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

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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. 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 –ONO2 is covalently attached and –ONO2 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 –ONO2 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).

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\textsuperscript{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 \textit{Ppard}\textsuperscript{+/−}, \textit{Ppard}\textsuperscript{+/−}/ 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.
Results

This study was undertaken to assess the expression of PPARs during intestinal carcinogenesis, and also to assess NO-ASA and PPAR-γ downregulates the expression of PPARs but not of PPAR-α.

The data were expressed as the mean ± SEM. Group means were compared using one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference test. Differences with a P value of < 0.05 were considered statistically significant.

Statistical analysis

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Materials and methods

Immunohistochemistry and TUNEL staining were performed as previously described (16). Antibodies (all from Santa Cruz, CA) and their final dilution were listed in Table I. For each sample, five randomly selected fields at 200 magnification were evaluated. The apoptosis index (AI), proliferation index (PI), and PPAR expression index (EI) were calculated by dividing the number of positive cells by the number of all epithelial cells in each case the number of positive cells by the number of all epithelial cells and multiplying it by 100.

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

<table>
<thead>
<tr>
<th></th>
<th>Wild-type mice</th>
<th>Min mice</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Normal epithelium</td>
<td>Tumor</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>Meta</td>
</tr>
<tr>
<td>Number of tumors</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PPAR-α</td>
<td>2.68 ± 0.8c</td>
<td>2.45 ± 0.73d (8.9)</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PI</td>
<td>9.6 ± 2.26</td>
<td>8.2 ± 2.57</td>
</tr>
<tr>
<td>AI</td>
<td>3.46 ± 0.36</td>
<td>3.58 ± 0.31 (2.7)</td>
</tr>
</tbody>
</table>

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; j versus k P < 0.0001; l versus n P < 0.02; all other differences are not statistically significant. 

PI, percentage of positive cells; AI, apoptosis index.
the potential effect of NO-ASA on it. Consequently, we studied both Min mice and also their congenic (wild-type) mice, C57BL/6Jþ/þ. Min is a mutant allele of the murine APC locus, encoding a nonsense mutation at codon 850. The homozygosity of Min mutation (APCMin/Min) leads to early embryonic lethality but heterozygous Min mutants (APCMin/þ) 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...
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
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 from 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.
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 PPAR 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 PPAR expression and apoptosis in tumors is significant; in addition, examination of successive thin tissue sections stained for PPARs and apoptosis suggests that the two parameters change in tandem. Of note, there are reports indicating that PPAR 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. PPAR6 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 carcino genesis 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 PPAR6 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 PPAR6 indeed contributes to intestinal carcinogenesis. They also suggest that there is a significant relationship between the overexpression of PPAR6 and apoptosis but do not distinguish between its cause and effect. Furthermore, they indicate that PPAR6 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 PPAR6.

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Conflict of Interest Statement. None declared.

References


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