Carcinogenesis vol.27 no.2 pp.232–239, 2006
doi:10.1093/carcin/bgi221
Advance Access publication August 25, 2005

NO-donating aspirin isomers downregulate peroxisome proliferator-activated receptor (PPAR)δ expression in *APC*$^{min/+}$ mice proportionally to their tumor inhibitory effect: Implications for the role of PPARδ in carcinogenesis

Nengtai Ouyang, Jennie L.Williams and Basil Rigas*

Division of Cancer Prevention, Department of Medicine, SUNY at Stony Brook, Stony Brook, NY 11794, USA

*To whom correspondence should be addressed. Division of Cancer Prevention, Life Sciences Building, Room 06, SUNY at Stony Brook, Stony Brook, NY 11794-5200, USA. Tel: +1 631 632 9035; Fax: +1 631 632 1992; Email: basil.rigas@stonybrook.edu

Nitric oxide donating aspirin (NO-ASA), consisting of a traditional ASA to which a NO-releasing moiety is covalently attached, is a promising chemopreventive agent against colon cancer. Its mechanism of action is not fully delineated. Here we examined its effect on the expression of the nuclear receptor PPARδ, whose role in colon carcinogenesis remains highly controversial. We studied histochemically the effect of the *meta* and *para* positional isomers of NO-ASA on PPARδ expression in *Min* (multiple intestinal neoplasia) and wild-type mice, and on cell proliferation and apoptosis. PPARδ, minimally expressed in wild-type mice, was significantly expressed in *Min* mice. *para* NO-ASA inhibited intestinal tumor incidence (59%) and PPARδ expression (55.3%) more than *meta* NO-ASA (38 and 41.5%, respectively). Neither isomer affected cell proliferation, but both induced apoptosis in *Min* mice (*para* 52.5% for normal mucosa and 70.3% for tumors; *meta* 31.4 and 21.9%, respectively). The changes in PPARδ expression correlated significantly with changes in apoptosis. Furthermore, NO-ASA induced areas of necrosis in intestinal tumors are probably resulting from the induction of atypical apoptosis. Our data suggest that NO-ASA suppresses intestinal tumorigenesis possibly in part through its inhibitory effect on PPARδ, the expression of which may contribute to intestinal carcinogenesis.

Introduction

NO-donating aspirin (NO-ASA) is emerging as a potentially important chemopreventive agent, due to its apparently excellent safety profile and enhanced potency compared with traditional aspirin (ASA). NO-ASA consists of a traditional ASA molecule to which $–ONO_2$ is covalently attached and $–ONO_2$ is the moiety that releases NO, which is considered responsible for much of its desirable pharmacological properties (Figure 1). There are three positional isomers of NO-ASA, *meta*, *ortho* and *para*, depending on the position of $–ONO_2$ in the benzene ring, with respect to the ester bond between the two benzene rings. Despite significant progress, the mechanism by which NO-ASA exerts its chemopreventive effect against colon cancer still remains incompletely understood (reviewed in Ref.1).

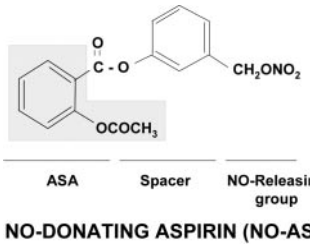

Fig. 1. The chemical structure of NO-ASA. NO-ASA consists of a traditional ASA molecule (shaded), the spacer and $–ONO_2$, which releases NO, the molecule considered responsible for much of its desirable pharmacological properties. There are three positional isomers of NO-ASA depending on the position of $–ONO_2$ in the benzene ring, *meta* (*shown here*), ortho and *para*, with respect to the ester bond between the two benzene rings.

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the superfamily of nuclear receptors that enable the cell to respond to extracellular stimuli through transcriptional regulation of gene expression (2,3). The three PPAR isoforms, α, γ and β/δ or simply δ, function as heterodimers with the 9-*cis*-retinoic receptor, their obligate partner, and regulate various developmental and metabolic pathways. Long-chain fatty acids, prostacyclin and several synthetic molecules activate PPARδ. Recent research is unraveling its role in diverse functions, including wound healing and control of inflammation (4).

The role of PPARδ in colon cancer has been unclear, as there are data suggesting that it either promotes or inhibits colon cancer; the sharpest controversy arises from the relevant animal studies [summarized in Ref. (5)]. The following observations strongly suggest that PPARδ enhances colon cancer formation: PPARδ inhibits differentiation, confers apoptotic resistance and promotes cell migration (6). PPARδ was elevated in colon cancer cells and was repressed by the *APC* (adenomatous polyposis coli) gene via the β-catenin/Tcf-4 response elements in its promoter (7). Moreover, non-steroidal anti-inflammatory drugs (NSAIDs), such as sulindac, disrupt the ability of PPARδ to bind to its recognition sequences, leading to the conclusion that PPARδ mediates their chemopreventive effect (7). Genetic disruption of PPARδ decreases the tumorigenicity of human colon cancer cells (8). Pharmacological activation of PPARδ accelerated intestinal adenoma growth in *Apc*$^{min/+}$ mice [henceforth denoted simply as *Min* (multiple intestinal neoplasia) mice] (9). Indirect evidence for its antitumorigenic effect was provided by findings that it causes differentiation in inflammatory conditions (10,11). The strongest evidence in this regard comes from animal studies. One of them, showed that the number of polyps was the same between *Min* mice that were *Ppard*$^{-/-}$, *Ppard*$^{+/-}$ or

Abbreviations: AI, apoptosis index; APC, adenomatous polyposis coli; ASA, aspirin; EI, expression index; FAP, familial adenomatous polyposis; *Min*, multiple intestinal neoplasia; NO-ASA, NO-donating aspirin; NSAID, non-steroidal anti-inflammatory drug, PI, proliferation index; PPAR, peroxisome proliferator-activated receptor; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling.

Pard$^{+/+}$. Thus PPARδ was considered nonessential for colon carcinogenesis, although data on polyp size suggested that it might be contributing towards maximal polyp growth (12). The most striking result was provided by a study demonstrating that in PPARδ deficient (*Ppard*$^{-/-}$) mice, both *Min* mutants and those with chemically induced cancers, colon polyp formation was significantly higher in those nullizygous for PPARδ (13). The conclusion was that PPARδ attenuates colon carcinogenesis instead of promoting it. Finally, Reed *et al*. (14) reported recently that PPARδ-null *Min* mice exhibited increased predisposition to intestinal tumorigenesis.

Given our limited understanding of the mechanism by which NO-ASA exerts its colon chemopreventive effect and the controversy surrounding the role of PPARδ in colon carcinogenesis, we examined the potential interaction between the two. Our data indicate that in *Min* mice the chemopreventive effect of NO-ASA isomers is accompanied by a reduction of PPARδ expression that is commensurate with the degree of chemoprevention. This effect is, in turn, accompanied by a quantitatively corresponding induction of apoptosis. Our findings suggest a potential mechanism for NO-ASA's effect on colon cancer and favor the notion that PPARδ participates in colon carcinogenesis.

Materials and methods

Animal study

Six-week-old female C57BL/6J *APC*$^{min/+}$ mice and the corresponding C57BL/6J$^{+/+}$ wild-type mice (of which the *Min* mice are a congenic derivative) were treated via intrarectal administration of NO-ASA for 21 days. Study groups (10 mice per group, randomly assigned) were as follows: Group 1, wild-type mice treated with vehicle; Group 2, wild-type mice treated with *meta*/NO-ASA 100 mg/kg/day; Group 3, wild-type mice treated with *para*/NO-ASA 100 mg/kg/day; Group 4, *Min* mice treated with vehicle; Group 5, *Min* mice treated with *meta*/NO-ASA 100 mg/kg/day and Group 6, *Min* mice treated with *para*/NO-ASA 100 mg/kg/day. A pelleted basal diet of LabDiet 5K20 (Jackson Laboratory, Bar Harbor, Maine) and water were available *ad libitum*. Mice were injected intraperitoneally with 5-bromo-2′-deoxyuridine (BrdU; BD Biosciences, San Jose, CA) 10 μg/g body wt 12 h before sacrifice. Results on tumor incidence in these mice have been recently reported (15).

Immunohistochemistry and TUNEL staining

Immunohistochemistry and TUNEL staining were performed as previously reported (16). Antibodies (all from Santa Cruz, CA) and their final dilution were as follows: polyclonal anti-PPARδ (sc-1987), anti-PPARα (sc-9000) and anti-PPARγ (sc-7196) antibodies, each at 1:50 dilution, and mouse monoclonal anti-PCNA antibody (sc-56) at 1:200 dilution. Anti-BrdU monoclonal antibody (NeoMarkers, Fremont, CA) was applied at 1:100 dilution. Epithelial cells with any nuclear staining for PPAR, proliferating cell nuclear antigen (PCNA) and BrdU or stained by the terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) method were scored as positive, and all others were scored as negative. For each sample, five randomly selected fields at ×200 magnification were evaluated. The apoptosis index (AI), proliferation index (AI) and PPAR expression index (EI) were calculated by dividing in each case the number of positive cells by the number of all epithelial cells and multiplying it by 100.

Statistical analysis

The data were expressed as the mean ± SEM. Group means were compared using one-way analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparisons procedure. Differences with a $P < 0.05$ were considered statistically significant.

Results

NO-ASA inhibits intestinal tumors in Min mice and downregulates the expression of PPARδ but not of PPARα and PPARγ

This study was undertaken to assess the expression of PPARδ during intestinal carcinogenesis, and also to assess

Table I. The effect of NO-ASA on tumor incidence, PPAR expression and cell kinetics in *Min* mice

	Wild-type mice			*Min* mice					
	Normal epithelium			Normal epithelium			Tumor		
	Vehicle	*Meta*	*Para*	Vehicle	*Meta*	*Para*	Vehicle	*Meta*	*Para*
Mean ± SEM; $N = 10$ for each group; (% change, compared with the control)									
Number of tumors	0	0	0				24.7 ± 3.8[a]	15.3 ± 2.4 (↓38%)	10.1 ± 1.4[b] (↓59)
PPAR-δ	2.68 ± 0.8[c]	2.45 ± 0.73[d] (↓8.9)	2.58 ± 0.96[e] (↓3.7)	28.4 ± 2.61[f]	22 ± 2.25[g] (↓22.6)	20.8 ± 2.49[h] (↓26.9)	27 ± 2.10[i]	15.8 ± 2.3[j] (↓41.5)	12.1 ± 1.4[k] (↓55.3)
PPAR-α	—	—	—	5.11 ± 0.56	4.20 ± 0.57 (↓17.8)	5.13 ± 0.58 (↑0.4)	1.83 ± 0.31	1.57 ± 0.20 (↓14.2)	1.86 ± 0.34 (↓1.6)
PPAR-γ	—	—	—	4.56 ± 0.50	4.6 ± 0.58 (↑0.8)	4.63 ± 0.60 (↑1.5)	1.50 ± 0.43	1.29 ± 0.36 (↓14.0)	1.63 ± 0.32 (↑8.7)
PI	9.6 ± 2.16	8.2 ± 2.57	8.8 ± 2.81	11.1 ± 1.46	10.2 ± 2.51	8.9 ± 2.28	76.2 ± 8.11	67.8 ± 6.32	63.7 ± 11.45
AI	3.46 ± 0.36	3.58 ± 0.31 (↑2.7)	3.52 ± 0.38 (↑4.3)	3.79 ± 0.38	4.98 ± 0.73 (↑31.4)	5.78 ± 0.83 (↑52.5)	2.69 ± 0.83[l]	3.28 ± 0.46[m] (↑21.9)	4.58 ± 0.54[n] (↑70.3)

a versus b $P < 0.05$; c versus f $P < 0.001$; d versus g $P < 0.001$; e versus h $P < 0.001$; i versus j $P < 0.002$; i versus k $P < 0.0001$; l versus n $P < 0.02$; all other differences are not statistically significant.
↓, decrease; ↑, increase.

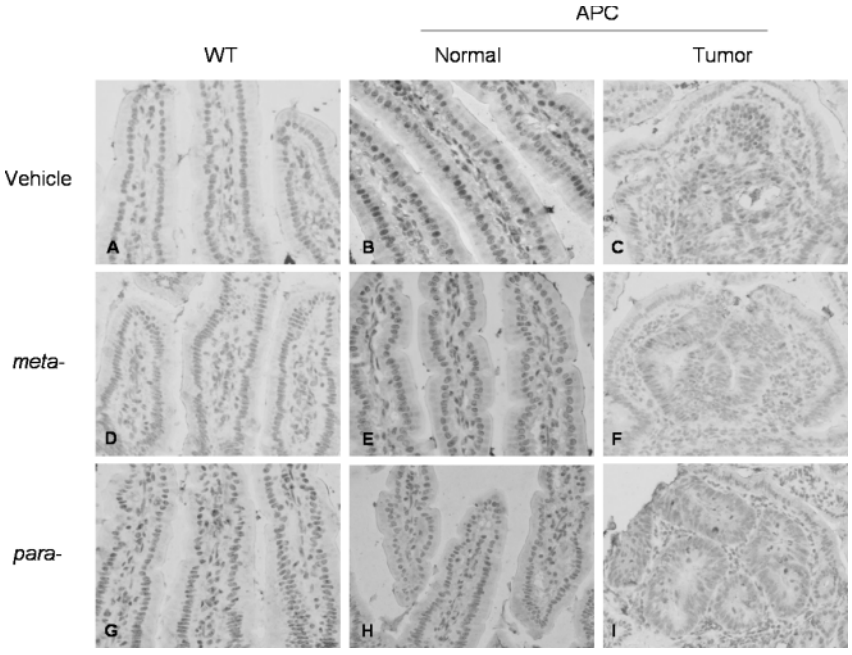

Fig. 2. PPARδ expression in murine intestinal mucosa following the treatment with NO-ASA. No PPARδ expression can be detected immunohistochemically in wild-type mice, including vehicle (**A**), *meta* NO-ASA (**D**) or *para* NO-ASA treated (**G**) animals. PPARδ is detected in control *Min* mice, mostly in the nuclei of villous epithelial (**B**) and tumor cells (**C**) but not in crypts. PPARδ expression is decreased in both epithelial (**E**) and tumor cells (**F**) of *meta* NO-ASA treated animals and more so in *para* NO-ASA treated animals (**H**) and (**I**). Magnification ×400.

the potential effect of NO-ASA on it. Consequently, we studied both *Min* mice and also their congenic (wild-type) mice, C57BL/6J$^{+/+}$. *Min* is a mutant allele of the murine *APC* locus, encoding a nonsense mutation at codon 850. The homozygosity of *Min* mutation (*APC*$^{Min/Min}$) leads to early embryonic lethality but heterozygous *Min* mutants (*APC*$^{Min/+}$) survive for 4–5 months and develop spontaneously tumors in the intestine (17). *Min* mice and patients with familial adenomatous polyposis (FAP) share significant traits: the mutation or loss of *APC* lead to the formation of multiple intestinal adenomas. However, mouse and human *APC* mutants also differ in many other respects; for example, *Min* mice develop mainly tumors of the small intestine, while FAP patients develop mainly tumors of the large intestine. The wild-type *APC* gene is a classical tumor suppressor at the cellular level (18). The mutation of the *APC* allele might lead to the loss of wild-type function, interfere with the wild-type function or have an increased or novel oncogenic activity.

Our previous work has demonstrated that the *ortho* and *para* positional isomers of NO-ASA have similar potencies in inhibiting cancer cell growth and each isomer is ∼100-fold more potent than the *meta* isomer (15). Thus we evaluated only the *meta* and *para* isomers of NO-ASA. As shown in Table I, wild-type mice, as expected, had no intestinal tumors and treatment with NO-ASA did not induce any tumor. In the *Min* mice, however, *meta* NO-ASA reduced tumor multiplicity by 38% and the *para* isomer was more effective, reducing it by 59% ($P < 0.05$) (15).

PPARδ staining was always nuclear (Figure 2). Most of the positive cells were found in the intestinal villi, with only a few positive cells encountered in the crypts. PPARδ was minimally expressed and virtually to the same extent among the three groups of wild-type mice. In contrast, the expression of PPARδ in the histologically normal mucosa of *Min* mice (vehicle-treated group) was ∼10-fold increased compared

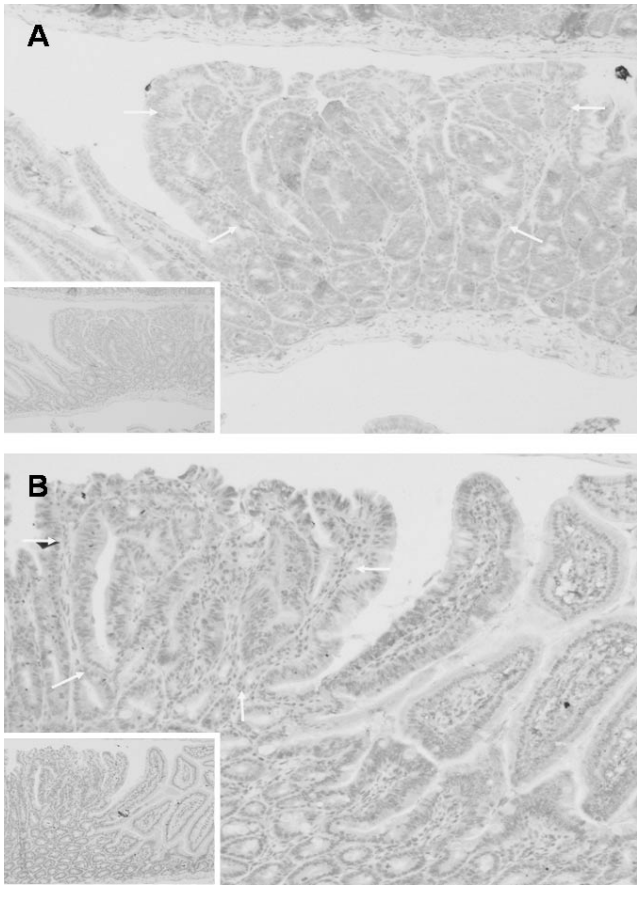

Fig. 3. PPARα and PPARγ expression in murine intestinal mucosa following treatment with NO-ASA. PPARα (**A**) and PPARγ (**B**) are expressed in both the nuclei and cytoplasm of villous epithelial cells and partly in tumor and crypt cells. Staining was carried out as described in Materials and methods. Insets represent negative controls with isotypic IgG. Arrows outline the tumors. Magnifications: A, ×200; B, ×100.

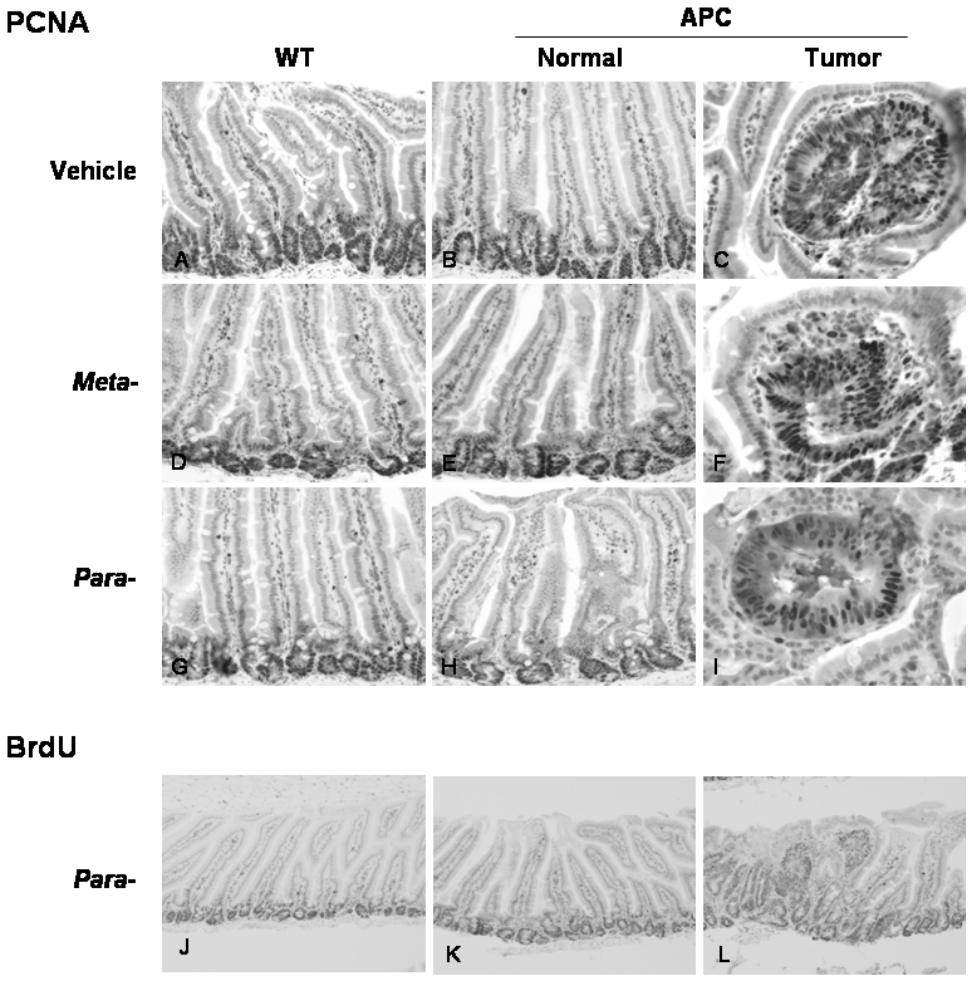

Fig. 4. PCNA and BrdU immunostaining of murine intestinal mucosa following treatment with NO-ASA. Staining was performed as described in Materials and methods. Upper panel: NO-ASA isomers (*meta* and *para)* failed to affect the percentage of PCNA positive cells in both wild-type and *Min* mice in normal epithelial (**A, B, D, E, G** and **H**) or tumor (**C, F** and **I**) cells. Similarly, when proliferation was assessed using the BrdU method (lower panel), no difference was found between wild-type control (**J**) and either normal epithelium (**K**) or tumors (**L**) of *Min* mice treated with *para* NO-ASA. Magnifications: C, F and I, ×400; A, B, D, E, G and H, ×200; J, K and L, ×100.

with that of wild-type mice (28.4 ± 2.6 versus 2.68 ± 0.8; $P < 0.001$; mean ± SEM for these and all subsequent values). The expression of PPARδ in tumors was similar to that in histologically normal intestinal epithelium (27 ± 2.1 versus 28.4 ± 2.6).

The two NO-ASA positional isomers inhibited the expression of PPARδ in both normal and neoplastic cells. The *meta* isomer suppressed the expression of PPARδ in histologically normal mucosa by 22.6% and in neoplastic tissue by 41.5%. The *para* isomer of NO-ASA suppressed PPARδ expression in histologically normal mucosa to a similar extent as the *meta* (26.9%) but nearly twice as much in neoplastic tissues (55.3%). The reduction in the number of tumors by each NO-ASA isomer and the respective suppression of PPARδ expression in neoplastic cells are strikingly similar: the *meta* isomer reduced tumor incidence by 38% and PPARδ expression by 41.5%, whereas the corresponding numbers for the *para* isomer are 59 and 55.3%.

In addition to PPARδ, we evaluated as a control the expression of two other members of the PPAR family, PPARα and PPARγ (Figure 3). Both were detected in the cytoplasm and the nuclei of epithelial cells. While their expression was undetectable in wild-type mice, it was clear-cut in *Min* mice, albeit limited to <10% of the cells, normal or neoplastic (Table 1). Tumor cells expressed these two nuclear factors much weaker than normal mucosa, their expression indices of tumors being roughly one-third of those of the corresponding normal mucosa. NO-ASA had no appreciable effect on their expression.

Effect of NO-ASA on proliferation and apoptosis in the intestinal mucosa: correlation with PPARδ expression

Chemopreventive agents, in general, modulate the cell kinetics of the tissue that they target. On the other hand, PPARδ has been reported to have an antiapoptotic effect in several systems, such as keratinocytes and colon cancer cells (19) and renal medullary interstitial tests, following hypertonic stress (20) Thus, it was important to evaluate the effect of NO-ASA on cell kinetics and examine whether this might be correlated with the expression of PPARδ.

As shown in Figure 4 and Table I, NO-ASA had no effect on cell proliferation in any of the animal groups that we evaluated, whether it was determined by assaying for the expression of PCNA or by in vivo labeling with BrdU. Similarly, it had no effect on the rate of apoptosis in the intestinal epithelium of wild-type mice (Figure 5A, B and C). In contrast, NO-ASA

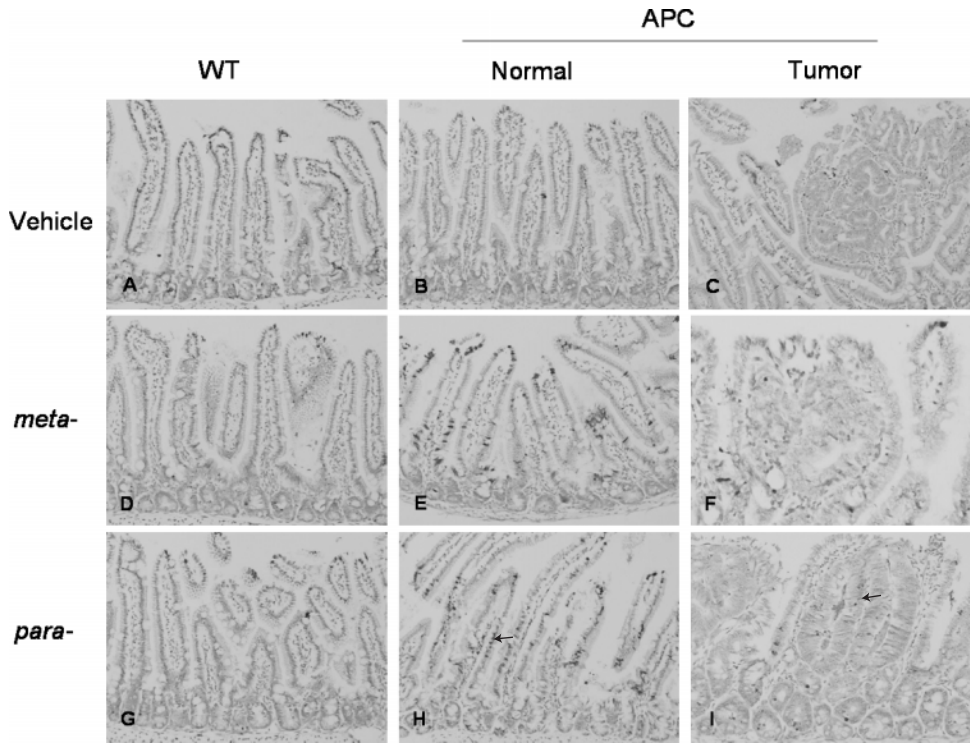

Fig. 5. Apoptosis in murine intestinal mucosa following treatment with NO-ASA. TUNEL staining, performed as described in Materials and methods, shows apoptotic cells in intestinal tissue of mice. In wild-type animals, the percentage of apoptotic cells in normal mucosa was not different between control (**A**) and *meta* NO-ASA (**D**) or *para* NO-ASA (**G**) treated animals. In *Min* mice, *meta* NO-ASA increased apoptosis modestly while *para* NO-ASA increased it significantly in normal mucosa (**E**) and (**H**) and tumors (**F**) and (**I**) compared with vehicle control (**B**) and (**C**). Arrows in H and I indicate apoptotic cells. Magnifications: C, F and I, ×200; all others, ×100.

induced apoptosis significantly in both histologically normal and neoplastic intestinal epithelial cells of *Min* mice, more prominently in the latter. Compared with tissues from vehicle-treated *Min* mice, *meta* NO-ASA increased apoptosis by 31.4% in the normal epithelium and 21.9% in the neoplastic tissues, while *para* NO-ASA increased it by 52.9 and 70.3%, respectively. In both cases, it is clear that the *para* isomer is more potent than the *meta*, in keeping with previous findings form cell culture systems (15). Of particular interest, there exists a statistically significant correlation between the percentage of changes in PPARδ expression and apoptosis induced by the NO-ASA isomers in neoplastic tissues in *Min* mice ($r = -0.41$, $P < 0.03$) (Figure 5G, H and I); this finding suggests a potential etiological association between the two events.

Relationship between PPARδ expression, tumor necrosis and apoptosis in NO-ASA treated Min mice

We noticed that some of the tumors in the NO-ASA treated groups of mice had relatively small necrotic areas. We examined in detail the expression of PPARδ as well as the rate of apoptosis around the necrotic areas. Figure 6 captures the evolution of this process. It is apparent that the area of necrosis, even in its nascent form is surrounded by an abundance of TUNEL positive cells, which appear at a much greater density than in neighboring tissues. On occasion, there were TUNEL positive areas within such necrotic areas. This suggests the existence of free 3′-OH ends of degraded DNA (that become TUNEL positive) and, by extension, the cellular origin of the area of necrosis, which in all likelihood represents coagulative necrosis.

Even though there is an apparent correlation between the induction of apoptosis by NO-ASA and the expression of PPARδ (Figure 5G, H and I), we sought to substantiate this in a direct way. Consequently, we studied two successive tissue sections of intestinal tumors displaying areas of necrosis, one stained for PPARδ expression and the next by the TUNEL method. Figure 7 makes this correlation obvious.

Discussion

Our data demonstrate that, compared with wild-type mice, the nuclear receptor PPARδ is overexpressed in the intestinal mucosa of *Min* mice and that two isomers of NO-ASA, which inhibit their intestinal neoplasia, inhibit to a commensurate degree the expression of PPARδ as well. This effect is accompanied by the induction of epithelial cell apoptosis, which correlates well with the antineoplastic effect of PPARδ.

The expression of PPARδ, minimal in the intestinal mucosa of wild-type mice, was markedly increased in the epithelial cells of histologically normal mucosa and neoplasms in *Min* mice. This finding suggests that the induction of PPARδ is an early event in the neoplastic process (appears maximally in the histologically normal mucosa). These data, however, cannot distinguish whether it is a mechanistically inconsequential result of carcinogenesis or an active player in this process. There is a degree of specificity in the induction of PPARδ, as neither PPARα nor PPARγ, the other two isoforms of this nuclear receptor, are induced to any significant extent compared with PPARδ. Of course, one should keep in mind that immunohistochemistry assesses only protein levels and not their functional status.

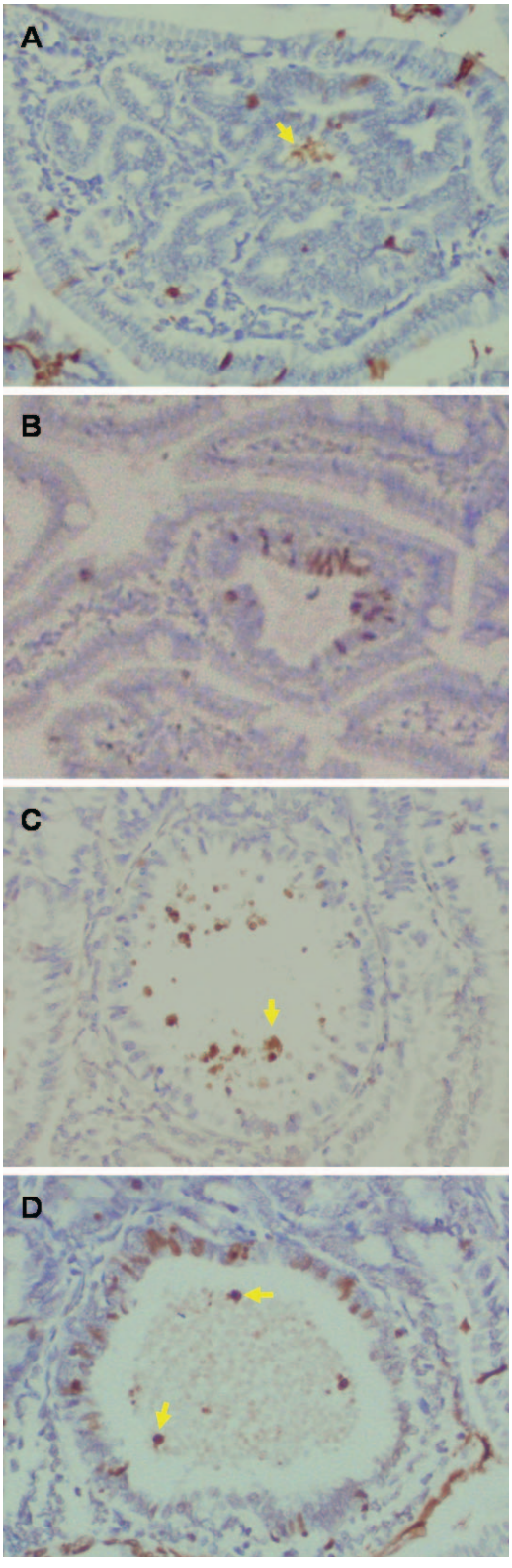

Fig. 6. The evolution of necrotic areas in NO-ASA treated intestinal tumors. Sections of tumors were stained by the TUNEL method, as described in Materials and methods. This series of slides from animals treated with *para* NO-ASA captures the evolution of this process. (**A**) the coalescence of TUNEL positive cells (arrow) represents the earliest stage. (**B**) abundant apoptotic cells at the margins of the developing area, in contrast to rarity of such cells in the surrounding tissue. (**C**) and (**D**) the necrotic area is increasing in size, but TUNEL positive cells persist at the margins of the necrotic area; multiple TUNEL positive areas within the necrotic areas (arrows) suggest its cellular origin. Magnification ×400.

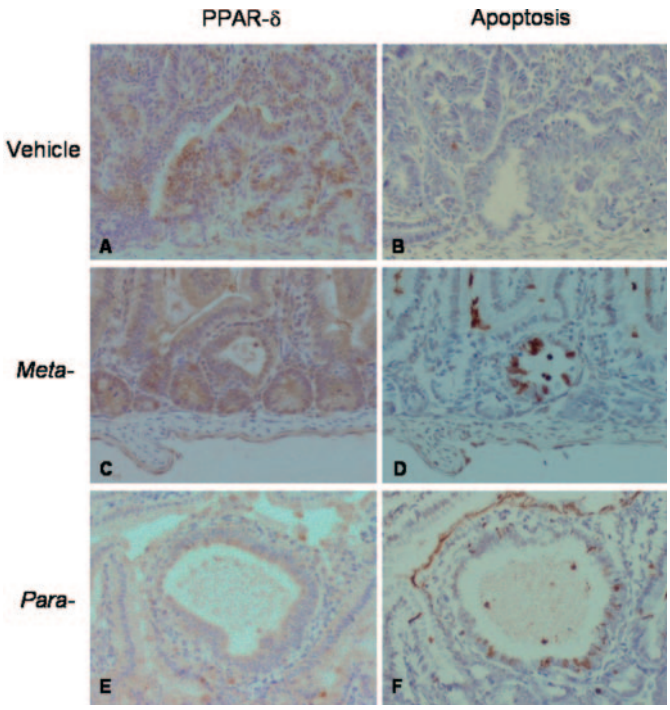

Fig. 7. The relationship of PPARδ and apoptosis in NO-ASA treated intestinal tumors. Successive sections of intestinal tumors from both treated and untreated animals were stained for PPARδ expression and apoptosis, as described in Materials and methods. The untreated tumor shows strong PPARδ expression (**A**) and rare apoptotic cells (**B**). After treatment with *meta* or *para* NO-ASA, tumors show decreased PPARδ expression (**C**) and (**E**) and increased apoptosis (**D**) and (**F**). Lower panel: The apoptosis index of tumors from NO-ASA treated mice is plotted versus the expression of PPARδ determined in successive tissue sections. The correlation between the two is statistically significant. Magnification ×400.

Both isomers of NO-ASA suppressed the expression of PPARδ in *Min* mice. This effect was specific in that neither of the NO-ASA isomers affected the expression of PPARα or PPARγ. Of the two isomers, the *para* was more potent than the *meta*. This difference reflected the known differential potency of these compounds in suppressing the growth of cancer cells, which was also manifested in these animals, in terms of inhibition of intestinal tumorigenesis (15). The degree of inhibition of PPARδ expression was very similar to that of tumor suppression. The correlation between NO-ASA's effect on PPARδ expression and its effect on tumor incidence is intriguing and, to a first approximation, indicates that the two events may be etiologically linked. These findings are consistent with reports that PPARδ mediates the effect of NSAIDs in colon cancer (7) and plays a central role in colon carcinogenesis (9). However, our findings, by no means constitute proof for this; they merely suggest a plausible mechanism that perhaps deserves further exploration.

The effect of NO-ASA on intestinal carcinogenesis was accompanied by changes in cell kinetics. Remarkably, the only detectable change was the induction of apoptosis, with proliferation remaining apparently unaffected. These compounds are known from *in vitro* studies to inhibit both processes, although the induction of cell death has been considered their dominant cell kinetic effect (21). This is the reason why we employed two methods, determination of PCNA and *in vivo* labeling with BrdU, to assess the effect of

NO-ASA on cell proliferation. Nevertheless, it is conceivable that our semiquantitative methods may have not detected small changes in proliferation.

There are three interesting aspects of the induction of apoptosis by NO-ASA. First, apoptosis was induced only in *Min* mice and not in their wild-type counterparts. Second, in tumors it correlated with the suppression of PPARδ expression. And, third, it correlated with the development of necrotic areas in intestinal tumors.

That apoptosis was induced only in *Min* mice suggests specificity of the effect and an apparent mechanistic association with the neoplastic process. The correlation between PPARδ expression and apoptosis in tumors is significant; in addition, examination of successive thin tissue sections stained for PPARδ and apoptosis suggests that the two parameters change in tandem. Of note, there are reports indicating that PPARδ suppresses apoptosis (22).

Apoptosis is classically thought of as a form of cell death leading to cellular disintegration such that it leaves no trace behind and thus spares a tissue the reaction accompanying cell necrosis. Nevertheless, there are exceptions to this and apoptosis is known, on occasion, to lead to necrosis (23,24). Our data indicate that this may be the case in the effect of NO-ASA on the intestinal mucosa of *Min* mice. Areas of tumor necrosis develop only in NO-ASA treated tumors and it is clear that foci of coalescing apoptotic cells evolve into a necrotic area. That dead cells account, at least partially, for these areas of necrosis is made clear by the detection of DNA fragments in them (TUNEL positive areas). NO-ASA is known to induce an atypical form of apoptosis that evolves rapidly into necrosis (21). In fact, in response to NO-ASA we have observed *in vitro* both classic apoptotic cells and necrotic cells (termed atypical cells). Thus it appears probable that NO-ASA induces necrotic cells that are responsible for the necrotic areas that we observed. Under these circumstances, both apoptotic and necrotic cells would be TUNEL positive, as both have cleaved DNA molecules (25). Documented further, such findings may substantiate the *in vivo* occurrence of the atypical cells, and this may explain some of NO-ASA's enhanced potency against cancer compared with its parent traditional ASA.

Taken together, these data suggest the following sequence of events. PPARδ is overexpressed in the context of intestinal carcinogenesis. NO-ASA suppresses its expression and this leads to enhanced apoptosis and perhaps atypical cell death, the latter may lead to the development of tissue necrosis within intestinal tumors. The end result is the suppression of carcinogenesis or at least this may be one of several pathways contributing to it. This conceptualization of our findings, speculative as it is, assigns a pathophysiological role to PPARδ in intestinal carcinogenesis and indicates that it should be a *bona fide* molecular target for cancer prevention and treatment. Our data, therefore, support the notion that PPARδ indeed contributes to intestinal carcinogenesis. They also suggest that there is a significant relationship between the overexpression of PPARδ and apoptosis but do not distinguish between its cause and effect. Furthermore, they indicate that PPARδ is one of the mechanistically important targets of NO-ASA and that this effect may account for some of its enhanced efficacy against colon cancer. The latter is consistent with the notion that NSAIDs prevent cancer, at least in part, via their effect on PPARδ.

Acknowledgements

This study has been supported by the NIH grant R01-CA92423.

Conflict of Interest Statement. None declared.

References

1. Rigas,B. and Kashfi,K. (2004) Nitric-oxide-donating NSAIDs as agents for cancer prevention. *Trends Mol. Med.*, **10**, 324–330.
2. Robinson-Rechavi,M., Escriva Garcia,H. and Laudet,V. (2003) The nuclear receptor superfamily. *J. Cell Sci.*, **116**, 585–586.
3. Michalik,L., Desvergne,B. and Wahli,W. (2003) Peroxisome proliferator-activated receptors beta/delta: emerging roles for a previously neglected third family member. *Curr. Opin. Lipidol.*, **14**, 129–135.
4. Tan,N.S., Michalik,L., Desvergne,B. and Wahli,W. (2003) Peroxisome proliferator-activated receptor (PPAR)-beta as a target for wound healing drugs: what is possible? *Am. J. Clin. Dermatol.*, **4**, 523–530.
5. Nahle,Z. (2004) PPAR trilogy from metabolism to cancer. *Curr. Opin. Clin. Nutr. Metab. Care*, **7**, 397–402.
6. Peters,J.M., Aoyama,T., Cattley,R.C., Nobumitsu,U., Hashimoto,T. and Gonzalez,F.J. (1998) Role of peroxisome proliferator-activated receptor alpha in altered cell cycle regulation in mouse liver. *Carcinogenesis*, **19**, 1989–1994.
7. He,T.C., Chan,T.A., Vogelstein,B. and Kinzler,K.W. (1999) PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell*, **99**, 335–345.
8. Park,B.H., Vogelstein,B. and Kinzler,K.W. (2001) Genetic disruption of PPARdelta decreases the tumorigenicity of human colon cancer cells. *Proc. Natl Acad. Sci. USA*, **98**, 2598–2603.
9. Gupta,R.A., Wang,D., Katkuri,S., Wang,H., Dey,S.K. and DuBois,R.N. (2004) Activation of nuclear hormone receptor peroxisome proliferator-activated receptor-delta accelerates intestinal adenoma growth. *Nat. Med.*, **10**, 245–247.
10. Tan,N.S., Michalik,L., Di-Poi,N., Desvergne,B. and Wahli,W. (2004) Critical roles of the nuclear receptor PPARbeta (peroxisome-proliferator-activated receptor beta) in skin wound healing. *Biochem. Soc. Trans.*, **32**, 97–102.
11. Michalik,L., Desvergne,B., Tan,N.S. *et al*. (2001) Impaired skin wound healing in peroxisome proliferator-activated receptor (PPAR)alpha and PPARbeta mutant mice. *J. Cell Biol.*, **154**, 799–814.
12. Barak,Y., Liao,D., He,W., Ong,E.S., Nelson,M.C., Olefsky,J.M., Boland,R. and Evans,R.M. (2002) Effects of peroxisome proliferator-activated receptor delta on placentation, adiposity, and colorectal cancer. *Proc. Natl Acad. Sci. USA*, **99**, 303–308.
13. Harman,F.S., Nicol,C.J., Marin,H.E., Ward,J.M., Gonzalez,F.J. and Peters,J.M. (2004) Peroxisome proliferator-activated receptor-delta attenuates colon carcinogenesis. *Nat. Med.*, **10**, 481–483.
14. Reed,K.R., Sansom,O.J., Hayes,A.J., Gescher,A.J., Winton,D.J., Peters,J.M. and Clarke,A.R. (2004) PPARdelta status and Apc-mediated tumourigenesis in the mouse intestine. *Oncogene*, **23**, 8992–8996.
15. Kashfi,K., Borgo,S., Williams,J.L., Chen,J., Gao,J., Glekas,A., Benedini,F., Del Soldato,P. and Rigas,B. (2005) Positional isomerism markedly affects the growth inhibition of colon cancer cells by nitric oxide-donating aspirin *in vitro* and *in vivo*. *J Pharmacol. Exp. Ther.*, **312**, 978–988.
16. Kozoni,V., Tsioulias,G., Shiff,S. and Rigas,B. (2000) The effect of lithocholic acid on proliferation and apoptosis during the early stages of colon carcinogenesis: differential effect on apoptosis in the presence of a colon carcinogen. *Carcinogenesis*, **21**, 999–1005.
17. Moser,A.R., Shoemaker,A.R., Connelly,C.S., Clipson,L., Gould,K.A., Luongo,C., Dove,W.F., Siggers,P.H. and Gardner,R.L. (1995) Homozygosity for the *Min* allele of *Apc* results in disruption of mouse development prior to gastrulation. *Dev. Dyn.*, **203**, 422–433.
18. Polakis,P. (1995) Mutations in the *APC* gene and their implications for protein structure and function. *Curr. Opin. Genet. Dev.*, **5**, 66–71.
19. Wang,D., Wang,H., Shi,Q., Katkuri,S., Walhi,W., Desvergne,B., Das,S.K., Dey,S.K. and DuBois,R.N. (2004) Prostaglandin E(2) promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta. *Cancer Cell*, **6**, 285–295.
20. Hao,C.M., Redha,R., Morrow,J. and Breyer,M.D. (2002) Peroxisome proliferator-activated receptor delta activation promotes cell survival following hypertonic stress. *J. Biol. Chem.*, **277**, 21341–21345.
21. Kashfi,K., Ryann,Y., Qiao,L.L., Williams,J.L., Chen,J., Del Soldato,P., Traganos,F. and Rigas,B. (2002) 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*., **303**, 1273–1282.
22. Shureiqi,I., Jiang,W., Zuo,X., Wu,Y., Stimmel,J.B., Leesnitzer,L.M., Morris,J.S., Fan,H.Z., Fischer,S.M. and Lippman,S.M. (2003) The 15-lipoxygenase-1 product 13-S-hydroxyoctadecadienoic acid down-regulates PPAR-delta to induce apoptosis in colorectal cancer cells. *Proc. Natl Acad. Sci. USA*, **100**, 9968–9973.
23. Assuncao Guimaraes,C. and Linden,R. (2004) Programmed cell deaths. Apoptosis and alternative deathstyles. *Eur. J. Biochem*., **271**, 1638–1650.
24. Okada,H. and Mak,T.W. (2004) Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat. Rev. Cancer*, **4**, 592–603.
25. Ben-Sasson,S.A., Sherman,Y. and Gavrieli,Y. (1995) Identification of dying cells–in situ staining. *Meth. Cell Biol*., **46**, 29–39.

Received June 28, 2005; revised August 8, 2005; accepted August 30, 2005