Nitric Oxide–Donating Aspirin Prevents Pancreatic Cancer in a Hamster Tumor Model

Nengtai Ouyang,[1] Jennie L. Williams,[1] George J. Tsioulias,[2] Jianjun Gao,[1] Michael J. Iatropoulos,[4] Levy Kopelovich,[5] Khosrow Kashfi,[3] and Basil Rigas[1]

[1]Division of Cancer Prevention, State University of New York at Stony Brook, Stony Brook, New York; [2]Department of Surgery, Mt. Sinai School of Medicine; [3]Department of Physiology and Pharmacology, City University of New York Medical School, New York, New York; [4]Department of Pathology, New York Medical College, Valhalla, New York; and [5]Chemoprevention Branch, Division of Cancer Prevention, National Cancer Institute, NIH, Bethesda, Maryland

Abstract

To evaluate the chemopreventive effect of nitric oxide–donating aspirin (NO-ASA), an ASA bearing a NO-releasing moiety, against pancreatic cancer, we studied six groups of female Syrian golden hamsters: groups 1 to 3 (n = 12 each) were given saline and groups 4 to 6 (n = 17) the carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP) s.c. in five weekly injections (the first, 70 mg/kg, and the remaining, 20 mg/kg each). Control and BOP-treated hamsters were fed a NO-ASA 3,000 ppm or conventional ASA 3,000 ppm or control diet for 19 weeks. Groups 1 to 3 had no tumors. Compared with the BOP/vehicle group, NO-ASA reduced the incidence (88.9%, P < 0.003) and multiplicity (94%, P < 0.05) of pancreatic cancer; ASA had no statistically significant effect. NO-ASA arrested the transition from PanIN2 to PanIN3 and carcinoma. The proliferation (proliferating cell nuclear antigen) / apoptosis (terminal deoxyribonucleotide transferase–mediated nick-end labeling) ratio of ductal cells increased with the histologic severity of the ductal lesion; NO-ASA suppressed it significantly during all stages except PanIN1A. $p21^{WAF1/CIP1}$, undetectable in normal cells, was progressively induced in neoplastic cells and suppressed by NO-ASA up to PanIN3. Nuclear factor-κB activation, absent in normal tissue, increased progressively (17-fold in cancer); NO-ASA suppressed it throughout and significantly in PanIN1B and PanIN2. Cyclooxygenase-2 expression, absent during early stages, was induced 6-fold in carcinoma and suppressed by NO-ASA in PanIN3 and carcinoma. Conventional ASA had no effect on these molecular markers. Thus, NO-ASA profoundly prevented pancreatic cancer and modulated multiple molecular targets in this model system; conventional ASA had no such effects. NO-ASA merits further evaluation as a chemopreventive agent against pancreatic cancer. (Cancer Res 2006; 66(8): 4503-11)

Introduction

More than 29,000 Americans are diagnosed annually with pancreatic cancer, the fourth leading cause of cancer deaths in the United States (1). Early diagnosis is difficult and by the time the disease is recognized clinically, it is usually too late to help the patient significantly. The magnitude of the clinical problem and its special features, which include diagnosis at far-advanced stages and the disappointing performance of current treatments, indicate that efforts should be directed at the prevention of this devastating disease.

Extensive work has made it clear that nonsteroidal anti-inflammatory drugs (NSAID) can significantly affect the development of several types of cancer. Cell culture and animal studies indicate that NSAIDs, including sulindac and indomethacin, inhibit pancreatic carcinogenesis (2–4). The epidemiologic evidence, however, on whether NSAID use affects the incidence of pancreatic cancer has been conflicting (5–9), with all possible outcomes (decreased, not changed, and increased risk) having been reported. As pointed out by Baron (10) in a recent editorial, in general the observed changes are only modest and the increased risk of one study (9) may reflect use of aspirin (ASA) in response to pancreatic cancer symptoms rather than vice versa. The discrepancy between epidemiologic studies and preclinical data could reflect the possibility that, unlike the situation in animals, NSAIDs may lack the potency to prevent pancreatic cancer in humans.

The clinical usefulness of NSAIDs combined with their potentially life-threatening toxicity has prompted intense efforts to improve their safety profile; NO-donating NSAIDs (NO-NSAID) represent such an approach (reviewed in ref. 11). NO-ASA, the most promising among them (Fig. 1), may evolve into a major chemopreventive agent based on the evidence for its superior efficacy and on its safety profile, which, albeit not extensively studied, seems excellent (11, 12). The exact mechanism of action of NO-ASA is not yet clearly understood (reviewed in ref. 11). Nevertheless, aspects of its mechanism of action pertinent to cancer include modulation of Wnt, mitogen-activated protein kinase, and nitric oxide synthase signaling, induction of phase II enzymes, and induction of oxidative stress that leads to cell death (13–17). Our previous work showed that in a cell culture system, NO-ASA was 700-fold more potent than conventional ASA in inhibiting the growth of human pancreatic cancer cells; this was the result of its antiproliferative and proapoptotic effects (18).

Based on these considerations, we evaluated the chemopreventive effect of NO-ASA against pancreatic cancer. We used the Syrian golden hamster model, which recapitulates many morphologic and molecular features of the human disease. In this model, pancreatic tumors develop following the administration of carcinogens, commonly a nitrosamine like *N*-nitrosobis(2-oxopropyl)amine (BOP), one of the few carcinogens that causes pancreatic neoplasia in rodents (19, 20). The tumors are ductal, fast growing, and have K-ras mutations and p53 changes; metastases are common; and the animals are often jaundiced and cachectic. Here, we report our findings, which show that NO-ASA is a potent chemopreventive agent against pancreatic cancer in this animal tumor model, modulating, in the process, cell kinetics and a number of potentially relevant signaling pathways.

Requests for reprints: Basil Rigas, Division of Cancer Prevention, State University of New York at Stony Brook, Life Sciences Building, Room 006, Stony Brook, NY 11794-5200. Phone: 631-632-9035; Fax: 631-632-1992; E-mail: basil.rigas@sunysb.edu.

doi:10.1158/0008-5472.CAN-05-3118

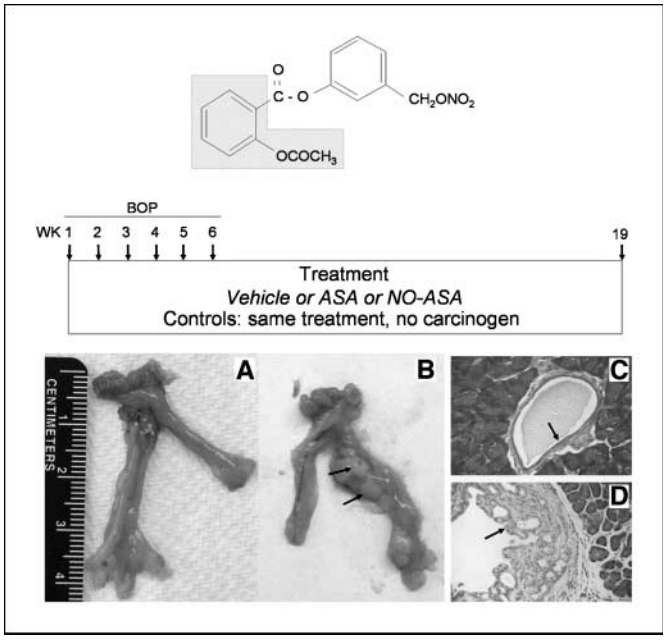

Figure 1. Treatment protocol and pancreatic tumors. *Top,* NO-ASA, composed of conventional ASA (*shaded*), is covalently linked to a moiety that releases NO. *Middle,* animal study design: 5-week-old female Syrian golden hamsters were fed a diet containing vehicle, NO-ASA, or ASA, each at 3,000 ppm, until sacrifice. One week later, weekly treatment with the carcinogen BOP started; the first dose was 70 mg/kg BW and the remaining four, 20 mg/kg each. *Bottom,* gross and microscopic specimens of hamster pancreas. *A,* the normal pancreas of a control (vehicle, no carcinogen) animal. *B,* several tumors are seen in a BOP, control diet–treated animal (*arrows*). *C,* normal pancreatic duct. *D,* ductal adenocarcinoma of the pancreas.

Materials and Methods

Animals and experimental design. Eighty-seven female Syrian golden hamsters (Charles River Laboratory, Kingston, NY), 5 weeks old with an initial average body weight of 70 g, were divided into six groups: groups 1 to 3, each consisting of 12 hamsters, were given saline as control; and groups 4 to 6, each consisting of 17 hamsters, were given carcinogen. The experimental design is shown diagrammatically in Fig. 1. Animals were housed two per plastic cage with sawdust bedding and kept under standard laboratory conditions (room temperature, 22 ± 2°C; relative humidity, 50 ± 5%; light/dark cycle 12/12 hours). All animals had access to pelleted diet (Dyets, Inc., Bethlehem, PA) and tap water ad libitum. After 1 week of acclimation, animals were placed on control, NO-ASA (NCX 4016) 3,000 ppm, or conventional aspirin (ASA) 3,000 ppm diets (made by Dyets), which were stored at 4°C until used. One week later, animals received the carcinogen BOP s.c. in 5 weekly injections, with the first dose being 70 mg/kg body weight and the remaining four 20 mg/kg each (21). Hamsters were observed daily and weighed once every 4 weeks. At the end of week 19, all surviving hamsters were euthanized.

Histologic examination. At necropsy, all organs of the thoracic and abdominal cavities were carefully examined *in situ* grossly. The three lobes of the pancreas with a short segment of the attached duodenum were resected (Fig. 1*C*). One third of one pancreatic lobe was snap-frozen in liquid nitrogen and the remaining pancreas was fixed in 10% phosphate-buffered formalin for 16 hours. The formalin-fixed pancreas was cut into small pieces at ~2 cm intervals and after dehydration each piece was embedded individually in paraffin blocks maintaining the same vertical orientation as in the intact organ. Four-micrometer-thick sections were processed routinely and stained with H&E. Two independent pathologists blinded to sample identity evaluated the sections of the pancreas and scored them according to the PanIN criteria, which include the following categories: normal, PanIN1A, PanIN1B, PanIN2, PanIN3, and carcinoma (22). Each sample was also scored based on the highest lesion found. Cancer incidence (presence or absence of pancreatic cancer in a hamster) and multiplicity (number of pancreatic cancers per hamster) were calculated based on these scores.

Immunohistochemistry. Two further sections of each pancreatic sample were placed side by side on a microscope slide, with one of them used as negative control. Paraffin-embedded sections were deparaffinized, rehydrated, and microwave heated for 15 minutes in 0.01 mol/L citrate buffer (pH 6.0) for antigen retrieval. Then, 3% hydrogen peroxide was applied to block endogenous peroxidase activity. After 15 minutes of blocking with horse serum, the primary antibody or control IgG were applied and incubated overnight at 4°C. Slides were washed thrice with PBS, each for 5 minutes. The biotinylated secondary antibody and the streptavidin-biotin complex (Vector Laboratories, Burlingame, CA) were applied, each for 30 minutes at room temperature with an interval washing. After rinsing with PBS, the slides were immersed for 5 minutes in the coloring substrate 3,3′-diaminobenzidine (DAB, Sigma) 0.4 mg/mL with 0.003% hydrogen peroxide, then rinsed with distilled water, counterstained with hematoxylin, dehydrated, and coverslipped. Antibodies used are as follows: proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA), cyclooxygenase-2 (COX-2; Cayman Chemical, Ann Arbor, MI), nuclear factor-κB (NF-κB) p65 (which recognizes activated NF-κB; Chemicon International, Temecula, CA), and $p21^{WAF1/CIP1}$ (PharMingen, San Diego, CA).

Terminal deoxyribonucleotide transferase–mediated nick-end labeling staining. Terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) staining was done using the *In situ* Cell Death Detection kit (Roche Applied Science, Indianapolis, IN) following the instructions of the manufacturer. Briefly, 4-μm-thick formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by hydrogen peroxide and tissue protein was hydrolyzed with proteinase K. Positive control are sections treated with DNase I 1,000 units/mL. Negative control are sections incubated with label solution (without terminal deoxynucleotidyl transferase enzyme). All other sections were incubated with TUNEL reaction mixture (fluorescein-labeled nucleotides) at 37°C for 1 hour in a humid chamber, incubated with converter-POD solution (antifluorescein antibody conjugated with POD) for 30 minutes at 37°C, treated with DAB, and counterstained with hematoxylin.

Scoring the expression of biomarkers. For each animal, ≥5 ducts per histologic type of PanIN ductal lesion (or ≥10 fields for carcinomas) were scored independently by two experienced investigators not aware of the identity of the specimens (×400). For PCNA, TUNEL, and $p21^{WAF1/CIP1}$ staining, cells with a brown nucleus were considered labeled and those with a blue nucleus unlabeled. For each, we calculated the percentage of positive cells over the total cells counted. For NF-κB and COX-2, we used the following semiquantitative scoring system. The extent of staining was graded as follows: 0, no staining; 1+, ≤25% of cells positive; 2+, 26% to 50% of cells positive; 3+, ≥51% of cells positive. The intensity of staining was scored as follows: 0, no staining; 1+, faint; 2+, moderate; 3+, strong. 1+, 2+, and 3+ were recorded as 1, 2, and 3 points, respectively. To compare differences in staining, an expression index (EI) was calculated by the following formula: EI = extent of staining × intensity of staining.

Statistical analysis. Data are expressed as mean ± SE and analyzed with ANOVA. $P \leq 0.05$ was considered statistically significant.

Results

NO-ASA markedly inhibits BOP-induced pancreatic carcinogenesis. During the period of observation, the body weights of hamsters did not differ significantly between the study groups at any time point (data not shown). Their survival rate was 100% in saline/vehicle and saline/ASA groups and between 83.3% and 94.4% in the remaining groups; these differences were not statistically significant.

All carcinogen-induced tumors originated from the pancreatic ducts; none were from acinar cells. The largest tumor in size was

Table 1. The chemopreventive effect of NO-ASA against pancreatic cancer in hamsters

	Incidence (% decrease)	Multiplicity (mean ± SE; % decrease)	Histologic score (%)					
			Normal	PanIN1A	PanIN1B	PanIN2	PanIN3	Cancer
BOP/vehicle (n = 15)	53.30%	1.00 ± 1.41	0	0	20.0	13.3	13.3	53.3
BOP/ASA (n = 15)	41.2% (22.7)	0.71 ± 1.10 (↓29%)	0	11.8	17.6	23.5	5.9	41.2
BOP/NOASA (n = 17)	5.9% (88.9)*	0.06 ± 0.24 (↓94%) †	0	11.8	41.2	29.4	11.8	5.9

*Statistically significant reduction compared with vehicle (P = 0.003) or ASA (P = 0.015); no significant difference between ASA and vehicle groups.
†Statistically significant reduction compared with vehicle (P < 0.05).

2 × 3 mm. All malignant tumors were ductal adenocarcinomas (Fig. 1). Macroscopically, six animals had changes suggestive of liver metastases. On histologic examination, however, only one was a metastatic lesion from the pancreas, the rest representing inflammatory changes. This animal belonged to the BOP/NO-ASA group. All pancreata from carcinogen-treated hamsters showed morphologic changes in the ductal epithelium. Similar to human pancreatic carcinogenesis, neoplastic changes occurred at multiple sites and various PanIN lesions were observed in the same pancreatic section; each pancreas was scored based on the highest lesion observed. Table 1 shows the incidence and multiplicity of pancreatic cancer in the various groups of animals. Compared with the BOP/vehicle group, NO-ASA dramatically reduced both the incidence (88.9%, P < 0.003) and multiplicity (94%, P < 0.05) of pancreatic cancer. The corresponding changes (22.7% and 29% reductions) induced by ASA were not significantly different.

The histologic findings in the six study groups are summarized in Fig. 2 and Table 1. All animals in the three groups of hamsters that did not receive the carcinogen had histologically normal pancreata without any evidence of neoplastic lesions. In contrast, the three groups of the carcinogen-treated animals showed varying degrees of ductal neoplastic changes. All animals from the BOP/vehicle group had abnormal histology at PanIN1B or higher. In fact, the majority of them displayed advanced lesions: two thirds had either fully developed carcinoma or carcinoma *in situ* with only one third showing lesions of intermediate severity (PanIN1B and PanIN2). The animals of the BOP/ASA group displayed lesions across all stages of neoplasia. Although ASA-treated animals had more lower-grade and fewer higher-grade lesions compared with BOP/vehicle controls, these differences did not reach statistical significance. The BOP/NO-ASA group showed a different distribution of pancreatic lesions. Only one animal (5.9%) showed a fully developed carcinoma, which was significantly different from the corresponding values for the BOP/vehicle (P < 0.003) and the BOP/ASA (P < 0.02) groups. For the remaining of the lesions, there was a clear trend for this group to have more lower-grade lesions than the BOP/vehicle, but these differences were not statistically significant. If carcinoma *in situ* (intraductal carcinoma, PanIN3) and invasive ductal carcinoma are combined, their frequency in BOP/NO-ASA–treated animals is less than one third of that in the BOP/vehicle group (17.6% versus 66.7%, P < 0.01). ASA failed to have such an effect (47.1% versus 66.7%, P > 0.45).

These results make it clear that NO-ASA arrested the process of carcinogenesis in its transition from PanIN2 to PanIN3 (and carcinoma), i.e., it affected the transition from benign to malignant. Of note, although NO-ASA had a dramatic effect on the formation of cancer, it had no effect on the lower-grade lesions, failing to completely protect even a single animal from the neoplastic (nonmalignant) effect of the carcinogen.

NO-ASA inhibits proliferation and induces apoptosis of ductal epithelial cells. To evaluate the mechanism by which NO-ASA exerts its profound chemopreventive effect against pancreatic cancer, we examined whether it modulates the cell kinetics of pancreatic ducts, the tissue of origin of this cancer. Thus, we monitored immunohistochemically both proliferation and apoptosis by assaying the fraction of ductal cells that expressed PCNA or were TUNEL positive, respectively. To assess the relative contribution to tissue homeostasis of these two antithetic processes, we calculated the ratio of proliferation and apoptosis for each pancreatic lesion that we studied.

As shown in Fig. 3, in control hamsters, the proliferation/apoptosis ratio increases progressively with the histologic severity of the ductal lesion, going from 18.17 ± 2.71 (mean ± SE, for this and all subsequent values) in PanIN1A to 28.31 ± 2.54 in carcinoma. This reveals a progressive suppression of apoptosis relative to proliferation (or vice versa). Of note, when we examined proliferation and apoptosis individually, there was a trend toward decreased proliferation and increased apoptosis but these changes were not statistically significant, perhaps reflecting the sample size and/or the magnitude of each effect alone.

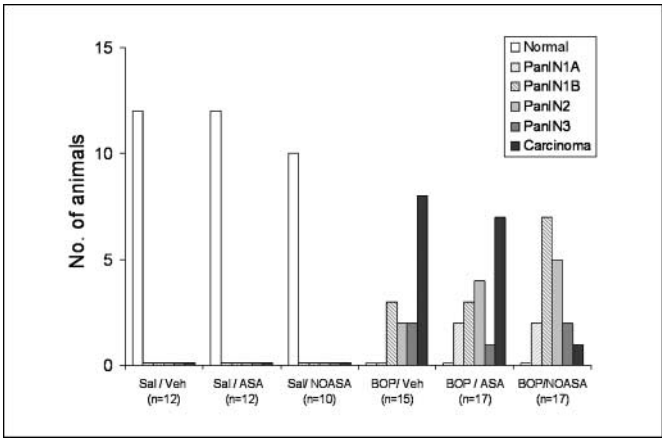

Figure 2. Pancreas histology in the study groups. The formalin-fixed pancreas was cut at ~2 cm intervals and tissue sections stained with H&E were evaluated using the PanIN criteria. Each pancreas was scored based on the highest lesion observed. *Columns,* numbers of animals in each histologic category.

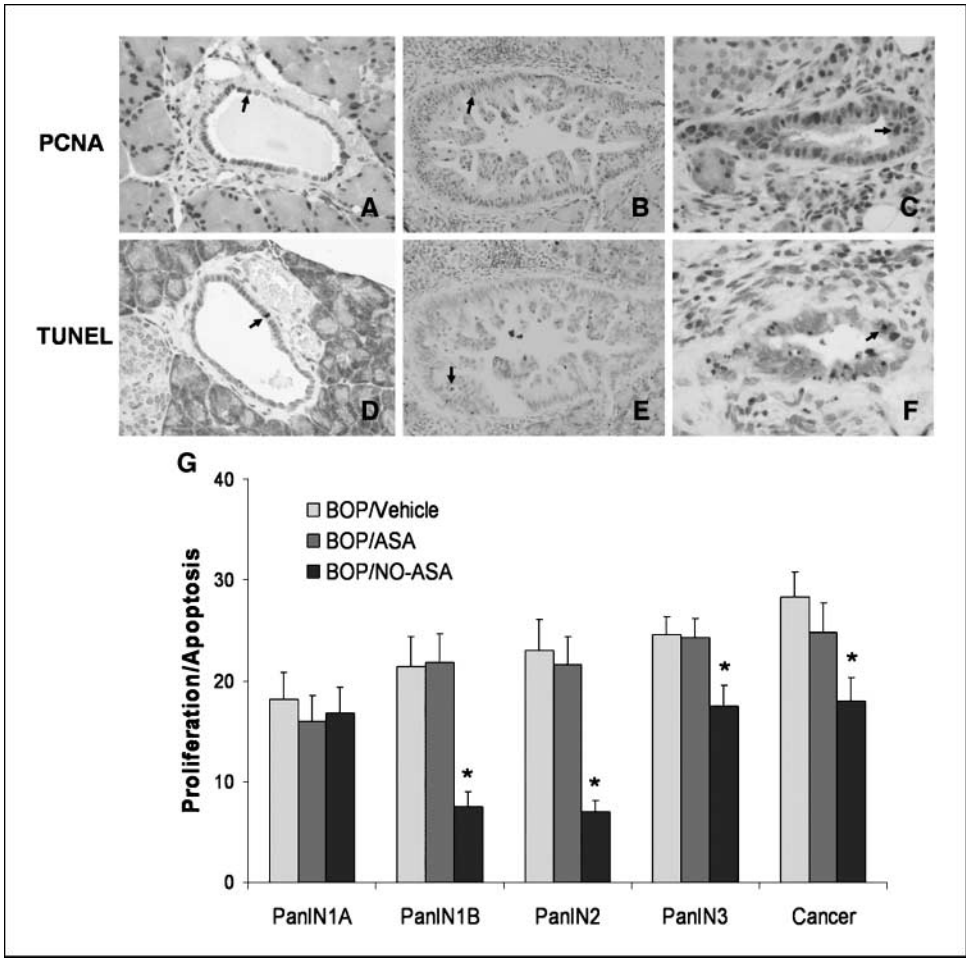

Figure 3. Effect of NO-ASA on proliferation and apoptosis during pancreatic carcinogenesis. *A* to *C,* PCNA immunohistochemical staining. *D* to *F* (corresponding serial sections of *A-C*), TUNEL staining for apoptotic cells in pancreatic tissues. The percentage of proliferating and apoptotic cells was determined as in Materials and Methods, and from these values, the proliferation/apoptosis ratio was calculated for each stage of pancreatic neoplasia and plotted in (*G*). All differences between BOP/vehicle and BOP/NO-ASA are statistically significant ($P < 0.0001$-0.01, except for PanIN1A). All differences between BOP/ASA and BOP/NO-ASA are statistically significant ($P < 0.0001$-0.02, except for PanIN1A and carcinoma). The differences between BOP/vehicle and BOP/ASA are not statistically significant ($P > 0.38$).

ASA-treated animals show an essentially identical pattern of progression of these values, with their small differences being statistically not significant. In contrast, the NO-ASA-treated hamsters display a dramatic reversal of this trend. Compared with BOP/vehicle, NO-ASA suppressed this ratio at all stages of carcinogenesis, although the reduction at PanIN1A (7.8%, compared with control) was not statistically significant. The ratio decreased significantly starting at the PanIN1B stage (65% decrease compared with control; $P < 0.0001$). A similar change was noted at the PanIN2 stage (69.5% decrease; $P < 0.0001$). Interestingly, however, the effect of NO-ASA on this ratio weakened somewhat at the subsequent two stages, PanIN3 (29% decrease; $P < 0.01$) and carcinoma (36.4% decrease; $P < 0.006$). Given that in cultured pancreatic cells, the dominant cell kinetic effect of NO-ASA was the induction of apoptosis (18), we can surmise that these changes in the proliferation/apoptosis ratio reflect a robust induction of apoptosis by NO-ASA. ASA had no significant effect on this ratio.

NO-ASA suppresses the expression of $p21^{WAF1/CIP1}$. To gain further understanding of the cell kinetic effect of NO-ASA on pancreatic ductal epithelial cells, we examined its effect on $p21^{WAF1/CIP1}$, which belongs to the Cip/Kip family of cyclin-dependent kinase (Cdk) inhibitors. Expression of $p21^{WAF1/CIP1}$ is induced by mitogenic stimulation and also by the tumor suppressor protein p53. In addition to inhibiting cell cycle progression by binding to Cdk/cyclin complexes, $p21^{WAF1/CIP1}$ may directly inhibit DNA synthesis by interacting with PCNA, a subunit of DNA polymerase δ (reviewed in ref. 23).

Normal ductal epithelial cells did not express detectable $p21^{WAF1/CIP1}$. However, neoplastic epithelial cells of all stages, from PanIN1A to carcinoma, stained positive for $p21^{WAF1/CIP1}$ (Fig. 4). Interestingly, as depicted in Fig. 4*E* and *F*, the positive cells are principally the "active" cells, whereas the "quiet" cells near the invasive cells are negative for $p21^{WAF1/CIP1}$ expression. NO-ASA markedly decreased $p21^{WAF1/CIP1}$ expression during the early stages (PanIN1A to PanIN2) of carcinogenesis by 70% to 90% compared with control ($P < 0.002$-0.0008); this decrease became much smaller and statistically not significant in PanIN3 and carcinoma (23% and 12%, respectively, compared with control). ASA failed to significantly change the expression of $p21^{WAF1/CIP1}$.

NO-ASA suppresses the activation of NF-κB during the early stages of pancreatic carcinogenesis. NF-κB plays an important role in carcinogenesis, providing, among others, an important conceptual link to inflammation (24). Several studies indicate that dysregulation of NF-κB may be a pivotal event in pancreatic carcinogenesis (reviewed in ref. 25). Consequently, we assessed the activation of NF-κB using an antibody that recognizes the part of the p65 NF-κB subunit that is attached to IκB. Such attachment sequesters NF-κB in the cytoplasm.

Ductal pancreatic cells from animals not exposed to carcinogen had no detectable NF-κB activation. Activated NF-κB was detected in both the cytoplasm and the nuclei of ductal epithelial cells and

tumor cells (Fig. 5). Such activation became apparent at the earliest stage of pancreatic carcinogenesis and increased progressively, becoming maximal at the carcinoma stage (17-fold over the level noted in PanIN1A). Compared with BOP/vehicle controls, NO-ASA inhibited this process during all stages of carcinogenesis (15.9-68.7% reduction). At PanIN1A, this reduction (46.5%) did not reach statistical significance, becoming, however, significant during PanIN1B (69%, $P < 0.0001$) and PanIN2 (68.7%, $P < 0.002$). In the last two stages, PanIN3 and carcinoma, this effect dissipated and the modest reduction of NF-κB activation by NO-ASA was not statistically significant (15.9% and 19.8%, respectively). In contrast, ASA failed to affect the activation of NF-κB to a statistically significant degree during all stages of carcinogenesis.

NO-ASA suppresses COX-2 expression in high-grade lesions. Overexpression of COX-2, initially described in colon cancer, has been the feature of many animal and human cancers, including pancreatic cancer (reviewed in ref. 26). Various reports have indicated that both conventional NSAIDs (e.g., ref. 4) and COX-2-specific inhibitors (27) prevent the development of pancreatic cancer. Thus, we examined the effect of NO-ASA on the expression of COX-2 during pancreatic carcinogenesis in these hamsters.

Hamsters that received no carcinogen showed no detectable expression of COX-2 in the pancreatic ducts (Fig. 6) or even in acinar cells (data not shown). Similarly, in BOP-treated animals, COX-2 was undetectable in PanIN1A and weakly so in PanIN1B ducts. Following that, there was a progressive increase in its expression becoming maximal at the carcinoma lesion (6-fold over that of the PanIN1B stage). Of note, the expression of COX-2 even in adenocarcinomas was not uniform, with only a fraction of the tumor cells staining positive.

NO-ASA inhibited COX-2 expression significantly only at the two most advanced stages, PanIN3 (49% reduction compared with control, $P < 0.007$) and carcinoma (57% reduction, $P < 0.009$). In PanIN1B and PanIN2, the differences between NO-ASA-treated and control groups were not statistically significant. ASA had a marginal inhibitory effect on COX-2 expression, the differences never reaching statistical significance ($P > 0.52$).

Discussion

Our findings document the profound chemopreventive effect of NO-ASA against pancreatic cancer. In addition, they show that conventional ASA failed to prevent pancreatic cancer and also provide an insight into the mechanism by which NO-ASA prevents pancreatic cancer in this animal model.

There are only a few models to study pancreatic cancer, some of them having been reported only recently (20, 28). Although no animal model of cancer can reflect all the features of its human counterpart, the Syrian golden hamster model that was used in this study captures many significant features of human pancreatic

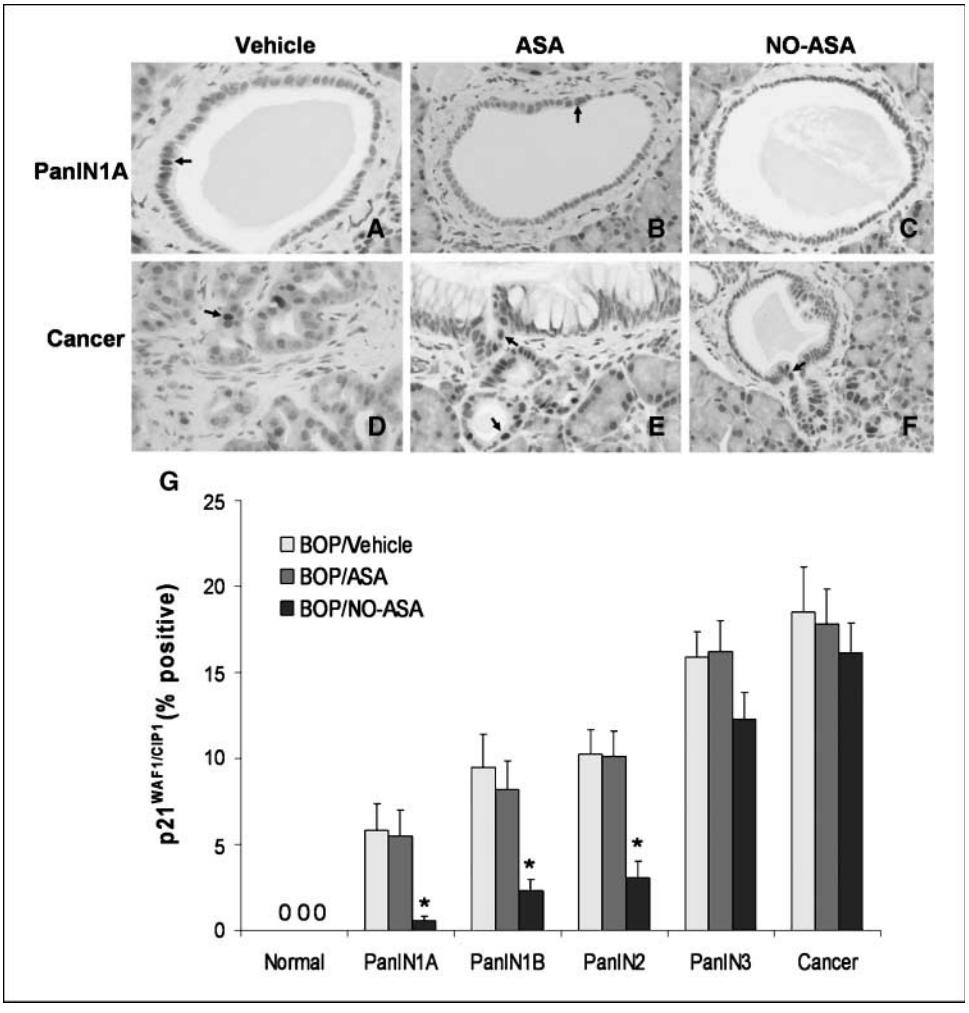

Figure 4. Effect of NO-ASA on $p21^{WAF1/CIP1}$ expression during pancreatic carcinogenesis. Nuclei of ductal epithelial cells from PanIN1A or carcinoma expressing $p21^{WAF1/CIP1}$ stain immunohistochemically brown (positive). Note that the early invasive growth cells are always $p21^{WAF1/CIP1}$ positive (*E* and *F*), in contrast to the neighboring "normal" or "quiet" cells, which are mostly negative. The expression of $p21^{WAF1/CIP1}$ was scored as in Materials and Methods and graphed. *Columns,* mean; *bars,* SE. All differences between BOP/vehicle and BOP/NO-ASA are statistically significant ($P < 0.0001$-0.01, except for PanIN13 and carcinoma). The differences between BOP/vehicle and BOP/ASA are not statistically significant ($P > 0.6$).

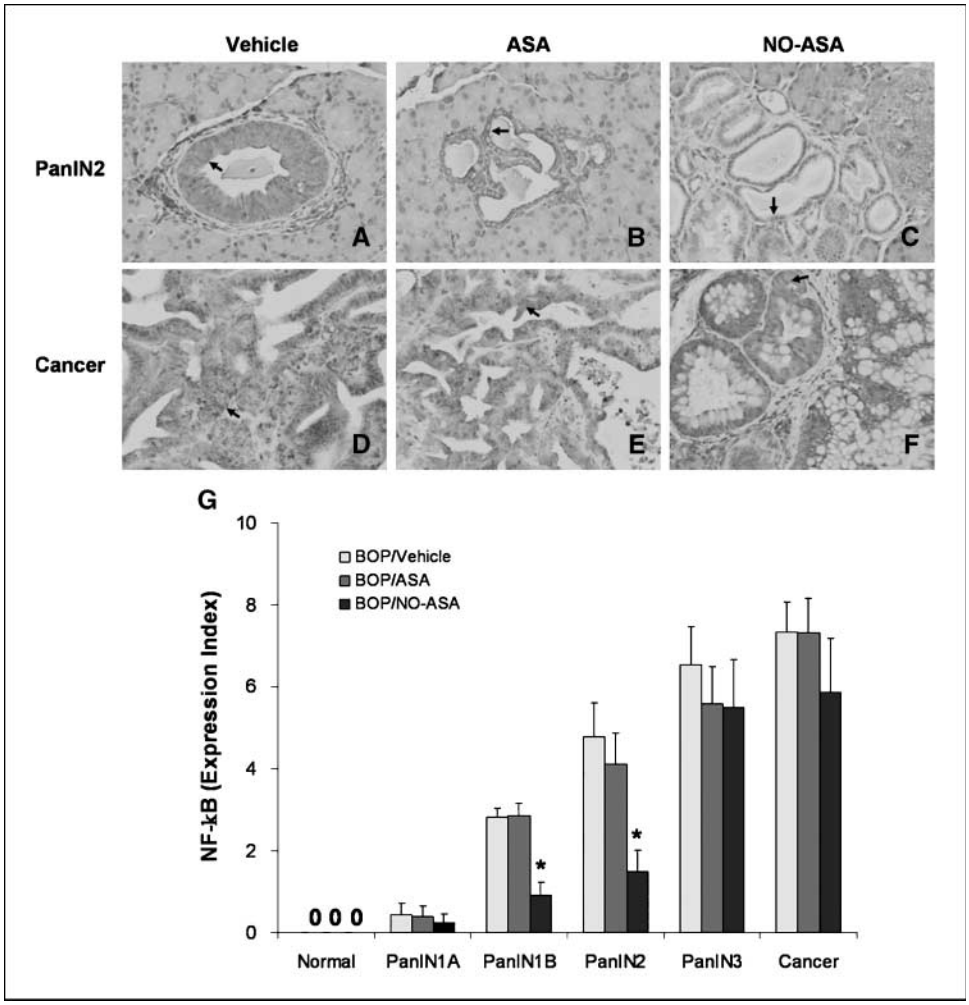

Figure 5. Effect of NO-ASA on NF-κB activation during pancreatic carcinogenesis. *A* to *F,* cytoplasmic and nuclear immunohistochemical staining for activated NF-κB (p65 subunit) in ductal epithelial cells from PanIN2 and carcinoma pancreatic tissue samples from hamsters treated with vehicle, ASA, or NO-ASA. NF-κB was scored as in Materials and Methods (expression index), and these values, calculated for each stage of pancreatic neoplasia, are plotted in (*G*). *Columns,* mean; *bars,* SE. Normal ducts expressed no activated NF-κB. The differences between BOP/vehicle and BOP/NO-ASA are statistically significant for PanIN1B ($P < 0.0001$) and PanIN2 ($P < 0.002$). Differences between BOP/ASA and BOP/NO-ASA are statistically significant for PanIN1B ($P < 0.0001$) and PanIN2 ($P < 0.002$). The differences between BOP/vehicle and BOP/ASA are not statistically significant ($P > 0.38$).

cancer. They include aspects of its histology (ductal tumors), molecular biology (K-ras mutations and p53 changes), and tumor biology (rapid growth and metastases). Thus, our findings can be considered of relevance to human pancreatic cancer, keeping of course in mind the limitations of such an extrapolation.

Both tumor incidence and multiplicity were profoundly suppressed by NO-ASA, being 90% and 94% lower than control, respectively. In contrast, the effect of conventional ASA was very weak, achieving no statistical significance (23% and 29%, respectively). The effect of NO-ASA is the strongest chemopreventive effect against pancreatic cancer reported to date. Nimesulide, a COX-2 inhibitor, reduced both the incidence and multiplicity of pancreatic cancer in golden Syrian hamsters using the same carcinogen (although a slightly lower cumulative dose of BOP was administered in this study compared with ours). However, the effect of nimesulide was substantially weaker compared with that of NO-ASA: Tumor incidence was reduced by 43% and multiplicity by 39% (27). In an earlier study, the same group using the same animal model observed a 48% reduction in tumor incidence with phenylbutazone and no significant effect with aspirin (only a tendency toward reduction akin to that observed by us), whereas indomethacin reduced significantly only tumor multiplicity (50% reduction; ref. 4). In a variation of the hamster tumor model, ethanol and a tobacco carcinogen were given to pregnant hamsters and ibuprofen was administered to the offspring, leading to a 50% reduction in the incidence of pancreatic cancer (29).

The current histologic classification of pancreatic carcinogenesis, marking the progression of the ductal epithelium from normalcy to malignancy, provides the opportunity to analyze the chemoprevention effect of NO-ASA in detail. In the control group, 66.6% had either carcinoma *in situ* (PanIN3) or adenocarcinoma. In the NO-ASA group, only 17.7% had these lesions, corresponding to a 73.4% reduction in the combined incidence of these two lesions. Furthermore, in the NO-ASA group, 53.6% of the animals had a low-grade lesion (PanIN1) and an additional 29.4% an intermediate grade lesion (PanIN2). Thus, NO-ASA essentially blocked the progression of these lesions from a benign stage to a malignant one, its greatest inhibitory effect concerning the transitions from PanIN1B to PanIN2 and from PanIN2 to PanIN3.

Concerning the mechanism of the chemopreventive effect of NO-ASA, our data clearly indicate a multitargeted effect. Pancreatic carcinogenesis was accompanied by a progressive change in cell kinetic variables. The ratio of proliferation/apoptosis that relates the primary (and antithetic) contributors to tissue homeostasis displayed a progressive increase with advancing neoplastic stages. NO-ASA inhibited this ratio significantly, its effect being greatest in the PanIN1B and PanIN2 stages, decreasing substantially in the PanIN3 and adenocarcinoma stages. It is unclear from our data which effect (antiproliferative or proapoptotic), if any, might have

predominated. These changes parallel the effect of NO-ASA on the neoplastic phenotype as discussed above. Moreover, they are consistent with *in vitro* findings by several groups, including our own, that NO-ASA inhibits proliferation and induces apoptosis in various cancer cell lines (30–32). It is worth noting that the diminution of the kinetic effect in the last two (malignant) stages of pancreatic carcinogenesis is consistent with the pattern displayed by other variables evaluated in this study.

An indirect insight into the mechanism underlying the cell kinetic changes induced by NO-ASA is provided by our study of the expression of $p21^{WAF1/CIP1}$ (23). This Cdk inhibitor is activated by p53 to produce cell cycle arrest in response to DNA damage. $p21^{WAF1/CIP1}$ blocks cell cycle progression, both by acting as a general inhibitor of Cdk/cyclin complexes and by inhibiting DNA replication by binding to PCNA, a subunit of DNA polymerase δ. Quantitatively, this was the most pronounced inhibitory effect of NO-ASA among all its targets evaluated in this study. The expression of $p21^{WAF1/CIP1}$ was progressively elevated during pancreatic carcinogenesis in these hamsters, becoming maximal at the last stage of adenocarcinoma. The suppressed expression of $p21^{WAF1/CIP1}$ paralleled the effect of NO-ASA on cell kinetics, suggesting that the two effects may be linked. Like other examples in biology, nitric oxide being a relevant one (33), $p21WAF1^{/CIP1}$ has a dual function in carcinogenesis (reviewed in ref. 34). Its ability to inhibit proliferation may contribute to its tumor suppressor function; a number of oncogenes repress $p21^{WAF1/CIP1}$ to promote cell growth and tumorigenesis. On the other hand, $p21^{WAF1/CIP1}$ also inhibits apoptosis and its repression may have an anticancer effect. Repression of p21 sensitizes tumor cells to apoptosis by anticancer drugs. Indeed, context determines the type of outcome. In our case, it is likely that (*a*) the progressive induction of $p21^{WAF1/CIP1}$ provides the neoplastic cell with a survival advantage

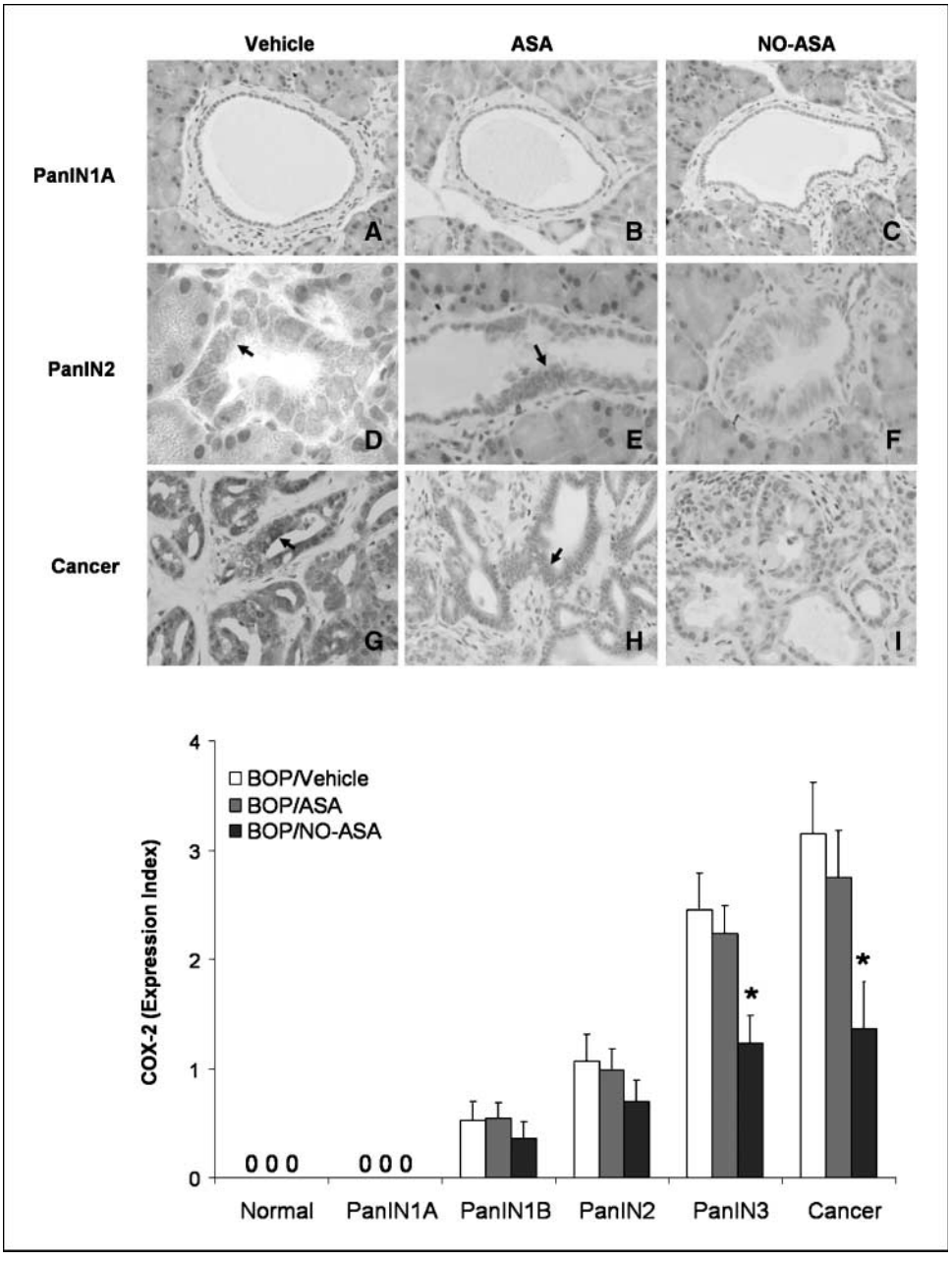

Figure 6. Effect of NO-ASA on COX-2 expression during pancreatic carcinogenesis. The expression of COX-2 was evaluated immunohistochemically (*top*), scored as in Materials and Methods (expression index) and graphed (*bottom*). *Columns,* mean; *bars,* SE. COX-2 was detected in neoplastic ductal epithelial and carcinoma cells and but undetectable in normal and PanIN1A ductal epithelium. A rather weakly positive staining appears in PanIN1B, becoming stronger in advanced stages (*D* and *G*). Differences between BOP/vehicle and BOP/NO-ASA are statistically significant only for PanIN13 and carcinoma ($P < 0.007$ and 0.009, respectively). The differences between BOP/vehicle and BOP/ASA are statistically not significant ($P > 0.52$).

and thus contributes to carcinogenesis and (*b*) its inhibition by NO-ASA contributes to the induction of apoptosis leading to inhibition of carcinogenesis. It is of interest to note that, in other systems, conventional NSAIDs had the opposite effect on $p21^{WAF1/CIP1}$ (35, 36), and this may in part account for the lack of efficacy of ASA in this animal model.

The activation of NF-κB during carcinogenesis is a prominent feature of human pancreatic cancer and is believed to represent a key molecular abnormality (37, 38). In this animal model, NF-κB activation is quantitatively the most pronounced change. Whether this impressive effect is also functionally consequential and thus contributes to the pathogenesis of cancer cannot be deduced from the present data. However, we have no evidence to the contrary either. NO-ASA showed a significant inhibitory effect on NF-κB activation. This effect was limited to stages where NO-ASA was effective. Of note, NO-ASA inhibits NF-κB activation in colon cancer cells (39).

A molecular target of chemoprevention that has been studied extensively in the last decade is COX-2. Its overexpression in both animal and human pancreatic cancer has been well documented and several preventive and therapeutic strategies revolving around COX-2 have been devised (26). Our data showed two important features of COX-2 overexpression during pancreatic carcinogenesis in our animal system. First, there is no detectable expression at its earliest stage, a finding analogous to that observed during colon carcinogenesis (40). Second, NO-ASA inhibited the expression of COX-2 only during the last two stages. Whether this effect contributed to the cancer chemoprevention effect of NO-ASA cannot be decided based on our data or what is known about the potential role of COX-2 in carcinogenesis (41).

Thus, it is apparent that NO-ASA exerts effects on multiple molecular targets in the ductal pancreatic cell; perhaps on more than the present study has revealed. These effects, spanning the entire process of carcinogenesis, are most pronounced in its middle stages and culminate in a dramatic reduction of cancer incidence and multiplicity, which is by far the strongest of any compound reported to date. The actual contribution of each of these changes to the chemoprevention effect of NO-ASA remains to be determined. As we have argued elsewhere regarding the mechanism of action of this compound (11), the choice between mechanistic dominance (one pathway is sufficient for effect) and mechanistic redundancy (effects on multiple pathways are required) is unresolved and our present study underscores this point. That inhibition of NF-κB and $p21^{WAF1/CIP1}$ occurs only during the precancerous stages suggests, however, that these two changes may be relevant to the effect of NO-ASA, which is to block the transition to PanIN3 and carcinoma stages. Once the process of carcinogenesis has advanced to these last two (malignant) stages, NO-ASA has no effect on them and this may perhaps be one of the reasons why these neoplasm progress all the way to carcinoma.

Of great interest has been the observation that the long-term administration of NO-ASA was not accompanied by any apparent side effects. Both the changes of body weight gain and the necropsy study of the hamsters failed to reveal signs of toxicity. This is consistent with the general experience with this compound, both in animals and humans (11). Another interesting finding has been the failure of conventional ASA to influence pancreatic carcinogenesis in this animal model. As already shown, ASA failed to affect any of the molecular targets that we studied and which potentially influence the process of carcinogenesis. This probably accounts for the negative outcome and underscores the fundamental mechanistic differences between ASA and NO-ASA.

In summary, our data indicate that NO-ASA has significant potential for pancreatic cancer prevention; provide a first approximation to its mechanism of action *in vivo*; and suggest that further detailed study of its role as a chemopreventive agent against pancreatic cancer is warranted.

Acknowledgments

Received 9/6/2005; revised 12/21/2005; accepted 2/1/2006.

Grant support: NIH grants CA92423, CA92423-S1, and CA34527.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. American Cancer Society. Cancer facts and figures 2005 Atlanta (Georgia): American Cancer Society; 2005.
2. Yip-Schneider MT, Sweeney CJ, Jung SH, Crowell PL, Marshall MS. Cell cycle effects of nonsteroidal anti-inflammatory drugs and enhanced growth inhibition in combination with gemcitabine in pancreatic carcinoma cells. J Pharmacol Exp Ther 2001;298:976–85.
3. Molina MA, Sitja-Arnau M, Lemoine MG, Frazier ML, Sinicrope FA. Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. Cancer Res 1999;59:4356–62.
4. Takahashi M, Furukawa F, Toyoda K, et al. Effects of various prostaglandin synthesis inhibitors on pancreatic carcinogenesis in hamsters after initiation with *N*-nitrosobis(2-oxopropyl)amine. Carcinogenesis 1990;11:393–5.
5. Anderson KE, Johnson TW, Lazovich D, Folsom AR. Association between nonsteroidal anti-inflammatory drug use and the incidence of pancreatic cancer. J Natl Cancer Inst 2002;94:1168–71.
6. Coogan PF, Rosenberg L, Palmer JR, et al. Nonsteroidal anti-inflammatory drugs and risk of digestive cancers at sites other than the large bowel. Cancer Epidemiol Biomarkers Prev 2000;9:119–23.
7. Langman MJ, Cheng KK, Gilman EA, Lancashire RJ. Effect of anti-inflammatory drugs on overall risk of common cancer: case-control study in general practice research database. BMJ 2000;320:1642–6.
8. Menezes RJ, Huber KR, Mahoney MC, Moysich KB. Regular use of aspirin and pancreatic cancer risk. BMC Public Health 2002;2:18.
9. Schernhammer ES, Kang JH, Chan AT, et al. A prospective study of aspirin use and the risk of pancreatic cancer in women. J Natl Cancer Inst 2004;496:22–8.
10. Baron JA. What now for aspirin and cancer prevention? J Natl Cancer Inst 2004;96:4–5.
11. Rigas B, Kashfi K. Nitric-oxide-donating NSAIDs as agents for cancer prevention. Trends Mol Med 2004;10:324–30.
12. Iconomou G, Kalofonos HP, Koutras A, Vagenakis AG, Rigas B. A pilot study of no-donating aspirin in patients with pancreatic cancer pain. J Support Oncol. In press 2006.
13. Nath N, Kashfi K, Chen J, Rigas B. Nitric oxide-donating aspirin inhibits β-catenin/T cell factor (TCF) signaling in SW480 colon cancer cells by disrupting the nuclear β-catenin-TCF association. Proc Natl Acad Sci U S A 2003;100:12584–9.
14. Spiegel A, Hundley TR, Chen J, et al. NO-donating aspirin inhibits both the expression and catalytic activity of inducible nitric oxide synthase in HT-29 human colon cancer cells. Biochem Pharmacol 2005;70:993–1000.
15. Hundley TR, Rigas B. NO-donating aspirin inhibits colon cancer cell growth via MAP kinase activation. J Pharmacol Exp Ther 2006;316:25–34.
16. Gao J, Kashfi K, Liu X, Rigas B. NO-donating aspirin induces phase II enzymes *in vitro* and *in vivo*. Carcinogenesis. In press 2006.
17. Gao J, Liu X, Rigas B. Nitric oxide-donating aspirin induces apoptosis in human colon cancer cells through induction of oxidative stress. Proc Natl Acad Sci U S A 2005;102:17207–12.
18. Kashfi K, Ryann Y, Qiao LL, et al. Nitric oxide-donating nonsteroidal anti-inflammatory drugs inhibit the growth of various cultured human cancer cells evidence of a tissue type-independent effect. J Pharmacol Exp Ther 2002;303:1273–82.
19. Birt DF, Pour PM, Nagel DL, Barnett T, Blackwood D, Duysen E. Dietary energy restriction does not inhibit pancreatic carcinogenesis by *N*-nitrosobis-2-(oxopropyl)amine in the Syrian hamster. Carcinogenesis 1997;18:2107–11.
20. Hall PA, Lemoine NR. Models of pancreatic cancer. Cancer Surv 1993;16:135–55.
21. Tsutsumi M, Kondoh S, Noguchi O, et al. K-ras gene mutation in early ductal lesions induced in a rapid production model for pancreatic carcinomas

in Syrian hamsters. Jpn J Cancer Res 1993;84:1101–5.
22. Hruban RH, Adsay NV, Albores-Saavedra J, et al. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. Am J Surg Pathol 2001;25:579–86.
23. Hershenson MB. p21Waf1/Cip1 and the prevention of oxidative stress. Am J Physiol Lung Cell Mol Physiol 2004;286:L502–5.
24. Algul H, Adler G, Schmid RM. NF-κB/Rel transcriptional pathway: implications in pancreatic cancer. Int J Gastrointest Cancer 2002;31:71–8.
25. Lin A, Karin M. NF-κB in cancer: a marked target. Semin Cancer Biol 2003;13:107–14.
26. Mann JR, DuBois RN. Cyclooxygenase-2 and gastrointestinal cancer. Cancer J 2004;10:145–52.
27. Furukawa F, Nishikawa A, Lee IS, et al. A cyclooxygenase-2 inhibitor, nimesulide, inhibits postinitiation phase of *N*-nitrosobis(2-oxopropyl)amine-induced pancreatic carcinogenesis in hamsters. Int J Cancer 2003;104:269–73.
28. Lowy AM. Transgenic models of pancreatic cancer. Int J Gastrointest Cancer 2003;33:71–8.
29. Schuller HM, Jorquera R, Reichert A, Castonguay A. Transplacental induction of pancreas tumors in hamsters by ethanol and the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Cancer Res 1993;53:2498–501.
30. Royle JS, Ross JA, Ansell I, Bollina P, Tulloch DN, Habib FK. Nitric oxide donating nonsteroidal anti-inflammatory drugs induce apoptosis in human prostate cancer cell systems and human prostatic stroma via caspase-3. J Urol 2004;172:338–44.
31. Tesei A, Ulivi P, Fabbri F, et al. *In vitro* and *in vivo* evaluation of NCX 4040 cytotoxic activity in human colon cancer cell lines. J Transl Med 2005;3:7.
32. Williams JL, Borgo S, Hasan I, Castillo E, Traganos F, Rigas B. Nitric oxide-releasing nonsteroidal anti-inflammatory drugs (NSAIDs) alter the kinetics of human colon cancer cell lines more effectively than traditional NSAIDs: implications for colon cancer chemoprevention. Cancer Res 2001;61:3285–9.
33. Wink DA, Mitchell JB. Nitric oxide and cancer: an introduction. Free Radic Biol Med 2003;34:951–4.
34. Gartel AL, Radhakrishnan SK. Lost in transcription: p21 repression, mechanisms, and consequences. Cancer Res 2005;65:3980–5.
35. Goldberg Y, Nassif II, Pittas A, et al. The antiproliferative effect of sulindac and sulindac sulfide on HT-29 colon cancer cells: alterations in tumor suppressor and cell cycle-regulatory proteins. Oncogene 1996;12:893–901.
36. Tseng WW, Deganutti A, Chen MN, Saxton RE, Liu CD. Selective cyclooxygenase-2 inhibitor rofecoxib (Vioxx) induces expression of cell cycle arrest genes and slows tumor growth in human pancreatic cancer. J Gastrointest Surg 2002;6:838–43; discussion 844.
37. Wang CY, Guttridge DC, Mayo MW, Baldwin AS. NF-κB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Mol Cell Biol 1999;19:5923–9.
38. Fujioka S, Sclabas GM, Schmidt C, et al. Inhibition of constitutive NF-κB activity by IκB αM suppresses tumorigenesis. Oncogene 2003;22:1365–70.
39. Williams JL, Nath N, Chen J, et al. Growth inhibition of human colon cancer cells by nitric oxide (NO)-donating aspirin is associated with cyclooxygenase-2 induction and β-catenin/T-cell factor signaling, nuclear factor-κB, and NO synthase 2 inhibition: implications for chemoprevention. Cancer Res 2003;63:7613–8.
40. Nobuoka A, Takayama T, Miyanishi K, et al. Glutathione-*S*-transferase P1-1 protects aberrant crypt foci from apoptosis induced by deoxycholic acid. Gastroenterology 2004;127:428–43.
41. Nath N, Labaze G, Rigas B, Kashfi K. NO-donating aspirin inhibits the growth of leukemic Jurkat cells and modulates β-catenin expression. Biochem Biophys Res Commun 2005;326:93–9.