pancreatitis in the pancreas of mice treated twice daily with PBS or 10 nmol/kg Ex-4 after caerulein administration as indicated above. Shown are the levels of the indicated transcripts relative to the control (saline)-treated group (day 0) as determined by real-time quantitative PCR normalized to the 18S rRNA content or cyclophillin mRNA levels (for STAT3, SOCS3, ndrg1, and ifitm3). Results are expressed as means ± SE. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ PBS versus Ex-4 treatment, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ saline (day 0) versus caerulein treatment, $n = 4$ in each treatment group.

type mice but not in *Glp1r*$^{-/-}$ mice (Fig. 2*D*). The expression levels of most pancreatitis-associated mRNA transcripts were comparable in *Glp1r*$^{+/+}$ versus *Glp1r*$^{-/-}$ pancreas; however, basal levels of suppressor of cytokine signaling (SOCS)-3 and STAT3 mRNA transcripts were lower in *Glp1r*$^{-/-}$ mice (Fig. 2*D*). Taken together, these findings demonstrate that structurally distinct GLP-1R agonists produce changes in pancreatic mass and gene expression, actions requiring a functional GLP-1 receptor.

Effect of Ex-4 pretreatment on susceptibility to caerulein-induced pancreatitis. To assess whether antecedent activation of the GLP-1R increases the severity of pancreatitis, mice were treated twice daily with Ex-4 for 1 week before exposure to caerulein (Fig. 3). A low dose of caerulein was employed for these studies (3 or 6 μg/kg) based on preliminary experiments designed to induce a detectable yet submaximal inflammatory response in the pancreas (supplementary Fig. 3). Ex-4 significantly increased pancreas weight (Fig. 3*B*) whereas caerulein alone produced a small increase in pancreas weight (Fig. 3*B*) and a modest increase in tissue edema (Fig. 3*C*). Caerulein consistently produced a low but detectable level of inflammation, as evidenced by increased pancreatic edema, elevated serum amylase, neutrophil infiltration, and upregulated egr-1 and c-fos expression in the exocrine pancreas (supplementary Figs. 3 and 4, and data not shown). Intriguingly, serum amylase levels were lower in mice pretreated with Ex-4 and then treated with 3 μg/kg caerulein (Fig. 3*D*). Ex-4 did not significantly modulate levels of most pancreatitis-associated mRNA transcripts regulated by caerulein administration with the exception of Reg IIIα and SOCS3 that were further induced in Ex-4–treated mice exposed to caerulein (Fig. 3*E*). Consistent with previous findings (Fig. 2*A*), treatment with Ex-4 for 1 week significantly increased the transcript levels of the anti-inflammatory protein PAP (Fig. 3*E*), in the presence or absence of caerulein.

Loss of GLP-1R signaling does not modify the severity of caerulein-induced pancreatitis. To determine whether endogenous GLP-1R signaling influences susceptibility to caerulein-induced pancreatitis, we assessed the effects of a submaximal dose of caerulein on gene expression and severity of pancreatitis in *Glp1r*$^{+/+}$ versus *Glp1*$^{-/-}$ mice. Pancreas weight, edema, and serum amylase were not significantly different in caerulein-treated *Glp1r*$^{+/+}$ versus *Glp1r*$^{-/-}$ mice (Fig. 4*B–D*). Furthermore, GLP-1R genotype had no effect on the expression of pancreatic genes known to be induced by caerulein, including *SOCS3*, *Mcp-1*, *egr-1*, *ATF-3*, and *c-fos* (Fig. 4*E*). Intriguingly, caerulein-induced levels of PAP, RegIIIα, c-myc, and junB mRNA transcripts were significantly greater in *Glp1r* $^{-/-}$ mice (Fig. 4*E*).

Ex-4 does not affect recovery from or severity of caerulein-induced pancreatitis. To determine whether GLP-1R agonists exacerbate the severity or prolong the recovery after initiation of acute pancreatitis, mice were treated with caerulein followed by 10 nmol/kg of Ex-4 twice daily for up to 6 days (Fig. 5*A*). Mice were killed for analysis at 1, 24, 72 h, and 6 days after the final injection of caerulein. Caerulein transiently but signifi-



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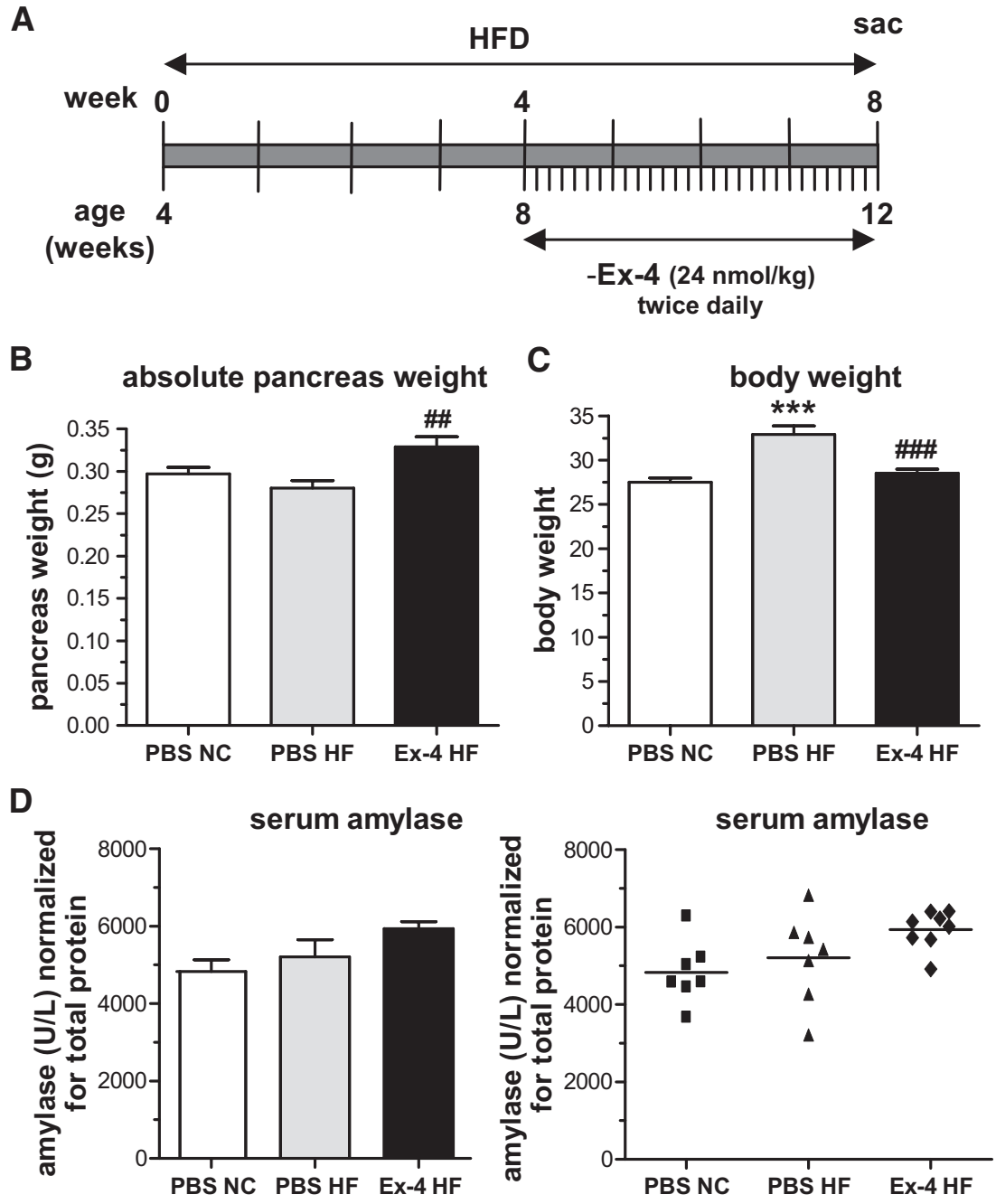

FIG. 6. Ex-4 modulates pancreatic gene expression in high-fat–fed mice. *A*: Schematic representation of experimental design. *B*: Pancreas weight, (*C*) body weight, (*D*) serum amylase, and (*E*) pancreas gene expression profiles of mice treated twice daily with PBS or 24 nmol/kg Ex-4 for 1 month on normal chow or high-fat diet as described (23). Results are expressed as means ± SE (*D*) (*left panel*) and individual serum amylase levels (*right panel*). *E*: Shown are the levels of the indicated transcripts relative to control (PBS-treated mice on normal chow) as determined by real-time quantitative PCR normalized to cyclophillin mRNA levels. Results are expressed as means ± SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ PBS-treated mice on normal chow versus high-fat diet. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ PBS- versus Ex-4–treated mice on a high-fat diet, $n = 7$ in each group.

cantly increased serum amylase (Fig. 5*B*, day 0), pancreatic edema (Fig. 5*C*, day 0), and relative pancreas weight (Fig. 5*D*, day 1). However, serum amylase and pancreatic edema returned to basal levels after 24 h, independent of Ex-4 treatment (Fig. 5*B* and *C*). Similarly, levels of mRNA transcripts encoding the proinflammatory proteins Mcp-1, interleukin (IL)-6, and ndrg1 were rapidly induced by caerulein but returned to basal levels within 24 h independent of Ex-4 treatment (Fig. 5*E*). Levels of STAT3 and ifitm3 transcripts were elevated in Ex-4–treated mice (Fig. 5*E*), and mRNA transcripts for the anti-inflammatory proteins PAP and SOCS3 remained elevated in Ex-4–treated mice. In contrast, mRNA transcripts for c-fos, c-myc, and junB were transiently increased in the pancreas of caerulein-treated mice but were not affected by concomitant Ex-4 treatment (Fig. 5*E*), whereas levels of pancreatic mRNA transcripts for mist1 and egr-1 were modestly but significantly higher in Ex-4–treated mice.

Ex-4 modulates pancreatic gene expression in mice with metabolic stress. To determine whether GLP-1R activation modified the expression of pancreatitis-associated

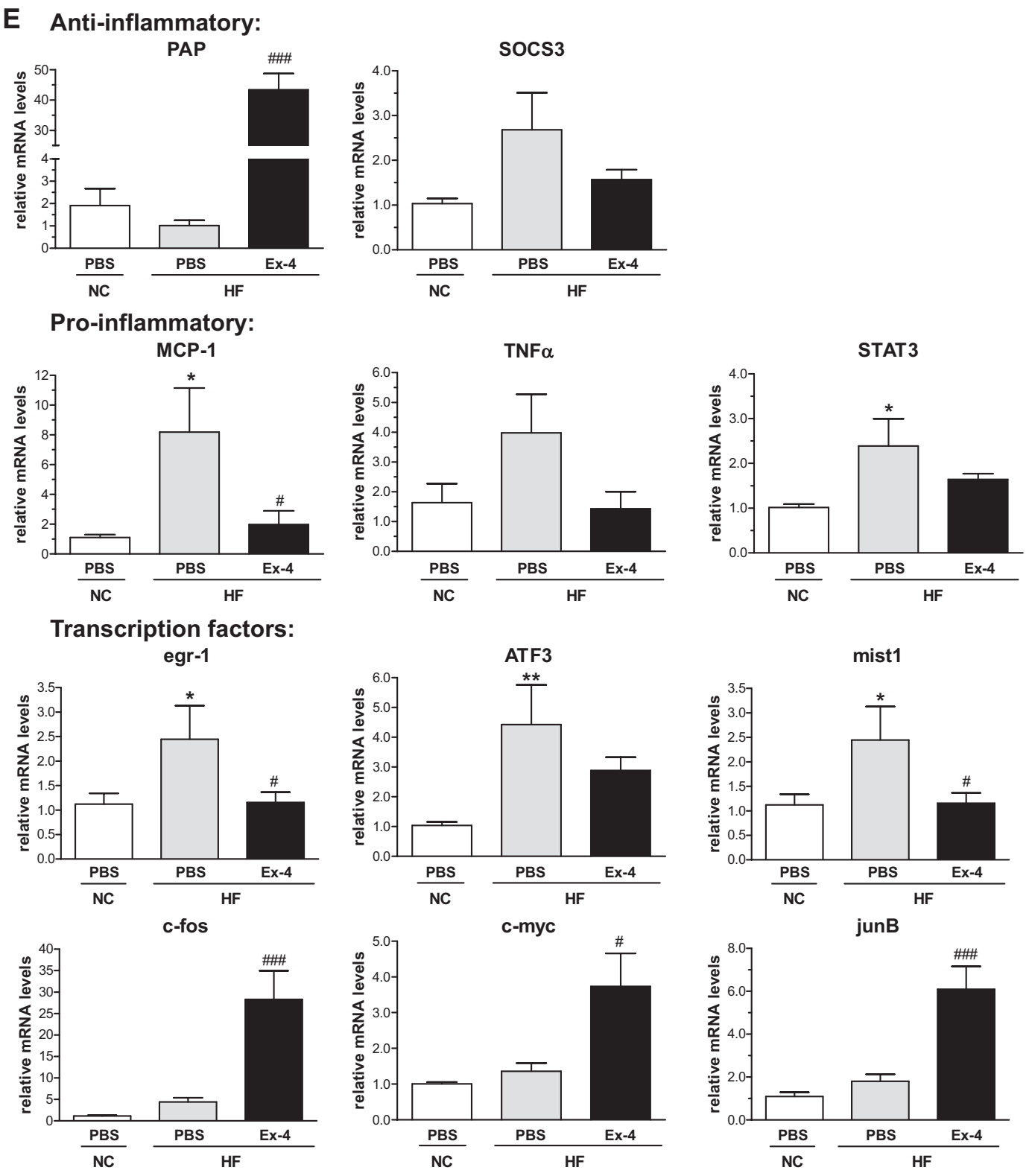

FIG. 6. Continued.

genes in the setting of a mild metabolic stress associated with the development of insulin resistance (19), mice were placed on a high-fat diet and treated twice daily with saline or Ex-4 (Fig. 6*A*). Consistent with previous observations, Ex-4 prevented weight gain (Fig. 6*C*) and significantly increased pancreas weight (Fig. 6*B*) but did not affect levels of serum amylase (Fig. 6*D*). Moreover, high-fat diet mice treated with saline, but not Ex-4, exhibited a significant increase in the transcript levels of pancreatitis-associated genes including Mcp-1, STAT3, egr-1, and ATF-3 as well as the exocrine-specific transcription factor mist1 (Fig. 6*E*). In contrast, Ex-4 (but not saline) administration significantly increased levels of mRNA transcripts for PAP, c-fos, c-myc, and junB (Fig. 6*E*).

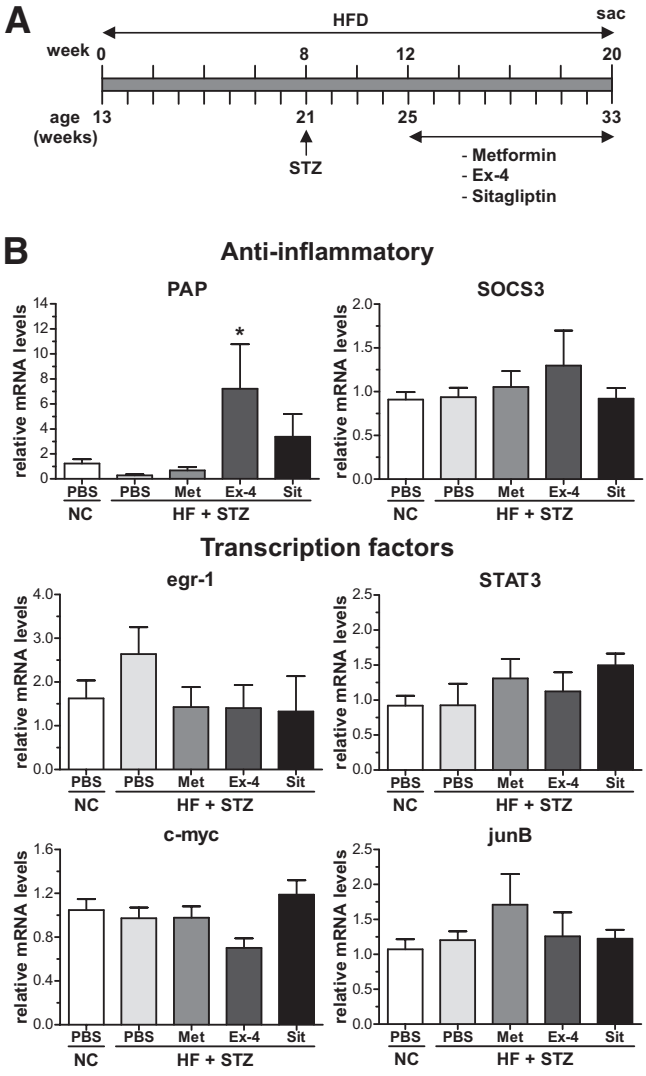

FIG. 7. Ex-4 increases PAP gene expression in diabetic mice. Mice on a high-fat diet for 8 weeks were treated with a single dose of STZ (100 mg/kg). After 4 weeks of hyperglycemia on a high-fat diet, mice were then treated with metformin (500 mg · kg^{-1} · day^{-1}), Ex-4 (3 nmol/kg), or sitagliptin (~370 mg · kg^{-1} · day^{-1}) for an additional 8 weeks on a high-fat diet. Mean glucose levels at end of study ranged from 10 to 14 to 16 mm for Ex-4– versus metformin- versus sitagliptin-treated mice. *A*: Schematic representation of experimental design. *B*: Expression profiles of pancreatitis-associated genes in the pancreas of mice treated as indicated above. Shown are the levels of mRNA transcripts relative to control (PBS-treated mice on normal chow) as determined by real-time quantitative PCR normalized to cyclophillin mRNA levels. Results are expressed as means ± SE. *P < 0.05 PBS- versus Ex-4–treated mice on a high-fat diet + STZ treatment, n = 4–6 in each group.

We next determined whether metformin, sitagliptin, or Ex-4, antidiabetic agents associated with enhanced GLP-1 receptor activation through various mechanisms (3,4,26), modulated pancreatitis-associated gene expression profiles in high-fat–fed diabetic mice. Ex-4, and to a lesser extent the DPP-4 inhibitor sitagliptin, but not metformin increased PAP mRNA transcript levels in diabetic mice (Fig. 7). Neither Ex-4, metformin, nor sitagliptin treatment modulated the expression of other genes associated with pancreatitis including SOCS3, egr-1, STAT3, c-myc, or junB (Fig. 7*B*). Finally, as Ex-4 was reported to exhibit high-affinity binding to guinea pig pancreatic acinar membranes and stimulate cyclic AMP formation through a distinct receptor that preferentially recognized Ex-4 relative to GLP-1 (27), we assessed cyclic AMP formation in slices from wild-type mouse pancreas. Forskolin but not Ex-4 rapidly stimulated cyclic AMP accumulation in pancreatic slices ex vivo (Fig. 8).

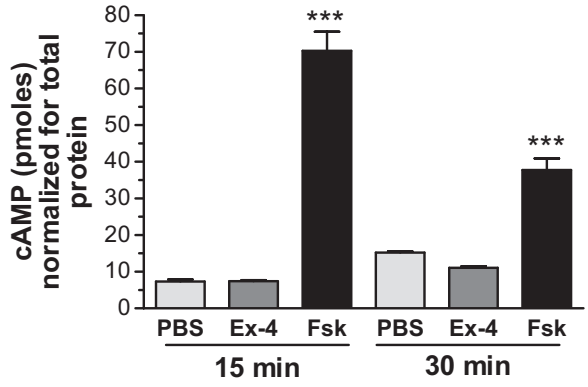

FIG. 8. Ex-4 does not increase cAMP levels in pancreatic fragments ex vivo. The entire pancreas was rapidly digested and treated with 100 nmol/l Ex-4 or 20 μmol/l forskolin for 15 or 30 min. Cell extracts were analyzed for cAMP content. cAMP levels were normalized to total protein in pancreatic extracts. Results are expressed as means ± SE. ***P < 0.001 PBS- versus forskolin-treated pancreas preparations, n = 3 in each group.

DISCUSSION

There are currently limited data regarding putative effects of GLPs on the function of the normal or inflamed exocrine pancreas. Intriguingly, coadministration of glucagon and careulein attenuated the increase in pancreatic weight and amylase expression seen in rats treated with careulein alone (28). Similarly, oxyntomodulin, a peptide structurally related to both glucagon and GLP-1, was shown to be 10-fold more potent than glucagon in the suppression of rat pancreatic exocrine secretion in the basal state or after administration of caerulein (29). Our results extend these findings by demonstrating that although acute and chronic GLP-1R activation modulates a gene expression program in the exocrine murine pancreas, GLP-1 receptor activation does not predispose to or exacerbate experimental pancreatitis in mice.

Several lines of evidence imply that exocrine cells express a functional GLP-1 and/or exendin receptor. Guinea pig pancreatic acini contain high-affinity binding sites for Ex-4, and Ex-4 binding was displaced from acinar cells by coincubation with native GLP-1 (27). Furthermore, both Ex-4– and GLP-1–stimulated cAMP formation but not amylase release in dispersed guinea pig pancreatic acini (27,30), and these stimulatory actions on cAMP were blocked by the GLP-1R antagonist exendin (9–39) (30,31). Similarly, Ex-4 stimulated cyclic AMP formation in rat pancreatic slices, and potentiated calcium ionophore-, neurotransmitter-, or CCK-induced amylase release in vitro (32). In contrast, we found that forskolin, but not Ex-4, rapidly increased cAMP formation in murine pancreatic fragments. Whether these findings reflect species-specific differences in the expression or signaling of functional pancreatic Ex-4/GLP-1 receptors requires further investigation.

We used the CCK agonist caerulein for studies of experimental pancreatitis as previous data demonstrated a functional interaction of CCK and GLP-1 signaling pathways in the endocrine and exocrine pancreas (32,33). Although the expression of most pancreatitis-associated genes was not further modified by administration of GLP-1R agonists, we consistently detected upregulation of PAP after chronic administration of Ex-4 to mice, in the presence or absence of caerulein administration. Similarly, liraglutide

also induced PAP expression, in a GLP-1R–dependent manner, and basal levels of pancreatic PAP mRNA transcripts were modestly reduced in *Glp1r*$^{-/-}$ mice.

PAP has been shown to be mitogenic, antiapoptotic, and anti-inflammatory and is strongly induced early in the course of inflammatory diseases such as pancreatitis, Crohn's disease, and ulcerative colitis (14). Moreover, inhibition of PAP gene expression in rats augments the severity of acute pancreatitis (34), and PAP knockout mice exhibit more extensive inflammation after induction of caerulein-induced pancreatitis (13), suggesting a protective role for PAP in the inflammatory response to cellular injury. Hence, the induction of PAP after GLP-1R activation may represent a compensatory mechanism that serves to limit damage to the exocrine pancreas.

Our studies of GLP-1R activation on gene expression and the development of and/or recovery from pancreatitis were motivated, in part, by clinical reports of acute pancreatitis in diabetic patients treated with GLP-1R agonists (8,10). Our findings do not support the hypothesis that GLP-1R activation sensitizes the murine pancreas to the development of pancreatic inflammation. Similarly, we did not detect any difference in the extent of pancreatic inflammation in *Glp1r*$^{-/-}$ mice. Taken together, the available data demonstrate that GLP-1R activation leads to increases in mass and changes in expression of pancreatitis-associated genes but does not modify pancreatitis susceptibility or severity in the murine pancreas. Whether GLP-1R activation modifies gene expression, enzyme secretion, or pancreatic inflammation in the human pancreas requires further analysis.

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