Cyclooxygenase-independent actions of cyclooxygenase inhibitors

IRMGARD TEGEDER, JOSEF PFEILSCHIFTER, AND GERD GEISSLINGER[1]

Pharmazentrum Frankfurt, Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt, 60590 Frankfurt am Main, Germany

ABSTRACT **Several studies have demonstrated unequivocally that certain nonsteroidal anti-inflammatory drugs (NSAIDs) such as sodium salicylate, sulindac, ibuprofen, and flurbiprofen cause anti-inflammatory and antiproliferative effects independent of cyclooxygenase activity and prostaglandin synthesis inhibition. These effects are mediated through inhibition of certain transcription factors such as NF-κB and AP-1. The respective NSAIDs might interfere directly with the transcription factors, but their effects are probably mediated predominantly through alterations of the activity of cellular kinases such as IKKβ, Erk, p38 MAPK, or Cdks. These effects apparently are not shared by all NSAIDs, since indomethacin failed to inhibit NF-κB and AP-1 activation as well as Erk and Cdk activity. In contrast, indomethacin was able to activate PPARγ, which was not affected by sodium salicylate or aspirin. The differences in cyclooxygenase-independent mechanisms may have consequences for the specific use of these drugs in individual patients because additional effects may either enhance the efficacy or reduce the toxicity of the respective compounds.—Tegeder, I., Pfeilschifter, J., Geisslinger, G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors *FASEB J.* 15, 2057–2072 (2001)**

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ASPIRIN-LIKE DRUGS, now referred to as nonsteroidal anti-inflammatory drugs (NSAIDs), have been used to treat arthritis since 1899, when the analgesic and anti-inflammatory effects of aspirin were first recognized. Despite their potential gastrointestinal and renal toxicities, they are among the most widely used therapeutic classes of compounds primarily because they are generally effective for the relief of pain and inflammation. More recently, aspirin and some other NSAIDs have received attention because of their protective effects against colon cancer (1–3) and cardiovascular disease (4–7). Recently, celecoxib, a selective COX-2 inhibitor, was approved by the FDA for the prevention of colon cancer in patients with familial polyposis coli, which is a hereditary precancerous disease due to a loss of the adenomatous polyposis coli (APC) tumor suppressor gene. NSAIDs may help to prevent or delay the onset of Alzheimer's disease (AD) (8, 9). It is generally accepted that the anti-inflammatory and analgesic efficacy of NSAIDs arises mainly from inhibition of the enzymatic activity of cyclooxygenases (COX), which convert arachidonic acid to prostaglandins (PGs) (10). Two COX isoforms have been described. COX-1 is constitutively expressed in almost all tissues and has characteristics of a housekeeping enzyme whose activity depends solely on the availability of its substrate. COX-1 supplies tissues with prostaglandins required to maintain physiological organ function, such as cytoprotection of the gastric mucosa and regulation of renal blood flow. Conversely, COX-2 behaves as an immediate early gene (11) and is subject to rapid regulation at the transcriptional level. COX-2 is up-regulated in peripheral tissues and the spinal cord in response to tissue injury and is thought to produce excessive amounts of prostaglandins that serve to sensitize nociceptors and stimulate the release of other inflammatory mediators. Prostaglandins also contribute to tumor growth by inhibiting apoptosis (12) and inducing the formation of new blood vessels that sustain tumor cell viability and growth (13, 14). COX inhibition may thus explain part of the antitumor activity of certain NSAIDs. However, some lines of evidence suggest that NSAIDs also modulate cyclooxygenase-independent signal transduction pathways, which may be involved in both the anti-inflammatory and antitumor activity of these drugs (15–17).

For example, doses of aspirin necessary to treat chronic inflammatory diseases are much higher than those required to inhibit PG synthesis. Whereas aspirin inhibits COX activity by acetylating the enzyme salicylic acid, which lacks the acetyl group and is ineffective as a COX inhibitor at therapeutic doses, it is nevertheless able to reduce inflammation (18–20). We have recently shown that R-flurbiprofen, which was generally considered the noncyclooxygenase-inhibiting 'inactive' counterpart of S-flurbiprofen, reduces inflammation (21), hyperalgesia (22), and nociception-induced release of PGE_2 from the spinal cord (23). In addition, R-flurbiprofen was reported to reduce tumor formation and progression in $APC^{Min/+}$ and TRAMP mice, which are in vivo models for colon and prostate cancer, respec-

[1] Correspondence: Pharmazentrum Frankfurt, Klinikum der Johann Wolfgang Goethe-Universität, Theodor Stern Kai 7, 60590 Frankfurt am Main, Germany. E-mail: geisslinger@em.uni-frankfurt.de

tively (24, 25). It is important to note that mechanisms that have been suggested to contribute to the antitumor activity of NSAIDs such as inhibition of cell cycle progression (17, 26), induction of apoptosis (27–29), and inhibition of angiogenesis (30, 31) have been observed only at high concentrations of the respective NSAIDs, which are 100- to 1000-fold higher than those needed to inhibit prostaglandin synthesis (**Table 1**). Furthermore, NSAIDs inhibit tumor formation and growth in COX-deficient cell lines (16, 32). These data leave little doubt that cyclooxygenase-independent mechanisms account for part of the effects of certain NSAIDs, at least at high doses.

CYCLOOXYGENASE-INDEPENDENT MECHANISMS OF NONSELECTIVE NSAIDs

Nuclear factor kappa B (NF-κB)

Cyclooxygenase-independent anti-inflammatory effects

Since Kopp and Ghosh (33) first reported that sodium salicylate and acetylsalicylic acid (aspirin) inhibit the activation of NF-κB, much interest has focused on this transcription factor as a potential target for certain NSAIDs. The vertebrate Rel/NF-κB transcription factor family includes five cellular proteins: c-Rel, RelA (p65), RelB, p50, and p52. As homodimers and heterodimers, Rel/NF-κB proteins bind to DNA target sites, collectively called κB sites, and directly regulate gene transcription. A single cell can have an array of diverse dimeric complexes, the most common of which is called NF-κB and consists of a p50/RelA heterodimer. Since NF-κB regulates the expression of proinflammatory enzymes, cytokines, chemokines, immunoreceptors, and cell adhesion molecules, it has often been termed a "central mediator of the immune response" (34, 35). Because of this key role, it was suggested that the inhibition of NF-κB observed might contribute considerably to the anti-inflammatory effects of salicylates (33, 36). However, this was not confirmed using the murine air pouch as a model of inflammation. In mice deficient in p105 (the precursor of the p50 component of NF-κB), aspirin and sodium salicylate retained their anti-inflammatory efficacy (37). Inhibition of inflammation was also independent of prostaglandin synthesis but was completely reversed by injection of an adenosine A2 receptor antagonist into the air pouch (37), suggesting that modulation of adenosine release or action was somehow involved. Indeed, aspirin and sodium salicylate caused cells to release adenosine into the inflammatory exudate, which was associated with a reduction of leukocyte accumulation (37).

In contrast to salicylates, the anti-inflammatory effects of dexamethasone, which is known to inhibit the activation of NF-κB (38–40), were completely abolished in p105 knockout mice (37). Thus, effects of salicylates and dexamethasone apparently were mediated through different mechanisms in this model. However, it cannot be excluded that NF-κB is a major target of NSAIDs in other models. Recently, we found that NF-κB was inhibited by R- and somewhat less by S-flurbiprofen, which was associated with a decrease in NF-κB-dependent gene transcription (21). Although R-flurbiprofen does not inhibit cyclooxygenase activity and is not epimerized to S-flurbiprofen in rats, it reduced zymosan-induced paw inflammation more potently than S-flurbiprofen and was almost as effective as dexamethasone, suggesting that NF-κB may be a primary target of R-flurbiprofen.

Inhibition of immune cell maturation and cytokine production

The functions of NF-κB in inflammation also include the regulation of immune cell differentiation and survival. The maturation of dendritic cells in particular depends on the activation of NF-κB (41). Immature dendritic cells differentiate on exposure to inflammatory signals such as cytokines. Matured cells are then able to initiate immune responses and are thought to contribute to the pathogenesis of autoimmune diseases by inappropriate presentation of self-antigens. When immature human myeloid dendritic cells were differentiated in vitro in the presence of aspirin or sodium salicylate, they were unable to stimulate T cell proliferation (42). This was associated with a change of cell surface marker proteins. Levels of CD83 and interleukin 12 (IL-12), both markers of mature dendritic cells, were markedly reduced, indicating that salicylates inhibited the differentiation process. The effect was mediated through inhibition of NF-κB and was independent of prostaglandin synthesis (42). The inhibitory effect occurred at concentrations (IC_{50}: 2.5 mM) similar to those found in plasma after administration of anti-inflammatory doses (1–2 mM). Thus, inhibition of immune cell differentiation may contribute to the anti-inflammatory effects of salicylates. Dendritic cell maturation was also inhibited by dexamethasone (43) whereas other NSAIDs, including ketoprofen, indomethacin, and NS398, a COX-2-selective agent, had no effect (42), which suggests that these NSAIDs did not inhibit NF-κB activation up to the concentrations tested in that study (100, 200, and 900 μM, respectively).

Inhibition of NF-κB in immune cells also results in a decrease in cytokine production (44–46). Aspirin and sodium salicylate were recently found to inhibit the transcription of IL-4 in CD4+ human T lymphocytes independent of NF-κB inhibition and PG synthesis (47). The effect occurred at concentrations of 1 mM and was caused by a reduced binding of a Ca^{2+}-inducible complex to an IL-4 promotor element upstream and not overlapping with the NF-κB binding site (47). Analysis of the sequence suggested that an Ets family member and/or a zinc binding protein might be involved in this complex (47). In contrast to salicylates, indomethacin and flurbiprofen had no effect (47). IL-4 is the prototypical cytokine of T helper 2 cells and plays a major role in the regulation of hematopoiesis and

TABLE 1. *Effect of different NSAIDs on cyclooxygenases, transcription factors, and diverse kinases*[a]

			Transcription factors				Kinases															
								MAP-kinase cascade					Cell cycle						Heat shock		Nuclear receptors	
	COX1-IC_{50} (μM)	COX2-IC_{50} (μM)	NF-κB (mM)	AP-1 (mM)	cMyc (mM)	STAT 1 (mM)	IKK (mM)	Erk1/2 (mM)	p38 (mM)	JNK (mM)	p90RSK (mM)	Akt/PKB (mM)	p70-S6K (mM)	Cdks (mM)	Cyclins (mM)	p21/p27 (mM)	p53 (mM)	NAG-1 (mM)	HSF-1 (mM)	Hsp70 (mM)	PPARγ (mM)	PPARδ (mM)
Acetylsalicylic acid (aspirin)	↓[b] (2.8)	↓[c] (26)	↓ (2–10)	↓ (2–10)	↓ (5)		↓ (2–5)	↓ (20)	↑ (10)	↑↓ (10, 2)	↓ (1–3)			↓ (1–10)	↓ (1–10)	↑ (1–10)	↑ (1–10)	↑ (10)	↑ (3)	↑ (3)	↔ (−10)	↔ (−10)
Sodium salicylate	↔[b] (>100)	↔[c] (>100)	↓ (5–20)	↓ (2–20)	↓ (5)	↑ (3–20)	↓ (10)	↓ (20)	↑ (20)	↑↓ (20, 2)	↓ (5–20)		↓ (2–10)	↓ (1–10)	↓ (1–10)	↑ (1–10)	↑ (1–10)	↑ (5)	↑ (20)	↑ (20)	↔ (−20)	↔ (−20)
Sulindac silfide	↓[d] (0.15)	↓[d] (0.0008)	↓↑ (0.2–1)				↓ (0.2)	↓ (0.04–0.6)		↑ (0.2)				↓ (0.2)	↓ (0.2)			↑ (0.05)		↑ (0.2–0.6)		↓ (0.1–0.2)
Ibuprofen	↓[b] (2.1)	↓[b] (1.6)	↓ (0.1–1)				↓ (0.1)	↓ (2)	↔ (−0.2)	↔ (−0.25)	↓ (1–2)							↑ (0.5)		↑ (3)	↑ (0.1)	
S-flurbiprofen	↓[b] (0.03)	↓[b] (0.9)	↓ (0.1–1)	↔ (−1)			↔ (−1)															
R-flurbiprofen	↔[b] (>40)	↔[b] (>100)	↓ (0.01–1)	↓ (0.1–1)			↔ (−1)															
Ketoprofen	↓[b] (0.3)	↓[c] (0.6)	↔ (−1)																			
Indomethacin	↓[b] (0.2)	↓[c] (0.7)	↔ (−2)		↑ (0.4)			↔ (−0.25)	↔ (−0.25)	↔ (−0.25)				↔ (−0.25)	↔ (−0.25)	↑ (0.4)	↑ (0.4)	↑ (0.1)		↔ (−0.3)	↑ (0.1)	↓ (0.2–0.4)
Celecoxib	↔[b] (>7)	↓[b] (0.9)	↑ (0.05)					↓ (0.1)				↓ (0.05)										
NS398	↔[b] (>10)	↓[b] (0.1)	↔ (−9)											↓ (0.1)	↓ (0.1)	↑ (0.1)		↔ (−0.1)			↔ (−1)	

[a] (↓) Inhibition, down-regulation, or reduction of activity (concentration or concentration range at which the effect was observed); (↑) stimulation, induction, or increase of activity (concentration or concentration range at which the effect was observed); (↔) no change of expression or activity (concentration up to which the effect was assessed); (↑ ↓) both inhibition and activation have been reported. [b] IC_{50} values were obtained with the whole blood assay, i.e., LPS-stimulated PGE_2 production in blood monocytes was used for COX-2 activity and TXB_2 release from clotting whole blood was used for COX-1 activity: ibuprofen (210); S- and R-flurbiprofen (23); indomethacin (211); celecoxib (212); NS398, modified whole blood assay (213); acetylsalicylic acid (214); sodium salicylate (215). [c] IC_{50} values were obtained by determining PGE_2 production of IL-1β-stimulated synovial fibroblasts [ketoprofen (216), acetylsalicylic acid (217)] or II-1β-treated A549 cells (sodium salicylate; 215). [d] IC_{50} values for COX-1 were obtained by determination of PGE_2 production in undifferentiated U937 cells that express COX-1 exclusively. COX-2 activity was assessed by determination of PGE_2 production in stimulated human osteosarcoma cells that express COX-2 exclusively (sulindac sulfide; 218). Note that IC_{50}s for COX-1 and COX-2 inhibition are given in μM and concentrations needed to produce other effects are given in mM. Abbreviations: COX, cyclooxygenase; NF-κB, nuclear factor kappa B; AP-1 activator protein 1; STAT, signal transducer and activator of transcription; IKK, I-kappa kinase; Erk, extracellular signal-regulated kinase; MAP-kinase, mitogen-activated protein kinase; JNK, Jun NH_2-terminal kinase; RSK, ribosomal S6 kinase; Akt/PKB, protein kinase B, p70-S6K, p70S6 kinase; Cdk, cyclin-dependent kinase; HSF, heat shock factor; Hsp, heat shock protein; PPAR, peroxisome proliferator-activated receptor.

immune responses (48). It is also involved in the pathogenesis of autoimmune diseases such as juvenile rheumatoid arthritis and Kawasaki syndrome, where the effectiveness of aspirin is well documented (49, 50). Thus, inhibition of IL-4 production may be an additional mechanism by which salicylates modulate immune function.

Decision between induction of apoptosis or cell survival

NF-κB is involved in the control of the transcription of many genes whose function extends beyond the immediate immune response. Similarly, several activators are not bacterial or viral pathogens. Therefore, rather than being a central mediator of immune responses, NF-κB probably represents a 'regulator of stress responses'. Hence, NF-κB also functions as a regulator of the apoptotic program either for induction of apoptosis or, more commonly, as its inhibitor. Whether NF-κB promotes or inhibits apoptosis appears to depend on the cell type and the type of inducer (**Fig. 1**). In numerous human cancer cells, NF-κB is persistently active, which results from a constitutive activation of upstream signaling kinases or mutations that inactivate IκB subunits (51). The persistent activation of NF-κB renders these cells more resistant to chemo- or radiotherapy (52, 53) or other agents that induce apoptosis. The salicylate-induced inhibition of NF-κB translocation might reconstitute the sensitivity of these cancer cells to apoptosis inducing treatment (54) and thereby contribute to the antitumor activity of salicylates. NF-κB activity is also required for the oncogenic transformation by ras and raf (55, 56). Mutations of ras are found in ~50% of colon cancers and play an important role in the multistage process of carcinogenesis. Salicylates were found to inhibit the growth of v-ras transformed fibroblasts. This effect depended on the ability to inhibit NF-κB but was independent of prostaglandin synthesis (57). In rat

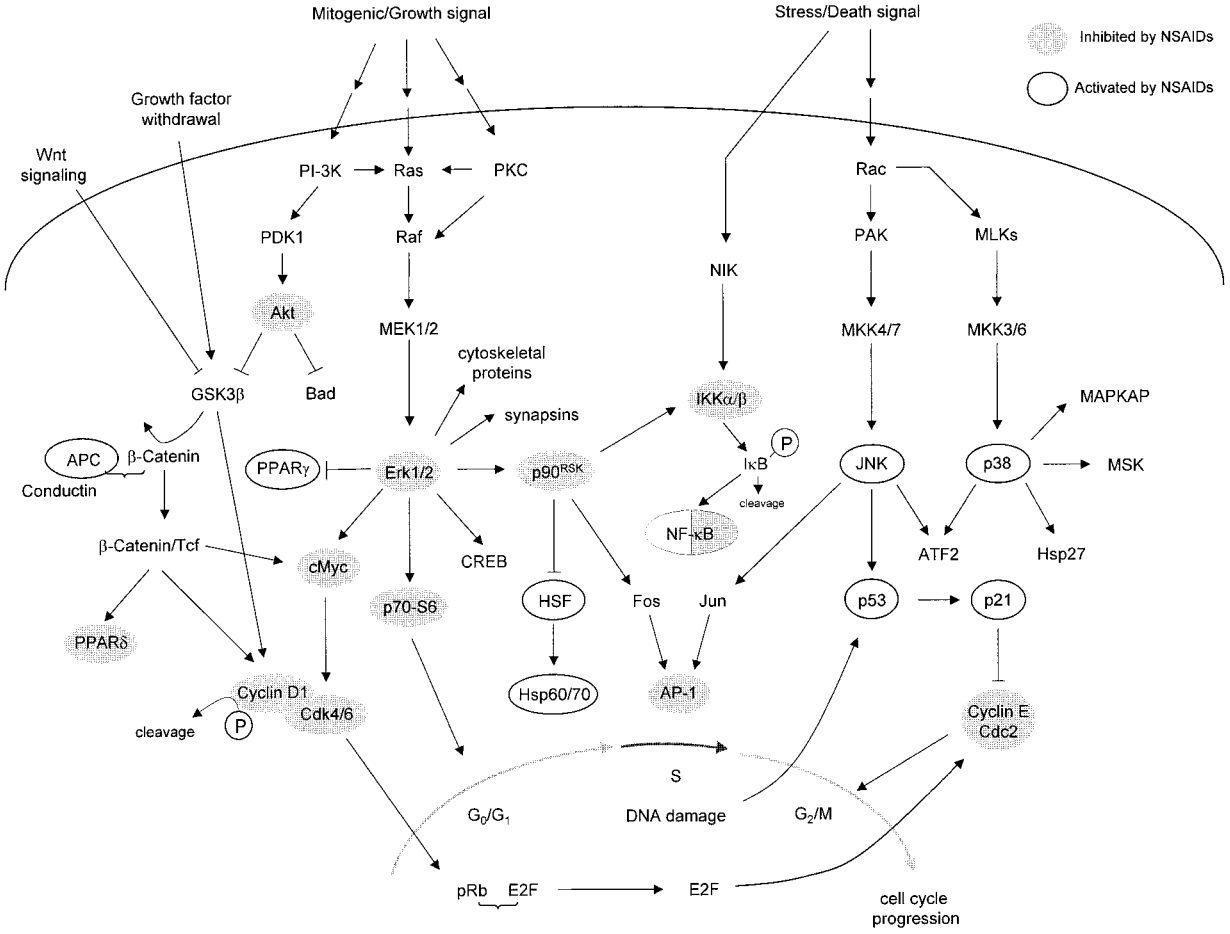

Figure 1. Activation or induction (→); inhibition (⊣); inhibited by NSAIDs (shaded oval); activated by NSAIDs (oval). Abbreviations: Akt/PKB, protein kinase B; APC adenomatous polyposis coli tumor suppressor gene; AP-1 activator protein 1; Cdk, cyclin-dependent kinase; CREB, cAMP response element binding protein; Erk, extracellular signal-regulated kinase; GSK3β, glycogen synthase kinase 3 beta; HSF, heat shock factor; Hsp, heat shock protein; IKK, I-kappa kinase; JNK, Jun NH_2-terminal kinase; MAPK, mitogen-activated kinase; MAPKAP, MAPK-activated protein kinase; MEK/MKK, mitogen-activated protein kinase kinase; MLK, mixed lineage kinase; MSK, mitogen- and stress-activated kinase; NIK, nuclear factor kappaB-inducing kinase; NF-κB, nuclear factor kappa B; PAK, p21-GTPase-activated kinase; PDK, phosphoinositol-dependent kinase; PI-3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; pRb, retinoblastoma protein; p70-S6K, p70S6 kinase; p90RSK, ribosomal S6 kinase; Tcf, T cell factor; Wnt (syn Wg), wingless

intestinal epithelial cells, ras-induced transformation led to an up-regulation of COX-2, which is constitutively expressed in > 80% of colorectal cancers and appears to contribute to colon carcinogenesis.

Conversely, NF-κB activation does not inhibit but promotes apoptotic cell death in a subset of neuronal cells including hippocampal neurons (58) and cerebellar granule cells (59). In these cells, salicylate and aspirin were shown to reduce glutamate-induced neurotoxicity, which was closely related to their ability to inhibit NF-κB activation (59). However, several lines of evidence suggest that NF-κB can also promote survival in neurons (60–62). For example, the β-amyloid peptide associated with AD appears to cause its neurodegenerative effects by down-regulation of NF-κB activity (63) probably through up-regulation of IκBα mRNA and protein expression (60). In contrast, exposure of astroglial cultures to β-amyloid peptide resulted in an activation of NF-κB and NF-κB-dependent gene transcription, including IL-1β and IL-6 (60, 64). Thus, alterations of NF-κB-directed gene expression may contribute to both the neurodegenerative and inflammatory response that occur in AD. The contradictory functions of NF-κB in cortical neurons and astroglial cells may partly explain the somewhat disappointing clinical results with NSAIDs in the treatment of AD (65–68). Unfortunately, some of these studies were performed with diclofenac (67) and indomethacin (68), which probably do not significantly inhibit NF-κB activation (see above).

The mechanisms by which NF-κB promotes cell survival are due in part to the up-regulation of anti-apoptotic genes such as members of the bcl and IAP families (69–71). As a result, salicylate-induced cell death was associated with a down-regulation of MCL-1, a Bcl-2 family member (72). Less is known about target genes that may be involved in cells where NF-κB induces apoptosis. Since NF-κB activates the genes encoding Fas and Fas ligand (73–77), NF-κB activation might sensitize cells to agents that act through or cooperate with the Fas triggered death pathway.

Mechanisms involved in NF-κB inhibition

Inhibitor κ-B kinase (IKK) complex NF-κB transcription factors are regulated primarily by interaction with inhibitor IκB proteins. Thus, in most cells NF-κB exists in the cytoplasm in an inactive complex bound to IκB. Most agents that activate NF-κB do so through a common pathway, which is based on the phosphorylation-induced, proteasome-mediated proteolysis of IκB. The key regulatory step in this pathway involves activation of an IKK complex. Liberated NF-κB then translocates from the cytoplasm to the nucleus, where it binds to the κB-sites in the promoter region of target genes and regulates their transcription.

IKK inhibition through salicylates When cells were stimulated with lipopolysaccharides (LPS) in the presence of sodium salicylate or aspirin, the LPS-induced proteolysis of IκBα was abolished, which suggests that the observed NF-κB inhibition was mediated through inhibition of the phosphorylation and/or the subsequent proteasome cleavage of IκB (33). The effects of sodium salicylate and aspirin were observed at concentrations of 2–20 mM and 2–5 mM, respectively (33). In serum, salicylate concentrations of 1–2 mM are required for anti-inflammatory activity whereas concentrations of > 6 mM are toxic (47, 78). At concentrations in the range of 2–20 mM, salicylate also inhibited the activity of several other cellular kinases. Thus, the specificity of the observed NF-κB inhibition has been questioned (79). Nevertheless, subsequent studies have confirmed that both aspirin and sodium salicylate inhibit LPS- or cytokine-induced nuclear translocation of NF-κB by preventing IκBα phosphorylation and degradation (80). Furthermore, it was shown that aspirin could directly bind to and inhibit the kinase activity of IKKβ by reducing its ability to bind ATP (81). The competitive inhibition of the ATP binding to IKKβ provides a potential explanation for the ability of sodium salicylate to inhibit the activity of multiple kinases. However, this ability makes it more difficult to decipher which pathways are mediating the observed effects. The simultaneous inhibition of multiple signaling pathways may be important for the ability of salicylates to inhibit tumorigenic cell growth.

IKK activity with other NSAIDs Other NSAIDs including ibuprofen (1–3 mM; ref 82, 83), sulindac, sulindac sulfide (1 and 0.2 mM, respectively; ref 84), and R- and S-flurbiprofen (0.01–1 mM and 1 mM, respectively; ref 21) were also able to inhibit NF-κB activation. Indomethacin (0.025–0.25 mM; see ref 42, 84), ketoprofen, and ketorolac (up to 1 mM; ref (42), however, were inactive (Table 1). Among the NSAIDs found to inhibit NF-κB, R-flurbiprofen and sodium salicylate are the only substances that do not significantly inhibit cyclooxygenase activity and thus do not cause gastrointestinal toxicity. In contrast to salicylate (81), ibuprofen (83) and sulindac (84), which were shown to inhibit IKKβ, R-flurbiprofen affected neither the phosphorylation, degradation, nor expression of IκB, suggesting that the effects of R-flurbiprofen were independent of IKK activity and IκB gene transcription (21). Thus, flurbiprofen apparently acts through a different mechanism than sodium salicylate. It is possible that R-flurbiprofen directly interacts with NF-κB, as has been described for glucocorticoids (38), or targets proteins that facilitate the nuclear translocation of NF-κB such as heat shock protein 70 (hsp70) (85, 86), which has been shown to play an important role in the immunosuppressive activity of 15-deoxyspergualin (87, 88).

Inhibition of activator protein 1 (AP-1)

Inhibition of NF-κB through R-flurbiprofen resulted in a reduced expression of COX-2 and tumor necrosis factor α (TNF-α), whereas inducible nitric oxide synthase (iNOS) was unaffected (21). Aspirin and sodium salicylate also reduced the expression of adhesion molecules in endothelial cells (80, 89) and chemokines

such as monocyte chemoattractant protein 1 and interferon γ (IFN-γ) -inducible protein 10 in monocytes and macrophages (90–92).

The transcriptional response to NF-κB activation depends in part on the coactivation of other transcription factors, since more than one transcription factor generally is required to induce effective gene transcription. Thus, although NF-κB participates in the transcriptional regulation of > 150 target genes, salicylates and other NSAIDs will probably affect only some of them. The selection of target genes might depend on the cell type, type of stimulus, and additional effects on other transcription factors, such as AP-1. AP-1 is a protein complex consisting of products of the jun and fos oncogene families, which are activated in response to a number of stimulants including UV irradiation, growth factors, TNF-α, and IL-1. Some of the genes known to be regulated by AP-1 are involved in the immune and inflammatory responses or tumor formation and progression. Thus, AP-1 and NF-κB target genes partially overlap and most target genes are activated by both AP-1 and NF-κB. However, for some genes the effects may be contrary. For example, iNOS transcription is activated by NF-κB (93) but negatively regulated by AP-1 (94). These opposite effects may explain why R-flurbiprofen, which inhibited both NF-κB and AP-1, had no effect on iNOS expression (21).

Aspirin and sodium salicylate have been shown to inhibit epidermal growth factor and UV-induced AP-1 activation in mouse epidermal cells (95). Furthermore, R-flurbiprofen was shown to inhibit LPS-induced AP-1 activation in mouse macrophages (21). In Epstein-Barr virus-infected tumor cells, expression of the EBV-latent membrane protein, the primary oncoprotein of EBV, was associated with increased activity of both AP-1 and NF-κB, which resulted in up-regulation of metalloproteinase 9 (MMP-9) and enhanced tumor invasiveness (96). Aspirin and sodium salicylate were able to reduce MMP-9 levels and tumor invasion through concomitant inhibition of NF-κB and AP-1 (97). Metalloproteinases also play an important role in the degradation of cartilage and bone in chronic arthritis (98, 99). High NF-κB and AP-1 DNA binding activity was found in the synovium of patients with rheumatoid arthritis and osteoarthritis (100), which resulted in an up-regulation of metalloproteinases (100). A major metabolite of aceclofenac was recently shown to inhibit the production of pro-MMP-1, pro-stromelysin, and pro-MMP-2 in rheumatoid synovial cells (101). These effects may contribute considerably to the therapeutic efficacy of the respective NSAIDs.

Alterations of the MAP kinase cascade

It is not known whether salicylates or R-flurbiprofen directly inhibit AP-1 or influence other upstream targets. AP-1 is a downstream target of MAP kinase family members including extracellular signal regulated kinases (Erk-1 and -2; p42/p44 MAPK), Jun kinases (JNKs), and p38 MAPK, which are believed to be activated in response to stress signals. Aspirin and sodium salicylate were shown to inhibit UV-induced AP-1 activity in mouse epidermal cells through blocking the activity of Erk-1 and -2 (102). Other studies have confirmed that activation of Erk-1 and -2 can be blocked by sodium salicylate and aspirin under certain circumstances (103, 104). Effective concentrations were in the range of 1–5 mM. It was recently shown that inhibition of angiogenesis by COX-2-selective and unselective NSAIDs was mediated through direct effects on endothelial cells involving inhibition of Erk2 activity and interference with its nuclear translocation (31). In contrast to Erk-1 and -2, p38 MAPK was reported to be activated by sodium salicylate in human fibroblasts (105) and was associated with induction of apoptosis. Since activation of p38 leads to a down-regulation of NF-κB activity in these cells (105), the apoptosis may have been mediated in part through inhibition of NF-κB. The same authors found that salicylate inhibited the TNF-α-induced activation of c-Jun NH_2-terminal kinase (JNK) in human fibroblasts (106). On the other hand, in HT-29 colon cancer and COS-1 cells, salicylate treatment resulted in activation of JNK (107). The contradictory results suggest that the effects of salicylate on MAPK family members may depend on the cellular context and may result from unspecific effects on kinase activity.

Inhibition of ribosomal S6 kinase

Another kinase shown to be inhibited by sodium salicylate is the ribosomal S6 kinase 2 (p90RSK2) (108); aspirin (1–3 mM) and ibuprofen (1–2 mM) had similar effects. This kinase plays a critical role as effector of the Ras-mitogen-activated protein kinase pathway and as regulator of immediate early gene transcription. Since p90RSK2 is phosphorylated and activated by Erk-1 and Erk-2, these MAPK family members may have been the primary targets of salicylates. Nevertheless, sodium salicylate inhibited p90RSK2 activity and thereby suppressed the phosphorylation and activation of the p90RSK2 substrates, cAMP response element binding protein (CREB) and IκBα. This resulted in an inhibition of CREB and NF-κB-dependent gene transcription. Effective concentrations of salicylate were in the range of 5–20 mM.

Activation of signal transducer and activator of transcription (STAT1)

In contrast to the inhibitory activity of salicylates on NF-κB and AP-1, salicylates were recently found to enhance the IFN-γ-induced activation of STAT1 (signal transducer and activator of transcription) in mouse macrophages (RAW 264.7). Salicylates alone did not enhance the activity of this transcription factor, but prolonged its tyrosine phosphorylation and enhanced its nuclear translocation and DNA binding activity after IFN-γ treatment of the cells (109). STAT1 is phosphor-

ylated by Janus kinases after ligand binding to cytokine receptors. It has multiple transcriptional functions. Upon activation, it drives the expression of many genes such as $p21^{waf1}$ (110), intercellular adhesion molecule-1 (111), or RANTES (112) but also suppresses the transcription of others such as collagenase 3 (113). These opposing characteristics apply to its role in facilitating the cross-talk between signal transduction pathways, as it participates in both synergistic activation and inhibition of gene expression. Using targeted gene disruptions in mice, it was shown that STAT1 mediates growth inhibitory signals and contributes to the host's rejection of tumors (114).

Regulation of the expression of a transforming growth factor β (TGF-β) family member

Another protein that might be involved in the antitumoral effects of NSAIDs was recently identified in HCT-116 colon cancer cells (115). The gene, designated 'NSAID-activated gene' (NAG-1) has an identical sequence with a recently described novel member of the TGF-β superfamily. In these studies, the protein was designated 'macrophage inhibitory cytokine' (116) and 'placental transforming growth factor β' (PTGF-β) (117). NAG-1 possesses proapoptotic and antitumorigenic activity. Incubation of HCT-116 cells with different NSAIDs, including sulindac sulfide, indomethacin, diclofenac, aspirin, and others, increased the expression of NAG-1 and initiated apoptosis, sulindac sulfide being the most potent NSAID tested (115). In antisense NAG-1 cells, NSAID-stimulated apoptosis was attenuated, suggesting that the proapoptotic effects of NSAIDs were mediated at least in part through NAG-1 up-regulation (115). Further studies demonstrated that NAG-1 (called PTGF-β in these studies) was regulated by the p53 tumor suppressor gene (118, 119), suggesting that NAG-1 induction by NSAIDs might depend on wild-type p53. Since NAG-1 expression also resulted in growth arrest and apoptosis in MCF-7 breast cancer cells (118), the NAG-1-mediated effects of NSAIDs are probably not restricted to cells of colonic origin.

Inhibition of cell cycle progression

Inhibition of p70S6 kinase

Salicylate was recently shown to inhibit the activation of p70S6 kinase at concentrations of 2–10 mM, which was associated with a down-regulation of c-*myc*, cyclin D1, cyclin A, and proliferating cell nuclear antigen (120). These target genes are known to play an important role in cell proliferation, and their down-regulation might contribute to salicylate-induced growth arrest. p70S6 kinase is a mitogen-activated kinase that is important for protein synthesis and G_1 cell cycle progression, and has been identified as a target of the immunosuppressant drug rapamycin (121). Inhibition of p70S6 kinase through sodium salicylate was associated with dephosphorylation on Thr-389, the major rapamycin-sensitive site (120). A rapamycin-resistant mutant of p70S6 kinase was also resistant to salicylate-induced dephosphorylation (120). Rapamycin inhibits cell cycle transition from G_1 to S, mediated partly through a reduction of cyclin D1 mRNA and protein stability (121). Likewise, salicylate inhibited the progression from G_1 to S and reduced cyclin D1 levels (120). The latter effect was also observed in human pancreatic cancer cells (122).

Expression and activity of cyclins and cyclin-dependent kinases

Progression through the various phases of the cell cycle is regulated mainly by cyclins and cyclin-dependent kinases (Cdks) (123). The Cdks associate with cyclins before they are activated in discrete phases of the cell cycle. Cdks that participate in regulating cell cycle progression include *1)* Cdk4 (124) and Cdk6 (125), which control the progression through G_1 together with D-type cyclins; *2)* Cdk2, which regulates the G_1/S transition in association with cyclin E (126–129); and *3)* Cdc-2, which in concert with type A and B cyclins controls the G_2/M phase transition (130–133). The function of cyclins is controlled primarily by changes in cyclin levels whereas Cdks are regulated through phosphorylation. The activity of the Cdk/cyclin complex is further negatively regulated by a number of Cdk inhibitors, including p21 (waf1) and p27 (kip1). Cdks hyperphosphorylate the retinoblastoma protein (pRb). pRb is present in quiescent cells in a hypophosphorylated state and sequesters E2F transcription factors (134). Phosphorylation of pRb at multiple sites releases E2Fs. These in turn activate the transcription of genes required for the progression of the cell cycle such as cdc2 and cyclin A (135). Recently, sodium salicylate (5–10 mM) was shown to inhibit the proliferation of vascular smooth muscle cells (SMCs) through up-regulation of the Cdk inhibitors $p27^{kip1}$ and $p21^{waf1}$. This was associated with a decrease in Cdk2 and to a lesser extent in Cdk6 activity, thus preventing hyperphosphorylation of pRb and cell cycle progression. Cyclin protein levels were unchanged (136). $p21^{waf1}$ is a major target gene of the tumor suppressor p53, which is activated and up-regulated upon cellular stress, particularly DNA damage and hypoxia. In parallel to $p21^{waf1}$, salicylate treatment of SMCs caused an up-regulation of p53 (136), suggesting that the observed effects might represent a stress response induced by high salicylate concentrations. Salicylate treatment also resulted in a modest inhibition of NF-κB activity (136). Although it has been shown that NF-κB activity is essential for SMC proliferation (137), its modest inhibition could not explain the profound inhibition of SMC proliferation in that study (136). Neither Cdk nor NF-κB inhibition was observed with indomethacin (136). Similar to salicylates, however, sulindac (1.2 mM) and sulindac sulfide (0.2 mM) reduced the proliferation rate of HT-29 colon carcinoma cells and caused them to accumulate in the G_0/G_1 phase (17). This was associated with reduced expression and reduced catalytic

activity of cyclin-dependent kinases (Cdc2, Cdk2, and Cdk4) (17).

Inhibition of the heat shock response

Apart from the effects on kinase activity, sodium salicylate has the property of partially inducing the human heat shock response (138, 139). This effect is probably mediated through activation of the heat shock transcription factor 1 (HSF1) from a latent cytoplasmic form to a nuclear, DNA binding state (139, 140). HSF1 can function as both an activator of heat shock genes and a repressor of cytokine genes. Treatment of LPS-stimulated monocytes with sodium salicylate resulted in an inhibition of cytokine gene expression similar to that observed after heat shock or overexpression of HSF1 (140). Similar effects were obtained with aspirin, sulindac, ibuprofen, and piroxicam, which also induced HSP70 mRNA expression (140). Thus, exposure to certain NSAIDs may lead to a switch in gene expression, with suppression of cytokine and induction of stress genes (141). HSF1 appears to play a regulatory role in these effects. Since p90RSK2 may function as a repressor of HSF1 (142), the observed inhibition of RSK2 through sodium salicylate (see above) might be involved in the salicylate-induced activation of HSF1.

Modulation of the activity of nuclear receptor family members

Activation of peroxisome proliferator-activated receptor γ (PPARγ)

The multitude of effects observed with sodium salicylate and aspirin argue in favor of the hypothesis that actions of salicylates at high concentrations are mediated through effects on several cellular kinases (79). Since the interest has focused on salicylates and, more recently, COX-2-selective agents, it is not clear whether the effects observed with salicylates are shared with the majority of other NSAIDs. The results obtained with indomethacin, however, which inhibits neither NF-κB (84, 143, 144), AP-1 (95, 145), p42/p44 MAPK (103), nor cell cycle regulatory proteins (136), suggest that there are substance specific differences.

This is further supported by the finding that indomethacin, but not sodium salicylate binds to and activates PPARγ, a member of a superfamily of nuclear hormone receptors that function as ligand-dependent transcription factors (146). Three distinct PPAR isoforms—PPARα, δ, and γ—have been isolated and characterized (147). PPARs bind to sequence-specific DNA response elements as a heterodimer with the retinoic acid receptor (148). PPARγ is highly expressed in adipose tissue and plays an important role in regulating genes involved in lipid utilization and storage, adipocyte differentiation, and insulin action. The antidiabetic thiazolidinediones such as troglitazone are high-affinity ligands of PPARγ (K_d 40 nM; ref 149). They enhance insulin sensitivity and promote preadipocyte differentiation in adipose tissue. Although the affinity of indomethacin to PPARγ is relatively low (K_d 100 μM; ref 150) vs. that of troglitazone, indomethacin was able to induce the differentiation of mesenchymal stem cells to adipocytes in vitro (150). Because of this feature, indomethacin has been used widely to study the process of adipocyte differentiation (151–154). Some other NSAIDs, including ibuprofen, fenoprofen, and flufenamic acid, also bind and activate PPARγ as assessed in reporter gene assays, but are less potent than indomethacin (150).

Some arachidonic acid metabolites, especially prostaglandin D_2 metabolites such as 15-deoxy-PGJ_2, are natural high-affinity PPARγ ligands (K_d 2 μM) (155–157) that promote adipocyte differentiation in vitro (158). Prostaglandin D_2 metabolites have not yet been identified in adipose tissue, but are major products of arachidonic acid metabolism in macrophages (159) and mast cells (160) raising the possibility that they might serve as endogenous PPARγ ligands in these cells. PPARγ is expressed in monocytes (161), bone marrow precursors (162), splenocytes, and helper T cells (163) and was shown to be up-regulated in activated macrophages (161, 164). Activation of PPARγ in response to 15-deoxy-PGJ_2 and synthetic PPARγ ligands resulted in inhibition of cytokine production in macrophages (161, 165, 166) and T cells (163) and reduced T cell proliferation (163). This effect was mediated in part by antagonizing the activities of the transcription factors AP-1, STAT, and NF-κB (161, 167). Thus, 15-deoxy-PGJ_2 can be expected to provide anti-inflammatory effects. This is supported by a recent study showing that 15-deoxy-PGJ_2 induces apoptosis in synoviocytes and suppresses adjuvant-induced arthritis in rats (168). Furthermore, inhibition of 15-deoxy-PGJ_2 synthesis through COX inhibitors was associated with an exacerbation of inflammation in the late stage of carrageenan-induced pleurisy in rats (169), i.e., at a stage dominated by mononuclear cells. Since this effect occurred with both indomethacin and NS398 (COX-2 selective), the unfavorable effect is apparently shared by selective and unselective NSAIDs. Since indomethacin is only a weak PPARγ agonist, reduction of the endogenous PPARγ ligand 15-deoxy-PGJ_2 is probably not offset by its activity.

Besides its role in adipogenesis and inflammation, PPARγ is highly expressed in the normal large intestine and in breast, colon, and prostate cancer (170–172). PPARγ agonists such as troglitazone and 15-deoxy-PGJ_2 were able to induce differentiation and apoptosis in tumor cells (170–173), suggesting that PPARγ suppresses tumor cell proliferation. This is further supported by the finding that human colon cancer is associated with loss-of-function mutations of PPARγ (174). Indomethacin was shown to reduce the clonogenic activity of prostate cancer cells (172) and increase the antiproliferative effect of 5-fluorouracil in colon cancer cells (175).

Inhibition of peroxisome proliferator-activated receptor δ (PPARδ)

Unlike the PPARα and PPARγ receptors, little is known about the physiological functions of PPARδ. Recently, PPARδ was shown to play an important role in the female reproductive process (176). PPARδ was activated by prostacyclin (PGI_2), which is the major PG subtype at the implantation site where levels of PPARδ were significantly increased (176). In COX-2 knockout mice, which exhibit multiple reproductive failures (including implantation defects), the stable PGI_2 analog carbaprostacyclin ($cPGI_2$) and PPARδ agonists were able to reverse the implantation defect (176). Cicaprost, however, which can activate IP_2 receptors but does not activate PPARδ, did not reverse the deficiency, suggesting that the actions of PGI_2 were mediated through activation of PPARδ (176). Since PGI_2 synthesis is inhibited by selective and nonselective NSAIDs, PPARδ activity can be expected to be reduced.

Besides its role in reproduction, PPARδ has recently been identified as one of the downstream targets of β-catenin. The β-catenin protein plays a critical role in embryonic development and oncogenic processes through its effects on E-cadherin-mediated cell adhesion and as an effector of Wnt-dependent signal transduction. In the absence of Wnts, β-catenin is phosphorylated by glycogen synthase kinase 3 (GSK-3β), which triggers ubiquitination of β-catenin and degradation in proteasomes. Conversely, Wnt signaling leads to an inhibition of GSK-3β and stabilization of cytosolic β-catenin (177), which is then transferred into the nucleus where it binds to members of the Tcf (T cell factor)/Lef (lymphoid enhancing factor) transcription factor family. The β-catenin/Tcf complex up-regulates the expression of β-catenin/Tcf-dependent genes including c-*myc* (178), cyclin D1 (179), PPARδ (180), and gastrin (181). The phosphorylation and degradation of β-catenin occurs only when β-catenin is complexed with conductin/axin and the APC tumor suppressor gene, which acts as a cofactor (182). Several studies have suggested that a major function of APC is to down-regulate cytosolic levels of β-catenin (183–185), thus preventing the formation of β-catenin/Tcf complexes and transcription of β-catenin-dependent oncogenes. An inactivating mutation of the APC gene is an early event in the development of colon and other cancers. Moreover, inherited mutations of APC cause familial adenomatous polyposis, which is characterized by the development of hundreds of colorectal adenomas that result in colon cancer in all affected individuals. The pivotal role played by APC/β–catenin interaction is underscored by the observation that in tumors where the APC gene is not lost or mutated, mutations are frequently found in β-catenin (186, 187). Accordingly, restoration of APC function in colorectal cancer cells with defective APC results in growth suppression and apoptosis (188).

Recently, sulindac and etodolac were shown to increase the expression of APC mRNA in the colon of rats treated with the colon-specific carcinogen azoxymethane (189). The increase in APC mRNA expression was associated with a reduction of preneoplastic lesions (aberrant crypt foci) (189). Furthermore, aspirin (190) and indomethacin (191) were shown to decrease intracellular β-catenin levels, associated in the case of aspirin with a reduction of the rate of tumor formation in APC Min/+ mice (190). PPARδ was recently identified as one of the target genes of the APC/β-catenin-Tcf pathway (180) and its expression was shown to be up-regulated in colorectal carcinomas (192). As mentioned, PGI_2 can act as an activator of PPARδ (176, 192). Thus, NSAID-mediated inhibition of eicosanoid metabolism is likely to result in a repression of PPARδ activity. This theory was supported by the finding that sulindac sulfide inhibited carbaprostacyclin ($cPGI_2$) -stimulated DNA binding activity of the PPARδ/RXR heterodimer, which was associated with induction of apoptosis in colorectal cancer cells (180). A similar effect was observed with the sulindac sulfide-related compound sulindac sulfone, which is devoid of COX inhibitory activity (193), suggesting that inhibition of PPARδ was in part mediated by a direct, prostaglandin-independent effect (180). Directly or indirectly, the suppression of PPARδ activity associated with the promotion of apoptosis can compensate for APC or β-catenin mutations, which result in an increased expression of PPARδ and constitute an early step in colon carcinogenesis. Thus, inhibition of PPARδ activity may contribute to the chemopreventive activity of NSAIDs.

Induction of the orphan nuclear receptor NFGI-B (Nur77)

Indomethacin was recently found to induce another member of the steroid-thyroid hormone receptor family, nerve growth factor-inducible B (NGFI-B), also termed Nur77 or TR3 (194). NGFI-B is one of the immediate early genes originally identified by virtue of its rapid activation by nerve growth factor (NGF) in PC12 pheochromocytoma cells (195) and by serum in fibroblasts (196). NGFI-B is rapidly synthesized in response to a variety of growth factors, phorbol esters, and treatments resulting in calcium influx. The protein is modulated by phosphorylation; the extent of phosphorylation depends on the stimulus. NGFI-B binds as monomer to a relatively simple response element, which implies it has the potential to activate a wide array of target genes (197). In contrast to other nuclear receptor family members, which need their respective ligands to gain transcriptional competence, NGFI-B is constitutively active when expressed (197). The action of NGFI-B has been shown to be necessary for activation-induced cell death in T cells and thymocytes (198–200). In response to apoptotic stimuli, NGFI-B translocates from the nucleus to mitochondria to induce cytochrome *c* release and apoptosis (201). The induction of NGFI-B by indomethacin was associated with induction of apoptosis in HCT-15 colon cancer cells (194). Since these cells lack COX-2 expression,

NFGI-B induction appears to be independent of COX-2.

CYCLOOXYGENASE-INDEPENDENT ACTIONS OF COX-2-SELECTIVE DRUGS

Activation of NF-κB through celecoxib

In contrast to most other NSAIDs, celecoxib and rofecoxib are not acidic compounds. Apart from their COX-2 selectivity, they differ chemically from other NSAIDs. We recently showed that celecoxib, in contrast to other NSAIDs, did not inhibit but activate NF-κB and NF-κB-dependent gene transcription (202), suggesting that its cyclooxygenase-independent actions may also differ from those of nonselective agents. NF-κB activation caused by high concentrations of celecoxib resulted in a complete loss of its anti-inflammatory efficacy (202). It is not yet known whether this NF-κB-activating effect is shared by all COX-2-selective agents or whether it is a special feature of celecoxib.

Celecoxib was repeatedly shown to reduce colon carcinogenesis in animals and humans (203–205). Thus, it apparently possesses antitumor activity similar to that of salicylates. These effects might be due in part to the activation of NF-κB since it has been reported that activation of NF-κB in colon cancer cells causes an up-regulation of FasL and facilitation of Fas-mediated killing (74) and a sensitization to apoptosis-inducing treatments such as chemo- or radiotherapy (206). This is supported by a recent study demonstrating that aspirin activates NF-κB in colon cancer cell lines by increasing the phosphorylation and subsequent degradation of IκB, which was associated with induction of apoptosis (207). Aspirin had no effect in cells of noncolonic origin (207), suggesting that the activation of NF-κB by aspirin is cell type specific and may explain why aspirin mainly prevents colon cancer. The apparent contradiction that aspirin may both inhibit and activate NF-κB could be explained by the cell type and stimulus, since NF-κB inhibition was observed mainly in immune cells after cytokine stimulation whereas NF-κB activation occurred in colon cancer cells only when aspirin was administered alone (207). In addition, the outcome is probably influenced by concomitant effects on cellular kinase activity or other transcription factors. Thus, the ability of celecoxib to activate NF-κB may limit its usefulness in inflammatory diseases, but may be an interesting feature concerning colon cancer or Alzheimer disease, where an activation of NF-κB has been shown to reduce β-amyloid-induced neuronal apoptosis (63).

Inhibition of Akt phosphorylation

Celecoxib was recently shown to induce apoptosis in COX-2-expressing, androgen-responsive (LNCaP) and nonresponsive (PC-3) prostate cancer cells (208). In contrast to an earlier report that attributed the apoptotic activity of the COX-2-selective inhibitor NS398 in LCNaP to a down-regulation of Bcl-2 (209), celecoxib did not alter Bcl-2 levels and its effects were not reversed by overexpression of Bcl-2 (208). In that study, celecoxib inhibited the phosphorylation of protein kinase B (PKB/Akt), thereby blocking its antiapoptotic activity. The effects of celecoxib on Akt were independent of phosphatidylinositol-3-kinase activity and not reversed by okadaic acid, which is an inhibitor of protein phosphatases 1 and 2A, suggesting a direct effect on Akt kinase.

We do not know whether celecoxib also affects other pathways that have been shown to be modulated by nonselective NSAIDs, especially salicylates. NAG-1 expression (see above) was not affected by most COX-2-selective inhibitors with the exception of SC-58125, which suggests that there might be additional differences between unselective and COX-2-selective NSAIDs apart from those concerning NF-κB. FJ

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