Dynamic Imaging of Pancreatic Nuclear Factor κB (NF-κB) Activation in Live Mice Using Adeno-associated Virus (AAV) Infusion and Bioluminescence*

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Background: NF-κB is an important signaling molecule in the development of acute pancreatitis.

Results: Adeno-associated virus-NF-κB-luciferase-infused mice showed a 77- and 140-fold increase in pancreas-specific NF-κB bioluminescence following caerulein and caerulein + LPS pancreatitis, respectively.

Conclusion: NF-κB activation can be examined in a live, dynamic fashion during pancreatic inflammation.

Significance: This technique offers a valuable tool to study real-time activation of NF-κB in vivo.

Nuclear factor κB (NF-κB) is an important signaling molecule that plays a critical role in the development of acute pancreatitis. Current methods for examining NF-κB activation involve infection of an adenoviral NF-κB-luciferase reporter into cell lines or electrophoretic mobility shift assay of lysate. The use of adeno-associated viruses (AAVs) has proven to be an effective method to transfecting whole organs in live animals. We examined associated viruses (AAVs) has proven to be an effective method of examining NF-κB activity in pancreatic acinar cells.
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include the transfection (or infection via viruses) of NF-κB-luciferase reporters. With these techniques, binding of NF-κB subunits to a nuclear response element drives transcription of the luminescent protein luciferase. The commonly used luciferase reporters are firefly (15) and Renilla luciferases (16). The development of secreted luciferases such as Gaussia (Gluc), secreted alkaline phosphatase, and Cypridina allows for serial determination of luciferase-based reporters from the same population of cells (17–20).

Bioluminescence (i.e. luciferase-based) imaging in vivo has emerged as a powerful tool in biomedical research for monitoring transgene expression, viral vector infection, tumor growth, and metastasis, as well as inflammation and gene therapy (21). Bioluminescence imaging is highly sensitive, cost-effective, noninvasive, and it facilitates real-time analysis in vivo. For this reason, luciferase-based reporters provide a major advantage over less sensitive reporters such as LacZ (22, 23) and eGFP (24).

The first globally expressed transgenic NF-κB reporter mouse line used a firefly luciferase, and the stimuli TNF-α, IL-1α, or LPS increased luminescence in most tissue, with the strongest activity observed in skin, lungs, spleen, Peyer patches, and the wall of the small intestine (25). In the current study, we first demonstrated that this mouse line poorly expressed NF-κB-luciferase in the pancreas and failed to induce robust pancreatic luminescence signals above background noise, even with a pancreatitis stimulus that is known to cause pancreatic NF-κB activation. Instead, novel gene delivery of NF-κB-luciferase through intrapancreatic duct infusion of an adenovirus (AAV6-NF-κB-luciferase) led to robust pancreas-specific NF-κB signals. We propose that this improved method can be used to measure pancreatic NF-κB activity in a dynamic fashion and can be adapted to examine NF-κB in other organ systems.

EXPERIMENTAL PROCEDURES

Reagents and Animals—All reagents were purchased from Sigma unless otherwise stated. NF-κB-luciferase transgenic reporter mice were from Taconic Farms (strain NF-κB-RE-luc; Taconic Farms, Hudson, NY). These mice were generated by microinjecting a transgene containing 6 NF-κB response elements, a CMVα promoter, and a basal SV40 promoter, which are upstream to a modified firefly luciferase called Luciferase 2P (Promega, pGL3, Fig. 1A). The transgene was microinjected into BALB/c] zygotes. The resultant mice were bred with a BALB/c] strain. Female Swiss Webster mice weighing 20–25 g (Charles River, Wilmington, MA) were used for the AAV6-NF-κB-luciferase infusion. They were fed standard laboratory chow, given free access to water, and randomly assigned to control or experimental groups.

Design and Purification of the AAV6-NF-κB-Luciferase Reporter Construct—The AAV6-NF-κB-luciferase plasmid was generated by cloning a pGL4.32[luc2P/NF-κB-RE/Hygro] vector (Promega number E8491) into a pAAV-MCS plasmid (Cell Biolab number VPK-410; Fig. 1B). The pGL4.32[luc2P/NF-κB-RE/Hygro] vector contains five copies of an NF-κB response element that drives transcription of the luciferase reporter gene luc2P (Photinus pyralis). As indicated on the Promega website, Luc2P is a synthetically derived luciferase sequence with humanized codon optimization that is designed for high expression and reduced anomalous transcription. Once cloned, the pAAV6-NF-κB-luciferase plasmid was cotransfected into HEK293 cells along with two helper plasmids: 1) pAAV-RC, which is used as a packaging plasmid carrying the serotype 6 rep and cap genes and 2) pHelper, a helper plasmid carrying the adenovirus helper functions (Fig. 1C). Cells were collected after 72 h and suspended in lysis buffer.

To purify the virus, cells were freeze/thawed three times to release the AAV6-NF-κB-luciferase. Cell lysates were treated with benzonase (0.05 units) at 37 °C for 30 min followed by 10% sodium deoxycholate at 37 °C for 30 min. To clean cell debris, lysates were spun at 2500 × g for 10 min. AAV6-NF-κB-luciferase was precipitated using a 1:4 mixture of 40% polyethylene glycol (PEG-800) and 2.5 M sodium chloride for 2 h at 0 °C. The solution was spun at 2500 × g for 30 min to collect the PEG precipitate. The PEG pellet was resuspended in HEPES buffer (50 mM), treated with an equal volume of chloroform (100%), spun at 2500 × g, and air dried for 30 min. Two phase partitioning was performed using 50% ammonium sulfate and 40% PEG, and the solution was spun at 2500 × g for 15 min. The ammonium sulfate phase was collected and dialyzed using a Slide-A-Lyser Dialysis Cassette (10K MWC0; Thermo Scientific, Rockford, IL) for 4 h. Dialysis was repeated a second time for 16 h. The AAV6-NF-κB-luciferase was concentrated using a concentrator filter tube (Millipore number UFC905024) and stored at −80 °C. Viral concentrations were quantified using the Quick-Titer AAV Quantitation Kit (Cell Biolabs, San Diego, CA). To verify that the construct was functional, HEK293 cells were infected with the AAV6-NF-κB-luciferase along with a plasmid expressing the constitutively active p65 NF-κB subunit (Fig. 1D).

Intrapancreatic Duct Infusion of AAV6—The procedure for retrograde infusion into the common bile duct (CBD) and pancreatic duct has been recently described (26–28). Briefly, Swiss Webster mice were anesthetized with isoflurane. A midline incision was made to reveal the abdominal cavity. The duodenum was flipped to reveal its distal side and held in place by ligatures. A 30-gauge needle was inserted through the anti-mesenteric aspect of the duodenum to cannulate the CBD. A small bulldog clamp was applied to the distal CBD (near the duodenum) to prevent backflow of the infusate into the duodenal lumen and to hold the cannula in place. A larger bulldog clamp was applied to the proximal CBD (near the liver) to prevent infusion into the liver and thus to direct flow to the pancreatic duct. One hundred microliters of AAV6-NF-κB-luciferase (titer 2.31 × 1012 pfu) were infused at 10 μl per min for 10 min using a P33 perfusion pump (Harvard Apparatus, Holliston, MA). Upon completion of the infusion, the bulldog clamps were released. The exterior abdominal wound was closed using 7-mm wound clips, and a single injection of buprenorphine (0.075 mg/kg) was given immediately after the surgery. Mice recovered on a heating pad for 30 min after the procedure. They were given free access to food and water after the surgery.

Bioluminescence Imaging and Quantification—Bioluminescence imaging was performed using an IVIS imaging system (Xenogen, Alameda, CA). Mice were anesthetized with 1–3%
isoflurane prior to a subcutaneous injection with D-luciferin (150 mg/kg). After 7 min, the mice were imaged. The regions of interest from displayed images were quantified using the LivingImage software 4.2 (Xenogen, Alameda, CA), represented as average radiance (photons/s/cm²/steradian). In vivo abdominal signals were normalized to a baseline value for each individual mouse. Ex vivo organ signal measurements were not normalized. In these particular experiments, mice had to have similar baseline luminescence values (i.e. before administration of stimuli).

**Experimental Pancreatitis Models**—Pancreatitis was induced in mice by administering hourly subcutaneous injections of caerulein (50 μg/kg body weight) for up to 12 h (29). In additional experiments, a more severe model of pancreatitis, and one that augmented non-pancreatic sources of NF-κB, was induced by administering 6 hourly caerulein injections followed by a subcutaneous injection of lipopolysaccharide (LPS; 10 mg/kg) as modified from Ding et al. (30). A second distinct and clinically relevant model of pancreatitis, mimicking post-ERCP (endoscopic retrograde cholangiopancreatography) pancreatitis, was induced by intraductal infusion of the radiocontrast iohexol (Omnipaque-300; 100 μl total per mouse; GE Healthcare) at 20 μl/min for 5 min. Animals receiving intraductal infusion of normal saline served as sham controls.

**Statistical Analysis**—Data were expressed as mean ± S.E. unless otherwise stated. Statistical analysis was performed using a Student’s t test. Statistical significance was defined as a p value ≤ 0.05. NF-κB-luciferase was measured as average radiance (photons/s/cm²/steradian).
RESULTS

Globally Expressing NF-κB-luciferase Transgenic Mice—The NF-κB-luciferase reporter mouse is a frequently employed tool to study activation of the transcription factor NF-κB in vivo (21, 25, 31, 32). In this transgenic mouse strain, the luciferase gene is placed downstream of the NF-κB response elements and a minimal CMV and SV40 promoter (Fig. 1A). Previous studies in isolated pancreatic acinar cells, as well as from in vivo pancreatic tissue, demonstrate that NF-κB activation occurs early in the course of pancreatic injury (5, 33, 34). To specifically exam-
FIGURE 3. NF-κB-luciferase transgenic mice show nonspecific elevations in the upper abdominal NF-κB signal following caerulein pancreatitis. A, schematic representation of the experimental time course for caerulein injections (50 μg/kg). B, representative bioluminescent images from NF-κB-luciferase transgenic mice at various time points following caerulein pancreatitis and (C) quantification of bioluminescence over time. n = 5 mice per condition. D, representative images of NF-κB-luciferase transgenic mice following 6 hourly injections of NS or 6 hourly injections of caerulein and one injection of LPS (1 mg/kg). E, the pancreas, liver, spleen, kidney, and heart/lung were removed and imaged for NF-κB bioluminescence (left). Quantification of organ bioluminescence is shown on the right.
ine pancreatic NF-κB activation in the whole mouse, we defined 4 regions of interest that outlined the (1) neck, (2) thorax, (3) upper abdomen, and (4) lower abdomen (Fig. 2A). The luminescent intensity values for the neck and thorax were 4- and 2.5-fold higher, respectively, than the collective average values of the upper and lower abdomen. We also observed some level of diurnal variation at baseline (Fig. 2C) and found that there was an artificial signal at the site of intraperitoneal injections of luciferin (Fig. 2D). For this reason, we subsequently only gave subcutaneous injections.

NF-κB-luciferase Transgenic Mice Demonstrate Mild Elevations in Upper Abdominal NF-κB Signal following Caerulein Pancreatitis—We next determined whether the transgenic NF-κB-luciferase mice could elicit a pancreatic signal in response to pancreatitis. A highly reproducible model of experimental pancreatitis in rodents is induced by giving hourly injections of high doses of the cholecystokinin analog caerulein (50 μg/kg; subcutaneously) for 12 h (Fig. 3A) (35, 36). Quantification of the NF-κB luminescent signal from the upper abdomen revealed a 2.2- and 1.8-fold increase with caerulein pancreatitis above normal saline-injected controls at 9 and 12 h, respectively (Fig. 3, B and C). Ex vivo imaging of the organs revealed that caerulein induced a higher signal in the pancreas compared with the other organs of the abdomen and thorax (Fig. 3E). However, the signal intensities in the pancreas were focally enhanced and were reminiscent of intrapancreatic lymph nodes. To test a more potent inducer of NF-κB activation, we administered the endotoxin lipopolysaccharide (10 mg/kg) 1 h after the sixth caerulein injection. LPS caused a global increase in NF-κB activation with large elevations in the heart, lungs, and spleen and only mild elevations in the pancreas (Fig. 3, D and E). Taken together, these results indicate that NF-κB transgenic mice poorly express NF-κB-luciferase in the pancreas and fail to demonstrate a pancreatic signal above background noise with a pancreatitis stimulus that is known to cause NF-κB activation.

Mice Infected with Intraductal AAV6-NF-κB-luciferase—To address the problem of specificity of signal to the pancreas we designed an adeno-associated virus serotype 6 (AAV6) carrying an NF-κB-luciferase reporter as described under “Experimental Procedures” (Fig. 1, B and C). Once purified, we infused the AAV6-NF-κB-luciferase (titer 10^{12} pfu) into the pancreatic duct of wild type mice and tracked the emergence of bioluminescence as the mice healed from the surgery (Fig. 4). The signals from the upper abdomen peaked 7–10 days after the infusion and reached a new baseline after 3 weeks. What was particularly noticeable was that the bioluminescence was restricted primarily to the upper abdomen (Fig. 5A), and it took on the shape of the pancreas. In contrast to the globally expressing NF-κB-luciferase transgenic mice, the AAV6-NF-κB-luciferase intraductally infused mice had less diurnal variation (Fig. 5B). However, there was still considerable inter-animal variability at baseline.

Pancreatitis Models Induce a Pancreas-specific NF-κB Signal in Vivo Using Mice That Underwent Intraductal Infusion of AAV6-NF-κB-luciferase—We next determined using caerulein hyperstimulation whether intraductally infused AAV6-NF-κB-luciferase mice could manifest a pancreas-specific signal in response to pancreatitis (Fig. 6, A and B). Quantification of the NF-κB luminescent signal from the upper abdomen revealed that caerulein-treated mice had a 15-fold peak in NF-κB luciferase 7 h after the first caerulein injection compared with nor-
mal saline-treated controls ($p < 0.05$; Fig. 6C). Comparing this time course with pancreatic histologic severity, the results confirm previously published findings (33, 34) that pancreatic NF-$\kappa$B is an early signal for pancreatic inflammation. In a separate batch of experiments with caerulein, the intra-abdominal organs were removed 8 h after the first hourly caerulein injection. The signal in the pancreas of the caerulein-induced mouse was 77.5-fold higher than in the pancreas of a normal saline-injected control mouse (Fig. 6, D and E). The spleen and stomach had no increased intensity, but there was a 12-fold increase in signal in the liver. A combination of 6 hourly caerulein injections followed by 1 injection of LPS, to provoke extra-pancreatic NF-$\kappa$B, led to a more intense upper abdominal signal (Fig. 6C) and ex vivo the pancreas-specific signal was 9-fold higher than with caerulein alone. However, in this case, the liver contributed the greatest intensity, which attests to the finding that there was likely some leakage of AAV6 into the hepatic ducts during the intraductal infusion. Nonetheless, the results indicate that with a pancreatitis-specific stimulus (i.e. caerulein alone), AAV gene delivery of NF-$\kappa$B-luciferase through intrapancreatic duct infusion leads to robust pancreatic NF-$\kappa$B bioluminescent signals in vivo.

隔离的腺泡细胞来自AAV6-NF-$\kappa$B-luciferase-infused小鼠具有高水平的NF-$\kappa$B活性（数据未显示）。这最可能是由于细胞因细胞的分离而引起的应激。因此，无法在细胞培养中通过在细胞中使用的体内-注入的 luciferase 来检查NF-$\kappa$B活性。

我们接着研究了一个模仿临床情况的新型模型，该模型可以模拟内镜逆行胆胰管造影（ERCP）引起的胰腺损伤。ERCP 是一种常见的消化道程序，在该程序中，通过肠镜的一个侧口，胆道和胰管被穿刺（通过肠镜的一个侧口），并注入少量的造影剂来拍摄影片。
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**A** Hourly caerulein injections

AAV6 Infusion

**B**

- NS
- Caer
- Caer + LPS

Pancreatic Histology

Baseline

- 6 hr
- 120 hr
- 168 hr

**C**

- Normal Saline
- Caerulein

Fold increase above baseline

| Time (hours) | Fold increase above baseline |
|--------------|-----------------------------|
| 0            | 5                           |
| 2            | 10                          |
| 4            | 15                          |
| 6            | 20                          |
| 8            | 15                          |
| 10           | 10                          |
| 12           | 5                           |
| 108          | 2                           |
| 120          | 1                           |

**D**

- Pancreas
- Liver
- Spleen
- Stomach

**E**

- NS
- Caerulein
- Caerulein + LPS

Avg Radiance (pCi/cm²/s)

| Organs     | NS | Caerulein | Caerulein + LPS |
|------------|----|-----------|-----------------|
| Pancreas   | 3.0×10⁷ | 2.0×10⁷ | 1.0×10⁷         |
| Liver      | 2.0×10⁷ | 1.0×10⁷ | 5.0×10⁶         |
| Spleen     | 1.0×10⁷ | 5.0×10⁶ | 2.5×10⁵         |
| Stomach    | 5.0×10⁶ | 2.5×10⁵ | 1.25×10⁴        |
ically visualize the ducts (37, 38). The most common complication of ERCP is pancreatitis, occurring in 4–7% of patients (39, 40). To recapitulate this scenario in mice, we performed an intraductal infusion of the radiocontrast iohexol (Omnipaque-300) in mice that had received intraductal AAV6-NF-κB-luciferase 5 weeks prior (Fig. 7A). Radiocontrast infusion led to a 13-fold increase in pancreatic NF-κB luciferase signals 4 h after surgery above the signals observed in a sham-operated mouse that received intraductal normal saline (p < 0.05; Fig. 7, B and C). These data provide complementary evidence that AAV6-

FIGURE 7. Radiocontrast-induced pancreatitis causes a pancreas-specific NF-κB signal in vivo using mice that underwent intraductal infusion of AAV6-NF-κB-luciferase. A, schematic representation of the experimental time course for intraductal radiocontrast infusion. B, representative bioluminescent images from AAV6-NF-κB-luciferase-infused mice following infusion of the radiocontrast iohexol (Omnipaque-300). C, quantification of the bioluminescent signal over 36 h. *, p < 0.05 compared with normal saline sham.

FIGURE 6. Caerulein-induced pancreatitis causes a pancreas-specific NF-κB signal in vivo using mice that underwent intraductal infusion of AAV6-NF-κB-luciferase. A, schematic representation of the experimental time course for caerulein injections (intraperitoneally, 50 μg/kg). B, representative bioluminescent images from AAV6-NF-κB-luciferase transgenic mice or wild type mice infused with AAV6-NF-κB-luciferase following caerulein pancreatitis (50 μg/kg) and hematoxylin and eosin-stained sections during a time course after the start of caerulein hyperstimulation. C, quantification of bioluminescence over time. D, the pancreas, liver, spleen, and stomach were removed and imaged for NF-κB bioluminescence. E, quantification of organ bioluminescence. *, p < 0.05 compared with normal saline-infused sham controls.
NF-κB-luciferase infusion is a sensitive and specific method for detecting pancreatic NF-κB activity.

DISCUSSION

In this study we provide the first demonstration of live, dynamic NF-κB signaling within the pancreas and successful pancreatic gene delivery of a bioluminescent reporter through AAVs. We validated the technique using two complementary pancreatic injury models. We believe that this tool will be particularly useful to examine a time course of NF-κB in the same mouse, thus minimizing the number of mice necessary for each experiment.

A growing body of evidence suggests that the NF-κB-dependent pathway is critical in the development and persistence of pancreatic inflammation and injury (36, 41–44). Thus, NF-κB signaling within the genome and should provide a long duration of effect.

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NF-κB-dependent pancreatic inflammation and injury models. We believe that this tool will be particularly useful to examine a time course of NF-κB in the same mouse, thus minimizing the number of mice necessary for each experiment.

Previous efforts to examine live, dynamic pancreatic NF-κB have been challenging. Using globally expressing NF-κB-luciferase mice, Gray et al. (45) found that mice fed a choline-deficient, ethionine-supplemented diet to induce pancreatitis had a prominent bioluminescent signal in the thorax and a broad nonspecific signal in the upper abdominal region. Specific organs emitting these signals could not, however, be well delineated. Subsequent studies in these NF-κB-luciferase reporter mice showed that administration of TNF-α, IL-1α, or LPS increased luminescence in a tissue-specific manner, with the strongest activity observed in skin, lungs, spleen, Peyer patches, and the wall of the small intestine (25). We found, however, that this transgenic mouse poorly expressed luciferase in the pancreas and failed to demonstrate a pancreatic signal above background noise with caerulein hyperstimulation pancreatitis.

Instead, we were able to detect specific pancreatic NF-κB activation dynamically in vivo by gene delivery of the NF-κB-luciferase reporter gene through intraductal infusion of AAV6. AAV6 and AAV8 appear to have the highest infection efficiency in the pancreas compared with other AAV serotypes and transduce pancreatic acinar cells, islets, and ducts (27, 46). In previous work, intrapancreatic ductal infusion of AAVs successfully yielded pancreas-specific infectivity (27, 46, 47). This route of delivery is preferred over hydrodynamic injection into the systemic circulation, because it is targeted to the pancreas. Unlike adenoviral vectors, AAVs offer the major advantage of evading an immunogenic response (48).

Bioluminescence imaging in vivo is a highly sensitive method for monitoring gene expression in luciferase reporter transgenic mice (49, 50). The noninvasive nature of this technology also allows convenient longitudinal studies and the ability to perform a paired analysis within the same mouse (51–53). Transient transfection of a plasmid with a luciferase reporter has been used to monitor NF-κB activation in the liver (54). However, reporter expression lasted for only a few days and was thus unsuitable for monitoring NF-κB activity during chronic pathological conditions. AAVs, on the other hand, integrate within the genome and should provide a long duration of effect.

In the current study, we demonstrate stable expression of an AAV6-NF-κB-luciferase reporter. A drawback of the technique, however, is that there is a smaller extra-pancreatic signal in the liver, even with caerulein alone, a stimulus that primarily evokes pancreatic inflammation. The hepatic signal was further pronounced when NF-κB was globally induced using LPS. It is also important to note that the intraductal infusion of AAVs causes incorporation of the reporter only within the native pancreatic cells. However, the signal does not account for the contribution of NF-κB, for example, from infiltrating immune cells during inflammation.

We used two relevant pancreatic inflammation models to induce pancreatic NF-κB activation. In the first model, caerulein-treated mice manifested an increase in signal after the 4-h time point, which is similar to what has been previously published (33, 34).

In this report, we also provide the first demonstration that pancreatic NF-κB is activated in an experimental model of post-ERCP pancreatitis. The results support the claim that NF-κB is crucial to a range of pancreatitis etiologies. In summary, we report here a novel technique of intraductal infusion of an AAV6-NF-κB-luciferase to examine pancreatic NF-κB activation in a live, dynamic fashion.

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