COX-2 is not required for the development of murine chronic pancreatitis

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Silva A, Weber A, Bain M, Reding T, Heikenwalder M, Sonda S, Graf R. COX-2 is not required for the development of murine chronic pancreatitis. Am J Physiol Gastrointest Liver Physiol 300: G968–G975, 2011. First published March 3, 2011; doi:10.1152/ajpgi.00497.2010.— Chronic pancreatitis is a severe inflammation of the pancreas associated with destruction of the parenchyma, fibrosis, and persistent abdominal pain. Cyclooxygenase-2 (COX-2) and COX-2-derived prostaglandins, key mediators of the inflammatory response, are elevated in patients with chronic pancreatitis. Previous studies investigated COX-2 as a therapeutic target. These reports showed a reduced pathology in COX-2-deficient mice with a better outcome. Here we compared the role of COX-2 in acute and chronic pancreatic inflammation using the same COX-2−/− mouse model of cerulein-induced pancreatitis. In a setting of acute pancreatitis, juvenile COX-2−/− mice exhibited a reduced histopathological score compared with wild-type littermates; on the contrary, adult mice did not show any difference in the development of the disease. Similarly, in a setting of chronic pancreatitis induced over a period of 4 wk, adult mice of the two strains showed comparable histological score and collagen deposition. However, the abundance of mRNAs coding for profibrotic genes, such as collagen, α-smooth muscle actin, and transforming growth factor-β was consistently lower in COX-2−/− mice. In addition, comparable histological scores and collagen deposition were observed in wild-type mice treated with a COX-2 inhibitor. We conclude that, in contrast to what was observed in the rat pancreatitis models, COX-2 has a limited and age-dependent effect on inflammatory processes in the mouse pancreas. These results suggest that COX-2 modulates the inflammatory process during the development of pancreatitis in a species-specific manner. Thus the pathophysiological roles of COX-2 and its therapeutic implications in patients with pancreatitis should be reexamined.

pancreatic inflammation; fibrosis; tissue damage

CYCLOOXYGENASE-2 (COX-2) IS the inducible isoform of the enzyme that converts arachidonate into prostaglandin (PG) G2, which is then converted to PGE2 in further steps (16). PGE2 is a known proinflammatory molecule that has been studied in many chronic diseases, including pain, osteoarthritis, and rheumatoid arthritis (2, 14), as well as in cancer (10, 19). In addition, COX-2 activity and its downstream metabolite PGE2 were described to possess mitogenic action, thus potentially driving cancer growth. Therefore, COX-2-selective, non-steroidal anti-inflammatory drugs may not only be used in the context of inflammation and pain, but were also suggested as adjuvant medication in chemotherapy of several types of cancers, including the ones of colon (13) and pancreas (1). However, due to the negative cardiovascular side effects, most of these drugs have been withdrawn from clinical use or are available only for selective indications. The need for such drugs, albeit without the negative side effects, is still prevalent.

In the pancreas, elevated COX-2 activity appears to correlate with chronic inflammation. Two reports described that COX-2 is strongly increased in pancreatic specimens from patients with chronic pancreatitis (CP) (5, 12). These studies revealed that COX-2 was localized to atrophic pancreatic acinar cells, islets, and duct cells, hence providing a potential target for treatment of patients with CP. In a proof-of-concept study on spontaneous CP in WBN/Kob rats, our laboratory showed that Vioxx (Rofecoxib), a specific COX-2 inhibitor, significantly suppressed inflammation and fibrosis and delayed the progression of the disease (11). Furthermore, we demonstrated that COX-2-derived PGE2 and TNF-α synergistically activate acinar cells to stimulate cytokine synthesis and secretion (18). We concluded that the ability of acinar cells to react to signals from invading inflammatory cells is a key factor contributing to the propagation and amplification of the proinflammatory response mediated by PGE2.

COX-2-deficient mice (COX-2−/−) are a well-established animal model that has been used successfully to determine the role of this enzyme in several chronic diseases. In most cases and various organs, the severity of inflammation was reduced (7, 8). However, loss of COX-2 in specific cell types has been reported to promote inflammation in a mouse model of Crohn’s disease (6, 21), suggesting that COX-2-dependent PGs can act also as anti-inflammatory signals in a selected physiological environment.

Two reports on experimental acute pancreatitis using COX-2−/− mice demonstrated that the absence of COX-2 activity reduced, albeit transiently, the levels of tissue injury and inflammation compared with wild-type (WT) animals (3, 17). Furthermore, it was shown that inhibition of COX-2 activity and its subsequent PGE2 synthesis decreased the activation in pancreatic acinar cells of NF-kB, a group of transcription factors with a key role in the development of pancreatitis (15).

In this report, we sought to further explore the role of COX-2 in the development of the inflammatory response in the context of CP. To this aim, we used a model of cerulein-induced CP in COX-2−/− mice (9, 20, 22) and mice treated with the specific COX-2 inhibitor Vioxx and evaluated the pathological and molecular aspects of the disease in absence of COX-2 activity.

MATERIALS AND METHODS

Animals. COX-2 WT and knockout animals were ordered from Taconic (Ry, Denmark). The animals were held under a light-dark regime (16:8 h), constant temperature, and free access to food and...
water at the University Hospital Zurich. Animals were kept according to institutional guidelines, and all experiments were performed following ethical approval from the local veterinary office.

**Experimental outline.** Acute pancreatitis was induced in young (4–5 wk) and adult (8–10 wk) animals with 12 hourly injections of cerulein (50 μg/kg, no. C-9026, SIGMA-Aldrich, Buchs, Switzerland) administered intraperitoneally. Animals were starved overnight before the onset of the experiments.

CP was induced in 8- to 10-wk-old animals with six hourly injections of cerulein (50 μg/kg), three times a week on alternate days, for a maximum of 4 wk (9, 20). Animals were harvested according to a standard operation procedure whereby mice were first anesthetized by isofluorane, then, under continuing inhalation anesthesia, a midline laparotomy was made, followed by dissecting a small piece of the pancreas near the spleen. This tissue was snap frozen immediately for further analysis of RNA. Blood was then sampled by heart puncture, and the remaining of the pancreas was prepared for histology. Groups of five to six animals were tested for each experiment.

WT C57/Bl6 mice, treated for 4 wk with cerulein, were fed powdered chow, with or without the addition of Vioxx, as described (11).

**Histology.** Standard procedures were used to fix tissues in 4% formalin and subsequently embedding in paraffin. Consecutive 5-μm-thick sections were prepared for hematoxylin-eosin (H&E) staining and immunohistochemistry. A histology score was determined on H&E-stained sections and included tissue destruction, edema, and inflammation. Single labeling of tissue sections was performed after antigen-retrieval in boiling citrate buffer, followed by antibody incubation and detection using ABC-Vectastain (Reactolab, Servion, Switzerland) developer. Primary antibodies used in this study were as follows: rabbit anti-Ki67, no. 16667, rat anti-mouse F4/80, no. 424.

![Fig. 1](http://ajpgi.physiology.org/)

**A:** Experimental acute pancreatitis in young wild-type (WT) and cyclooxygenase-2-deficient (COX-2/−) mice. Mice, 4–5 wk old, were injected 12 times with cerulein and harvested 12 and 24 h after the first injection. A: amylase levels indicating tissue damage were either not different (12 h) or reduced after 24 h in WT animals. B: the histology score was significantly lower in COX-2/− mice after 12 h. C–F: transcript levels of the cytokines/chemokines TNF-α, IL-6, monocyte chemotactant protein (MCP)-1, and transforming growth factor (TGF)-β, respectively, were quantified. G, H, J: immunohistochemical detection of Ki67-positive cells in untreated pancreas (G), 24 h after the initial cerulein injection (H), and double staining of Ki67 (brown, nuclei) with amylase (red, cytosol) to verify Ki67 positivity in acinar cells from animals after chronic cerulein treatment (J). Insets: enlarged view of positive cells: acinar cells in G and J, inflammatory cells in H. Scale bar: 50 μm. K and L: proliferation was quantified by counting Ki67-positive cells on ×40 high-power fields. K: number of nonacinar Ki67-positive cells. L: number of acinar cells positive for Ki67. Values are means ± SE. *P < 0.05.
ab6640, both from Abcam (Cambridge, UK), and rabbit anti-human amylase, Sigma no. A-8273 (Buchs, Switzerland). Double labeling for Ki67 and amylase was performed as follows. Ki67 was first stained using diamino-benzidine (no. K3468, DAKO;Cytomation) as a chromogen. Sections were then boiled again, labeled with anti-amylase antibody, and developed with an anti-rabbit IgG phosphatase-coupled antibody using fuchsine dye as the second chromogen (no. K0625, DAKO;Cytomation).

Quantification of stained cells was performed by counting 10 representative fields for each mouse section using a ×40 objective.

**Transcript analysis.** RNA was extracted from pancreata, as previously described (4). Real-time PCR was run on a Taqman 7000 (Applied Biosystems, Rotkreuz, Switzerland) under standard conditions. Transcript levels were normalized using 18S RNA as a reference and expressed as fold induction relative to the value of untreated control animals, set as one (11). The following Taqman probes (Applied Biosystems) were used: TNF-α, Mm00443258_m1; IL-6, Mm00446190_m1; monocyte chemoattractant protein-1 (MCP-1), Mm00441242_m1; transforming growth factor (TGF)-β, Mm00441724_m1; COX-1, Mm00477214_m1; COX-2, Mm00478374_m1; Coll3a1, Mm00802331_m1; α-smooth muscle actin (α-SMA), Mm01546133_m1; cyclin D1, Mm04332359_m1; cyclin E1, Mm00432367_m1; 18S, 4308923 (Vic).

**RESULTS**

**Age-dependent reduction of acute pancreatitis in COX-2−/− mice.** We initially evaluated the role of COX-2 in a mouse model of acute pancreatic inflammation to establish the phenotypic characteristics of COX-2−/− and WT mice with the same genetic background. The first set of experiments was performed according to a published protocol (3) using 4- to 5-wk-old mice analyzed 12 and 24 h after the initial cerulein injection. Serum amylase, an indicator for pancreatic tissue damage, increased in a comparable manner in both genotypes at the 12-h time point and remained elevated in COX-2−/− mice 24 h after the initial cerulein injection, while amylase levels in WT mice decreased by one-half (Fig. 1A). While the analyzed parameter did not indicate decreased tissue damage in the absence of COX-2 activity, the histological score significantly improved in COX-2−/− 12 h after the first injection. However, the phenotype was transient, and 12 h later (24-h time point), the score was similar in both strains (Fig. 1B).

Analysis of cytokine transcripts from harvested pancreata revealed that TNF-α and the chemokine MCP-1/CCL2 was comparable in the two strains at both time points investigated (Fig. 1, C and E), while IL-6 and TGF-β were downregulated in COX-2−/− mice at the 24-h time point compared with WT animals (Fig. 1, D and F). Next, we evaluated whether regeneration of pancreatic cells was induced under these experimental conditions. To this aim, expression and nuclear translocation of the proliferation marker Ki67 was investigated by immunohistochemistry. Ki67-positive cells were rarely observed in pancreas of control mice (Fig. 1), but were numerous in cerulein-treated animals 24 h after the first injection (Fig. 1H). Quantification of Ki67+ cells revealed that a large proportion of replicating cells (∼90%) was of inflammatory origin (Fig. 1H), and their number markedly increased at 24 h. No difference was observed in the two mouse strains. A lower number of acinar cells, identified by morphological criteria, were also found positive for Ki67. To verify localization of Ki67 in acinar cells, double-staining with pancreatic amylase was performed on sections derived from chronically treated animals (Fig. 1J). In WT mice, the number of replicating acinar cells increased over time (Fig. 1L); inter-
Interestingly, in COX-2−/− mice, the number of replicating cells was significantly higher than WT 12 h after cerulein injection; however, it did not further increase 24 h after induction of pancreatitis.

In a next step, we evaluated the development of acute pancreatitis in adult mice in the absence of COX-2 activity. For this aim, 8- to 10-wk-old WT and COX-2−/− mice were injected 12 times at hourly intervals and killed 12 h after the first injection. As indicators of pancreatic damage and inflammation, serum amylase, histopathology, and gene expression regulation were analyzed. Compared with untreated control mice, serum amylase strongly increased after cerulein treatment; however, no statistically significant difference between COX-2−/− and WT mice could be observed (Fig. 2A). Similarly, the histopathological score, which was significantly lower in COX-2−/− young animals at this time point, was comparable to that of WT mice (Fig. 2B). In addition, the transcript levels of proinflammatory cytokines, as well as of the profibrotic genes TGF-β, collagen, and α-SMA, were also comparable in the two mouse types, indicating that, in adult mice, the absence of COX-2 did not alter the disease progression in a setting of acute pancreatitis (Fig. 2C). In addition, transcript levels of COX-1, the noninducible cyclooxygenase producing PGE2, which might compensate for the lack of COX-2, were not altered. Importantly, the cerulein-induced upregulation of TNF-α and MCP-1 transcript was reduced in adult mice compared with the previously examined juvenile animals, suggesting that, in adult mice, the damage of pancreatic tissue under acute conditions was not severe enough to elicit a COX-2-dependent reaction. Hence, in the following experiment, a sustained pancreatic damage was induced by chronic application of cerulein.

**Fig. 3.** Establishment of chronic pancreatic inflammation in the mouse pancreas. WT animals were injected repetitively over several weeks with cerulein or saline (NaCl). A: cerulein treatment upregulated COX-2. B: α-SMA mRNA in a time-dependent manner. Values are means ± SE. C and D: pancreas sections after 4 wk of cerulein treatment. C: Sirius stain to visualize collagen fibrils. D: hematoxylin-eosin (H&E)-stained section. E: Ki67 staining to visualize cell proliferation. F: H&E stained control section of untreated pancreas. Scale bar: 50 μm.
COX-2 is dispensable for the development of chronic cerulein-induced pancreatic inflammation. Our laboratory showed previously that COX-2 is a crucial factor in the establishment of inflammation and fibrosis in the WBN/Kob rat model of spontaneous chronic pancreatic inflammation (11). Here, we wanted to dissect the molecular role of COX-2 in the progression of the pathology using the mouse model of cerulein-induced CP.

First, we established a time course to define the length of treatment with cerulein injections required to induce a visible and measurable CP in WT mice. Administration of six hourly cerulein injections, three times a week, upregulated COX-2 mRNA levels in a time-dependent manner and reached ~30-fold upregulation after 4 wk of treatment. This suggests that COX-2-dependent processes are active at this time point of the disease progression (Fig. 3A). In addition, both α-SMA mRNA
and collagen fibrils accumulated after 2–4 wk of cerulein treatment (Fig. 3, B and C), indicating the establishment of a fibrotic process. Additional histopathological features were the presence of inflammatory cells, acinar-to-duct metaplasia, and periacinar fibrosis (Fig. 3D). Induction of acinar cells positive for Ki67 (Fig. 3E) indicated an active repair process. Saline-injected control animals did not display any morphological changes (Fig. 3F).

Since we observed that the pancreatic pathology gradually developed within 4 wk, the following experiments comparing WT and COX-2−/− mice focused on a 4-wk cerulein treatment. At the end of the treatment, analyses of histopathology, inflammation levels, and tissue regeneration were performed.

A blinded analysis of morphological changes in pancreata showed no differences between WT and COX-2−/− mice. Furthermore, quantification of collagen fibrils by Sirius red staining and enumeration of F4/80+ macrophages did not reveal any difference between cerulein-treated WT and COX-2−/− mice (Fig. 4, A–C).

In line with the latter observation, transcript levels of the proinflammatory cytokines TNF-α, IL-6, and MCP-1/CCL2 did not differ between the animals (Fig. 4, D and E). In contrast, the profibrotic genes TGF-β, collagen, and α-SMA were selectively downregulated in COX-2−/− mice after 4-wk treatment compared with the WT mice, suggesting a partial dependence of fibrotic processes on COX-2 (Fig. 4, G–J).

To compare the relative transcript levels between acute and chronic pancreatic inflammation, age-matched mice treated and killed after 12 hourly injections are shown (Fig. 4, D–J). In both WT and COX-2−/− mice, IL-6 and MCP-1 were initially highly upregulated and then decreased at 4 wk post-cerulein treatment. In contrast, all of the profibrotic genes analyzed were strongly upregulated only in the later phase of the treatment when the fibrotic process took place.

To further evaluate whether pancreatic repair during CP was affected by the absence of COX-2, we quantified Ki67-positive proliferating cells. While in the acute phase, most of the Ki67-positive cells were of inflammatory nature, in the chronic state, acinar cells were the predominant cell population exhibiting proliferative activity (Fig. 4K). In all cases, there was a trend, albeit not statistically significant, in COX-2−/− animals showing fewer Ki67+ cells. Furthermore, cyclin D1 and cyclin E1 mRNA levels were significantly lower in COX-2−/− animals compared with WT mice after 4 wk of cerulein treatment (Fig. 4L), suggesting that COX-2 activity promotes repair in chronic pancreatic inflammation.

Finally, we evaluated whether pharmacological inhibition of COX-2 activity in WT mice influenced the progression of CP in a similar manner as genetic removal of COX-2. To this aim, mice were fed Vioxx or control chow during the 4 wk of cerulein treatment. Similarly to what was observed in COX-2−/− animals, the histological score was identical in control and Vioxx-treated mice (Fig. 5A). Furthermore, Ki67 staining indicated comparable cell proliferation in the two experimental groups (Fig. 5B). In addition, we evaluated the expression of profibrotic genes, as well as cytokines. As previously detected in COX-2−/− mice, transcription of collagen mRNA was significantly lower in Vioxx-treated animals, while other genes, including α-SMA and TGF-β, did not differ from those of control mice. Moreover, Vioxx treatment inhibited the induction of cytokine mRNA, including IL-6 and COX-2 mRNA. Last, collagen deposition was quantified on the protein level, and, once more, there was no difference between the

Fig. 5. Effect of COX-2 inhibitor on chronic pancreatic inflammation. WT mice were fed with the COX-2 inhibitor Vioxx or control regimen and treated with multiple injections of cerulein during a period of 4 wk. A: histological score. B: Ki67-positive cells of nonacinar and acinar origin. C: relative expression levels of collagen, α-SMA, TGF-β, TNF-α, IL-6, and COX-2. Values were normalized to untreated WT mice. D: morphometric analysis of collagen fibers using Sirius red staining. Values are means ± SE. *P < 0.05.
treated and control mice, corroborating the results from COX-2−/− animals.

**DISCUSSION**

In this report, we explored the contribution of COX-2 to acute and chronic inflammation in the mouse pancreas. Although individual parameters were affected in the absence of COX-2 activity in both settings, tissue injury and inflammatory reaction were only transiently lower in COX-2−/− mice compared with WT controls, thus suggesting that pancreatic inflammation does not depend considerably on this enzyme and its products.

**Acute pancreatitis.** COX-2 is a proinflammatory enzyme active in many diseases. Two groups have focused on acute pancreatitis in mouse models and demonstrated a beneficial effect on the disease progression following COX-2 inhibition or depletion (3, 17). In these reports, mice were used at 4–5 wk old, which is quite an early age compared with the 8- to 10-wk-old adult mice commonly used in both acute and CP models. Indeed, this early age does not resemble the physiology of adult mice, as lymphoid and nonlymphoid organs have not reached their final state of development. To establish our protocols, we repeated the previously published experiment in 4- to 5-wk-old mice using the same knockout strain and cerulein conditions, as reported (3). Our results confirmed the previously published effects of COX-2 on pancreatic inflammation. However, these effects could potentially stem from, or could be explained with, developmental deficits of lymphoid tissues in these juvenile mice. Therefore, we analyzed 8- to 10-wk-old adult mice, commonly used in the cerulein models of pancreatitis. In this experimental condition, the phenotype observed in COX-2−/− mice was indistinguishable from that of WT animals. The interpretation of these results can be discussed in two ways: 1) tissue injury is stronger in juvenile mice, hence COX-2-dependent inflammation is more evident at this mouse age; 2) the development of the immune system is COX-2 dependent, and the absence of COX-2 leads to a more limited response. In support of the first hypothesis, tissue injury, as determined by serum amylase, was higher in young than in adult WT animals. However, comparison of histological scores revealed that tissue injury and inflammation were reduced only in the young COX-2−/− mice, supporting the hypothesis that COX-2 may influence the maturation and development of the innate immune system. Further analyses will be required to elucidate the role of COX-2 in the context of immune system development. Thus, integrating our own results with that of the current literature, we conclude that COX-2 exerts a limited role during the early phase of acute experimental pancreatitis in the mouse.

**CP.** Our primary goal of this study was to verify in two independent mouse models of deficient COX-2 activity our previous observations obtained in the WBN/Kob rats, where application of a COX-2 inhibitor caused an impressive improvement of pancreatic inflammation (11). In the present report, we found a number of parameters that were influenced by COX-2 activity in the setting of CP in adult mice. Particularly, the mRNA levels of the profibrotic genes TGF-β, α-SMA, and collagen were significantly lower in absence of COX-2 activity compared with control mice. These observations support our laboratory’s previous conclusion that COX-2 is required for the establishment of fibrotic processes (11). However, the histopathological score, one of the most indicative parameters for assessing the severity of the disease, and tissue deposition of collagen fibrils were comparable in the absence of functional COX-2 and control mice, indicating that the absence of COX-2 activity is not advantageous to limit the progression of pancreatitis. How can this discrepancy be explained? It is known that the products of COX-2 activity, the PGs, promote proliferation. Furthermore, inhibitors of COX-2 not only impair proliferation but also induce apoptosis in human colorectal cancer cell lines (13). Under the condition of chronic pancreatic inflammation, we observed a higher rate of proliferation and increased cyclin expression in WT animals. Hence repair and regeneration appear to be partially influenced by COX-2. Thus, if COX-2 has a mitogenic beside a proinflammatory activity, damaged tissue might be more efficiently repaired. These two activities may balance out the process of chronic destruction and repair, with a phenotype that is not distinguishable in COX-2−/− and WT mice. Thus, based on our results using both genetic and pharmacological approaches to block COX-2 activity, we conclude that targeting COX-2 does not improve the progression of CP in mouse models.

Considering the different outcome of CP observed in rat models during COX-2 inhibition, further studies are required to understand the role of COX-2 in human patients suffering from CP and to assess the clinical relevance of pharmacological inhibitors of COX-2 in the progression of this disease.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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