Indomethacin Decreases Arachidonic Acid Uptake in HCA-7 Human Colon Cancer Cells

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Abstract. Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce the incidence of colorectal cancer. However, evidence is accumulating that NSAIDs have anti-cancer effects in addition to inhibiting cyclooxygenase (COX)-mediated prostanoïd biosynthesis. We now show that indomethacin, a popular NSAID, significantly reduced the $[^3H]$-arachidonic acid uptake in HCA-7 human colon cancer cells. Interestingly, no decrease in the uptake of $[^3H]$-arachidonic acid occurred when the cells were treated with aspirin, diclofenac, and sulindac even though the concentrations of these NSAIDs were high enough to inhibit COX-2 activity. These findings suggest that indomethacin has a novel anti-cancer effect that may be independent of COX-2 inhibition.

Keywords: indomethacin, arachidonic acid, human HCA-7 colon cancer cell

The up-regulated expression of cyclooxygenase-2 (COX-2) is well reported in a variety of malignancies, especially in colorectal cancer (1). An elevated level of COX-2 is responsible for the increased biosynthesis of prostanoïds that is believed to cause the malignancy in this disease. Prostanoids are derived from the action of COX-2 utilizing arachidonic acid as a substrate. The chemo-preventative effect of nonsteroidal anti-inflammatory drugs (NSAIDs) against colon cancer is now well known evidence in prevention and/or possible treatment of this disease. NSAIDs exert their effects principally by inhibiting COX activity, thereby decreasing the biosynthesis of all prostanoids, including a major product of COX, prostaglandin E$_2$ (PGE$_2$). It is reported, therefore, that the risk of developing colorectal cancer is decreased by 40% – 50% in persons regularly taking NSAIDs (1). However, COX-independent activities of NSAIDs that may be involved in anti-tumor effects are also reported (2). Thus, to study the other possible effects of NSAIDs on colon cancer development, we examined the effect of indomethacin, a potent and popular NSAID, on the uptake of arachidonic acid in the HCA-7 human colon cancer cell line since arachidonic acid is a major substrate for COX to produce variety of prostanoids such as PGE$_2$. We now report that indomethacin, but not other NSAIDs such as aspirin, diclofenac, and sulindac, significantly decreased the amount of arachidonic acid taken up by the cells, suggesting that indomethacin has a novel mechanism of action besides the inhibition of COX-2.

HCA-7 human colon cancer cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin.

Cells were cultured in 24-well plates and 16 h prior to the experiments, switched to DMEM containing 0.033 μCi/well of [5, 6, 8, 9, 11, 12, 14, 15-$^3$H]-arachidonic acid (GE Healthcare, Buckinghamshire, UK) and 0.5% fatty acid-free bovine serum albumin (Sigma). Cells were pretreated with final concentrations of either 0.1% dimethyl sulfoxide (vehicle), 10 μM to 1 mM of indomethacin; other NSAIDs (3 mM aspirin, 100 μM diclofenac, or 100 μM sulindac); or 1 μM PGE$_2$ (Cayman, Ann Arbor, MI, USA) for 16 h at 37°C. Cells were then washed twice with phosphate-buffered saline and
lysed with 1% Triton X-100. The radioactivity of [3H]-arachidonic acid incorporated in lysed samples was determined by liquid scintillation spectrometry and expressed as a percentage of the total radioactivity labeled, approximately 80,000 dpm/well in vehicle-treated control cells.

COX-2 activity was assayed according to the manufacturer’s instructions utilizing the peroxidase component of COX (Cayman, 760151). Briefly, 100 U/ml of purified COX-2 (Cayman, 60120) was incubated with NSAIDs for 15 min at room temperature. The peroxidase activities of NSAID-treated COX-2 were assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-etramethyl-p-phenylenediamine at 590 nm.

To examine the effect of indomethacin on the uptake of arachidonic acid in the HCA-7 human colon cancer cell line, cells were treated with [3H]-arachidonic acid and with increasing concentrations of indomethacin from 10 μM to 1 mM for 16 h, and the amount of [3H]-arachidonic acid incorporated into the cells was measured. As shown in Fig. 1, treatment with indomethacin significantly produced a dose-dependent decrease in [3H]-arachidonic acid uptake with an IC₅₀ of approximately 34 μM. No obvious morphological changes or cell detachment and cell growth inhibition were observed following treatment of the cells with up to 300 μM of indomethacin at least for 16 h (data not shown). However, treatment with 1 mM indomethacin caused approximately 50% of the cells to detach from the culture dish, probably representing a toxic effect.

As mentioned in the introductory portion of this study, it is well established that NSAIDs can prevent the development of cancer, especially colorectal cancer. Thus, it is of interest to know if the effect of indomethacin on reducing the uptake of arachidonic acid is a common feature of NSAIDs. We therefore examined the effects of aspirin, diclofenac, and sulindac, the other typical NSAIDs, on the uptake of arachidonic acid in HCA-7 cells. Consistent with Fig. 1, treatment with 100 μM indomethacin for 16 h significantly reduced the uptake of arachidonic acid as compared to control treatment (Fig. 2A). However, treatment of the cells with 3 mM aspirin, 100 μM diclofenac, or 100 μM sulindac for 16 h had virtually no effect (Fig. 2A). To confirm if the ineffectiveness of the other NSAIDs was due to the concentrations used, we performed COX-2 activity assays using the same concentrations used in Fig. 2A. As shown in Fig. 2B, although sulindac had relatively weak activity, all the NSAIDs examined significantly inhibited the activity of COX-2. Thus, the concentrations of NSAIDs used in Fig. 2A were sufficient to inhibit COX-2 activity, that is, were

![Fig. 1.](image1.png) Effect of indomethacin pretreatment of HCA-7 cells on the uptake of [3H]-arachidonic acid. HCA-7 cells were labeled with medium containing [3H]-arachidonic acid and then treated with either vehicle (0 indomethacin) or 10 μM to 1 mM of indomethacin for 16 h. The radioactivity of the [3H]-arachidonic acid incorporated was expressed as a percentage of the total radioactivity. Data are normalized to the vehicle-treated control (0 indomethacin) as 100%. Data are the means ± S.E.M. of more than three independent experiments each performed in duplicate. *P<0.05, analysis of variance, as compared with vehicle treatment (0 indomethacin). AA: arachidonic acid.

![Fig. 2.](image2.png) Effects of NSAIDs pretreatment of HCA-7 cells on the uptake of [3H]-arachidonic acid (A) and on COX-2 activity (B). A: HCA-7 cells were labeled with medium containing [3H]-arachidonic acid and then treated with vehicle (cont), 100 μM indomethacin (ind), 3 mM aspirin (asp), 100 μM diclofenac (dic), or 100 μM sulindac (sul) for 16 h. The radioactivity of the [3H]-arachidonic acid incorporated was expressed as a percentage of the total radioactivity. B: The COX-2 activity was assayed according to the manufacturer’s instructions utilizing the peroxidase component of purified COX-2 (Cayman, 760151). Data are normalized to the vehicle-treated control (cont) as 100%. Data are the means ± S.E.M. of more than three independent experiments each performed in duplicate. *P<0.05, analysis of variance, as compared with the control. AA: arachidonic acid.
Indomethacin Reduces AA Uptake in HCA-7

HCA-7 cells are known to express COX-2 and produce PGE2 (3). Thus, it is possible that the decrease in the uptake of arachidonic acid in HCA-7 cells was due to inhibition of COX-2, resulting in decreasing production of PGE2 by indomethacin. We, therefore, treated exogenous PGE2 with indomethacin to examine if this inhibitory effect of indomethacin on arachidonic acid uptake is restored. As shown in Fig. 3, concomitant treatment with exogenous PGE2 neither altered the basal [3H]-arachidonic acid uptake nor the inhibitory effect of indomethacin. These findings strongly suggest that indomethacin has an additional effect, reducing the uptake of arachidonic acid, besides the inhibition of COX-2 activity.

We have previously shown that treatment of LS174T cells, another human colorectal cancer cell line, with indomethacin results in a down-regulation of EP2 prostanoid receptor expression (4). Interestingly, LS174T cells were found to have not only a low level of COX-2 but also no detectable PGE2 production (3). Thus, the down-regulation of EP2 receptor expression by indomethacin in LS174T cells may occur via a COX-2-independent mechanism. As mentioned in introductory portion of this report, NSAIDs including indomethacin have been found to mediate a variety of COX-independent mechanisms (2) such as stimulation of the peroxisome proliferator-activated receptor-γ (PPARγ) with an EC50 of approximately 40 μM (5). Since the expression of EP2 receptor mRNA and its protein has been shown to be regulated by a PPARγ agonist (6), it is likely that the down-regulation of EP2-receptor mRNA expression by indomethacin in LS174T cells involves the activation of PPARγ. Unlike LS174T cells, HCA-7 cells are known to express COX-2 and produce PGE2 (3). Thus, it is natural to consider the decrease in the uptake of arachidonic acid caused by indomethacin in HCA-7 cells to be due to inhibition of COX-2. However, as shown in Fig. 2, despite their ability to inhibit COX-2, the other tested NSAIDs did not reduce the uptake of arachidonic acid, suggesting that the effect of indomethacin is independent of COX inhibition. This is further confirmed by the result that concomitant treatment of exogenous PGE2 with indomethacin did not block the effect of indomethacin on [3H]-arachidonic acid uptake (Fig. 3). In addition, treatment of the cells with sulindac had a slight, although not significant, effect on arachidonic acid uptake. The structural similarity between sulindac and indomethacin explain this. Since sulindac was designed as a prodrug, it requires transformation to become active. Thus, sulindac might have a relatively weak ability to inhibit COX-2 activity as shown in Fig. 2B. However, treatment of HCA-7 cells with more than 100 μM sulindac, as well as more than 3 mM aspirin or 100 μM diclofenac, evoked non-negligible cytotoxicity (data not shown) so we could not perform further examinations.

Although the inhibiting effect of indomethacin on arachidonic acid uptake may be COX-2 inhibition independent, reducing arachidonic acid uptake could potentially reduce the synthesis of prostanoids such as PGE2 because arachidonic acid is known as a major substrate for COX. This is corroborated by the previous reports that treatment with exogenous arachidonic acid increased PGE2 production in osteoblastic cell lines (7) as well as in HEK cells transfected with COX and membrane-associated PGE2 synthase (8). Thus, reduction of arachidonic acid uptake by indomethacin itself may be independent of COX-2 activity since concomitant treatment of PGE2 with indomethacin did not restore the arachidonic acid uptake (Fig. 3), but it may reduce PGE2 synthesis by acting in concert with COX-2.

It is also reported that leukotriene B4 (LTB4) has an important role in the progression of human colon cancer (9). Besides its role as a substrate for COX, arachidonic acid is also used as a substrate for lipoxygenase (LOX) to produce leukotrienes including LTB4. Thus, the effect of reducing arachidonic acid uptake by indomethacin would have additional effect other than COX inhibition, which is reducing the production of LTB4 by reducing the LOX substrates that would eventually reduce the colon cancer progression.

One of the mechanisms regulating the concentrations of intracellular fatty acids including arachidonic acid is

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**Fig. 3.** Effect of concomitant treatment of PGE2 with indomethacin on the uptake of [3H]-arachidonic acid. HCA-7 cells were labeled with medium containing [3H]-arachidonic acid and then concomitant treatment of either vehicle (0.1% Me2SO) or 1 μM PGE2 with either vehicle (cont) or 100 μM indomethacin (ind) for 16h. The radioactivity of the [3H]-arachidonic acid incorporated was expressed as a percentage of the total radioactivity. Data are normalized to the vehicle-treated control (vehicle/cont) as 100%. Data are the means ± S.D. of three independent experiments. *P<0.05, analysis of variance, as compared with the vehicle-treated control (vehicle/cont). AA: arachidonic acid.
controlled by an import/export system involving fatty acid transport protein (FATP). Fatty acids are transported across cell membranes by the action of FATP. Intriguingly, FATP mRNA expression is up-regulated by a PPARγ agonist (10). Moreover, a PPARγ agonist stimulates lipolysis to release fatty acids from the membrane (11). It is possible, therefore, that the decreased uptake of arachidonic acid on the treatment of HCA-7 cells with indomethacin involves the activation of PPARγ followed by the export and/or lipolysis of the initially incorporated labeled arachidonic acid. This is supported by the IC$_{50}$ of indomethacin for the inhibition of arachidonic acid uptake in Fig. 1. As mentioned above, the EC$_{50}$ of indomethacin to activate PPARγ is approximately 40 μM, very similar to the IC$_{50}$ of approximately 34 μM in Fig. 1. Obviously further study will be needed, for example, if arachidonic acid is the only fatty acid to be affected by indomethacin. However, to the best of our knowledge, a decrease in the uptake of arachidonic acid caused by indomethacin has not been reported previously. In addition to inhibiting COX-2, indomethacin decreases the uptake of arachidonic acid and may reduce the amount of substrate available for COX-2, further inhibiting prostanoid biosynthesis. These findings may shed light on an additional mechanism of the anti-cancer effects of indomethacin.

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References