Annu. Rev. Pharmacol. Toxicol. 2002. 42:553–83

THE SPINAL PHOSPHOLIPASE-CYCLOOXYGENASE-PROSTANOID CASCADE IN NOCICEPTIVE PROCESSING

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Key Words intrathecal, PLA_2, hyperalgesia, COX, isozymes, pain

■ **Abstract** Intrathecal phospholipase A_2 (PLA_2) and cyclooxygenase-2 (COX-2), but not COX-1, inhibitors attenuate facilitated pain states generated by peripheral injury/inflammation and by direct activation of spinal glutamate and substance P receptors. These results are consistent with the constitutive expression of PLA_2 and COX-2 in spinal cord, the spinal release of prostaglandins by persistent afferent input, and the effects of prostaglandins on spinal excitability. Whereas the acute actions of COX-2 inhibitors are clearly mediated by constitutively expressed spinal COX-2, studies of spinal COX-2 expression indicate that it is upregulated by neural input and circulating cytokines. Given the intrathecal potency of COX-2 inhibitors, the comparable efficacy of intrathecal versus systemic COX-2 inhibitors in hyperalgesic states not associated with inflammation, and the onset of antihyperalgesic activity prior to COX-2 upregulation, it is argued that a principal antihyperalgesic mechanism of COX-2 inhibitors lies with modulation of constitutive COX-2 present at the spinal level.

INTRODUCTION

Nonsteroidal antiinflammatory drugs (NSAIDs) have been long used to treat mild or moderate pain following injury, disease, or minor surgery (see e.g., 1). They are currently the most widely utilized class of analgesic compounds (see e.g., 2) and play important roles in the management of postsurgical pain (see e.g., 3, 4), as well as chronic pain states including arthritis and cancer (see, eg., 5, 6). The reliability of these agents can be appreciated by noting that, in terms of "numbers-needed-to-treat" (NNT) to establish clinically significant effects, agents such as ibuprofen and diclofenac have NNT values of 2–3. (For every 2–3 patients receiving a drug, one patient will have at least 50% relief.) In contrast, codeine, a widely used opiate analgesic, has an NNT of 17 (7). This reliability argues for an action directed at fundamental mechanisms in the genesis of facilitated pain states.

0362-1642/02/0210-0553$14.00

ANTIHYPERALGESIC VERSUS ANALGESIC ACTIONS OF NSAIDS

Preclinical studies have emphasized that NSAIDs do not typically elevate the normal pain threshold in animal models as measured in threshold escape paradigms, such as the hot plate, tail flick, or paw mechanical pressure, but NSAIDs commonly normalize the exaggerated pain behavior (hyperalgesia) that is observed after tissue injury or inflammation (such as the acetic acid induced writhing or paw carrageenan tests) (8). Two early observations provided evidence that the antihyperalgesic characteristics of NSAIDs were mediated by a peripheral action. (*a*) Agents, classified as nonnarcotic, nonsteroidal antipyretic agents (9) shared the ability to inhibit the cyclooxygenase-mediated synthesis of prostaglandins (10, 11). (*b*) Prostaglandins, elaborated locally following tissue injury, sensitized peripheral nerve endings and facilitated pain behavior in animal models (12). These properties were taken to support the unifying hypothesis that NSAIDs acted in models of peripheral inflammation in which the hallmark of nociception was a "hyperalgesic state" induced by the local release of prostaglandins (13, 14). It was appreciated, however, that there were clear dissociations between the antiinflammatory and analgesic activity of the several agents (15), suggesting that actions other than those associated with peripheral inflammation might account for the antihyperalgesic characteristics. Moreover, early research pointed to a spinal action for cyclooxygenase (COX) inhibitors and prostanoids (16), and subsequent work has essentially substantiated and expanded upon this finding.

To appreciate the spinal action for cyclooxygenase inhibitors, we first consider the current thinking in regard to the systems underlying the spinal processing of nociceptive information initiated by peripheral injury. In subsequent sections, we consider in detail the properties of the spinal phospholipases from which the arachidonic acid (AA) arises, the COX isozymes that convert the arachidonic acid to prostanoids and finally the prostanoids and their receptors that mediate the facilitated processing that arises when this cascade of events is activated by peripheral tissue injury and inflammation.

BIOLOGY OF THE SPINAL CASCADE INDUCED BY TISSUE INJURY

Tissue injury and inflammation results in an enhanced response to subsequent noxious stimuli (hyperalgesia). Current work emphasizes that this exaggerated response arises in part because of a sensitization of the peripheral terminal (as noted above) and partly by the initiation of a facilitated state of processing of afferent input in the spinal cord (17). Changes in spinal responsiveness are mediated by (*a*) injury-induced afferent input and (*b*) injury-induced appearance of circulating factors. Figure 1 provides a schematic representation of the organization of this dorsal horn cascade.

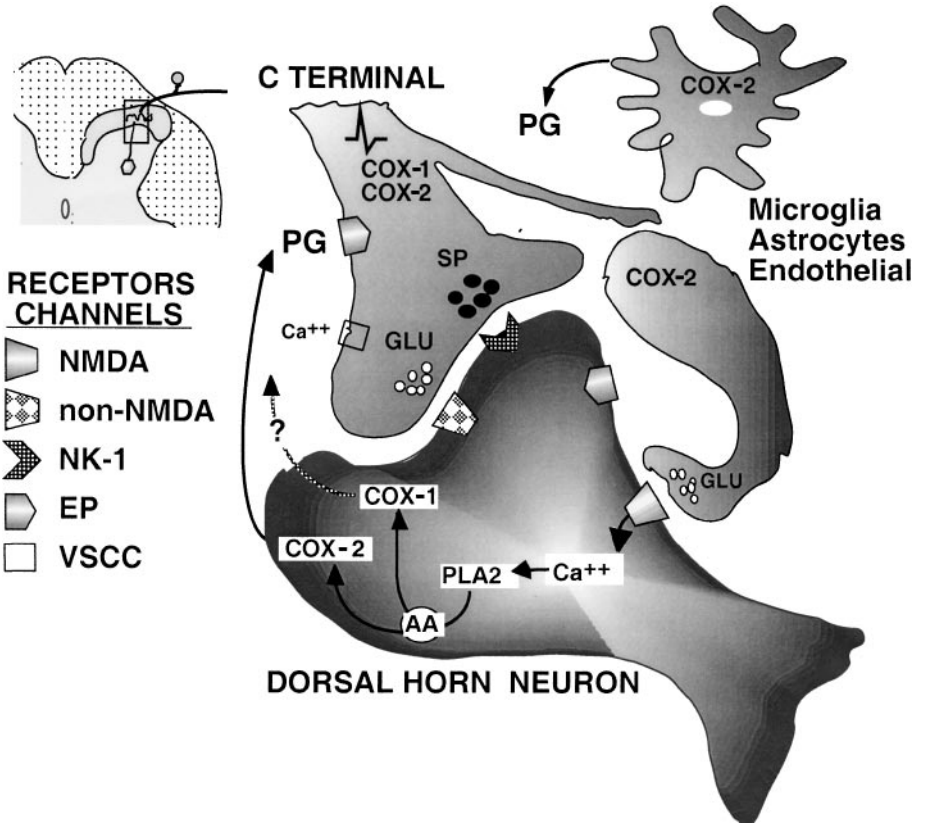

Figure 1 Illustration of the dorsal horn at the level of the primary afferent synapse in the substantia gelatinosa. The schematic presents potential structures constitutively containing both cyclooxygenase (COX) isozymes (COX-1 and COX-2), from which prostaglandins (PG) may derive to alter spinal nociceptive processing. These structures are believed to be both neuronal and nonneuronal (microglia, astrocytes, endothelial cells). Brief but persistent activation of small afferent C fibers yields release of several excitatory afferent transmitters, including substance P (sP) and glutamate (GLU). This results in a depolarization and increased intracellular calcium ($[Ca]^{2+}$), which in turn results in the activation of a number of intracellular enzymes, including phospholipase A_2 (PLA_2). PLA_2 results in an increase in cytosolic arachidonic acid (AA), which then enters the COX cascade leading to the formation of a variety of PGs that gain access to the extracellular space. Prostanoids acting via prostanoid E receptors (EP) can increase intracellular calcium in sensory afferents and depolarize dorsal horn neurons. The former effect would facilitate afferent transmitter release. Behavioral pharmacology has emphasized that the hyperalgesia and the evoked release of PGs from spinal cord is blocked by COX-2, but not COX-1, inhibitors. This system is not static. Thus, there is an enhanced expression of mRNA and protein for COX-2 that is initiated by persistent afferent input generated by peripheral tissue injury and inflammation. In addition, peripheral injury leads to circulating factors such as cytokines and LPS, which can initiate COX-2 upregulation in neuronal and nonneuronal elements. (See text for details and citations.)

Neural Linkages

Peripheral tissue injury and local inflammation initiates persistent activity in small primary afferents. Repetitive afferent stimulation evokes a cascade leading to a behaviorally defined hyperalgesia, as indicated by a decreased response threshold or an increased magnitude of responding (18). Spinal components of the cascade generated by tissue injury are initiated by an afferent-evoked release of amino acids (glutamate/aspartate) and peptides (substance P), activating N-methyl-D-aspartate (NMDA), non-NMDA, and Neurokinin-1 (NK1) receptors (19). Stimulation of these receptors results in activation of spinal kinases, phospholipases, and synthases. Kinases phosphorylate a variety of channels (calcium), receptors (NMDA), and enzymes and enhance their functionality (20). Phospholipases form arachidonic acid (AA) (21, 22), which is acted upon by COX to yield products converted into prostanoids. The behavioral relevance of these events is indicated by the observations that spinal prostanoids increase spinal excitability (23, 24), while blockade of spinal NMDA or NK1 receptors (23–26), kinases (27), COX, or PLA_2 (see below) attenuates many behaviorally defined expressions of hyperalgesia.

Circulating Factors

Inflammatory reactions associated with tissue injury or local irritants result in the release of cytokines, such as TNF-α or IL-1β. These products, when delivered either systemically (28, 29) or intrathecally (30, 31), can evoke hyperalgesic states. At the spinal level, these cytokines can influence spinal function by a direct action on spinal cells (neurons, microglia, astrocytes) (32, 33).

The organization of spinal systems mediating postinjury hyperalgesia is complex. However, several lines of evidence emphasize the relevance of the spinal PLA_2-COX-prostanoid cascade in the evolving pain state.

SPINAL PHOSPHOLIPASE A_2 (PLA_2) ISOZYMES

Arachidonic acid concentrations in cell cytosol are low under normal conditions and formation of arachidonic acid is generally considered to be the rate-limiting step in prostanoid synthesis. Arachidonic acid is found in the sn-2 position of membrane phospholipids, and PLA_2 hydrolizes the sn-2 ester bond to generate free arachidonic acid and lysophospholipids. The PLA_2 family can be divided into at least 10 groups based on amino acid structure and other characteristics. Focusing on biological properties, three distinct classes of PLA_2 have been characterized: (*a*) calcium-dependent cytosolic PLA_2 ($cPLA_2$), (*b*) calcium-independent cytosolic PLA_2 ($iPLA_2$), and (*c*) secretory PLA_2 ($sPLA_2$) (21).

$cPLA_2$

$cPLA_2$ (Group IV PLA_2) is found in the cytosol of practically all cell types studied, and the enzyme is translocated to nuclear/endoplasmic reticulum membranes in

response to increased intracellular calcium (34, 35). $cPLA_2$ possesses a preference for phospholipids containing AA (36) and phosphorylation of $cPLA_2$ by mitogen-activated protein kinases increases its activity (35).

$sPLA_2$

$sPLA_2$ (Group IB, IIA, IIC, V and X PLA_2) are low molecular weight, structurally related isozymes that require millimolar concentrations of calcium for activation. They are not selective for a particular fatty acid at the sn-2 position and release arachidonic acid in addition to other fatty acids from phospholipids. Importantly, these isozymes are released into the extracellular space and can induce phospholipid cleavage in adjacent cells (37).

$iPLA_2$

$iPLA_2$ (Group VI PLA_2) is involved in fatty acid remodeling rather than AA liberation. $iPLA_2$ incorporates AA and other fatty acids into membrane phospholipids (see e.g., 38). There are studies suggesting that the AA-releasing PLA_2s ($cPLA_2$, $sPLA_2$) use different AA pools for the release. Thus, by regulating fatty acid remodeling, $iPLA_2$ may regulate AA available for PLA_2 cleavage (39, 40).

Constitutive Spinal Localization of PLA_2 Isozymes

PLA_2 immunoreactivity is found in the dorsal horn and motor neurons of rat and monkey (41, 42) and is also present in rat spinal cord as shown by Western blotting (43). $iPLA_2$ mRNA is constitutively expressed in human spinal cord (44), and we have recently shown that mRNA for $cPLA_2$, $iPLA_2$, and $sPLA_2$ (IIA and V) are present in normal rat spinal cord (45).

Induction of Spinal PLA_2 Isozymes

Increase of $cPLA_2$ protein and mRNA has been shown in murine astrocyte cultures (46, 47) after lipopolysaccharide (LPS) and IL-1β stimulation, and in monocytes after zymosan treatment (48). $sPLA_2$ mRNA in brain increases after systemic LPS (136), and elevation of $sPLA_2$ protein levels occurs after LPS and IL-1β stimulation of astrocyte cultures (49–51). A recent study did not, however, reveal upregulation of spinal $cPLA_2$, $sPLA_2$ activity, or $cPLA_2$ protein levels after intraplantar Freuds complete adjuvant (FCA) (43).

PLA_2 Pharmacology

Presently, no isoform-specific PLA_2 inhibitors are commercially available. The most common PLA_2 inhibitors used are AATFMK, MAFP, and BEL. AATFMK (arachidonyl trifluromethylketone: AACOCF3) is a $cPLA_2$ and $iPLA_2$ inhibitor (see e.g., 52), and at higher concentrations it may also inhibit COX (53, 54). MAFP (methyl arachidonyl fluorophosphonate) inhibits $cPLA_2$ and $iPLA_2$ (see e.g., 52), though it has been reported that MAFP also acts as an inhibitor in the enzymatic hydrolysis of anandamide (55, 56) and as an antagonist of the cannabinoid CB1

receptor (55). BEL [bromoenol lactone, also referred to as haloenol lactone suicide substrate (HELSS)] is a relatively selective iPLA$_2$ inhibitor. BEL is, however, known to inhibit other important effectors in signal transduction such as phosphatidate phosphohydrolase (38). More specific sPLA$_2$ inhibitors, including LY311727 (3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propane sulfonic acid), have recently been described (57).

Spinal PLA$_2$ in Regulating Hyperalgesic Behavior

The most straightforward approach to studying the role of specific PLA$_2$ isoforms in spinal pain processing is to inhibit their activity by IT administration of selective inhibitors. Unfortunately, as noted above, no absolute type-specific PLA$_2$ inhibitors are widely available. Nevertheless, IT administration of AATFMK and MAFP results in a dose-dependent antihyperalgesia (45), indicating a possible role of spinal PLA$_2$ isozymes in facilitated processing.

SPINAL CYCLOOXYGENASE (COX) ISOZYMES

Cyclooxygenases are membrane-bound enzymes with 63% of their amino acid sequences in common. The enzymes have two catalytic activities: a cyclooxygenase activity mediating PGG$_2$ formation from arachidonic acid and a peroxidase activity involved in the reduction of PGG$_2$ to PGH$_2$. PGH$_2$ is converted to final active products by individual prostaglandin (PG) synthetases (58, 59). Two COX isozymes have been sequenced and cloned: COX-1 and COX-2 (72 kDa) (see e.g., 60). The COX-1 gene is stably expressed in most tissues and functions as a "housekeeping gene" (61). In contrast, COX-2 is generally not constitutively present in peripheral tissues, except in kidney and vas deferens (62, 63), but it is highly inducible by a variety of mechanisms. The COX-2 mRNA contains consensus sequences for several nuclear transcription factors including NF-kappaB, providing potent mechanisms for induction. The gene also displays repeated motifs in the untranslated regions (ATTTA) common to immediate early genes rendering the mRNA less stable (60), whereas during catalysis COX-2 undergoes suicide inactivation (64). Such properties indicate an effective regulation of enzyme expression and activity.

Constitutive Location of Spinal COX Isozymes

In contrast to the periphery, both COX-1 and COX-2 mRNA and protein are expressed constitutively in dorsal root ganglia (DRG) and in spinal dorsal and ventral gray matter as shown by in situ hybridization (65, but see 66), Northern blotting (67–70), immunohistochemistry (68, 71), and immunoblot techniques (67, 71, 72) (see Table 1). Under basal conditions, COX-1 and COX-2 have been found in neurons and in nonneuronal cells such as astrocytes. In DRGs, COX-1 immunoreactivity is observed in the cytoplasm, nuclear membrane, and axonal

TABLE 1 In vivo COX-1 and COX-2 localization in spinal cord (constitutive expression)[a]

		Localization			Cell			
Method	**Species**	**Dorsal horn**	**Ventral horn**	**DRG**	**Neurons**	**Astrocyte**	**Perivascular, endothelial**	**Refs**
COX-1								
NB, WB	rat	x						69
IHC	rat	(x)	np	x	x			71
WB	rat	x*	x*					76
NB		x*	x*					67
WB	rat	x*	x*					72
WB, IHC	mouse	x*	x*		np	x		75
IHC	rat			x	x			65
NB, WB	mouse	x*	x*					73
COX-2								
NB,WB	rat	x						69
IHC	rat	x	x		x			71
WB	rat	x*	x*					76
WB	rat	x*	x*					67
IHC, WB	rat		x		x		x	77
IHC	rat	x	x		x	x		78
WB	rat	x*	x*					72
IHC	mouse	np	x		x	x		75
IHC,WB, RPA	rat	x			x			43
WB	rat	x	x	x				79
IHC	rat	x	x	x	x	x	x	74
NB, WB, IHC	mouse	x	x		x		x	73
NB	human	x*	x*					80

[a]Abbreviations: * = homogenate of whole spinal cord assayed; (x) = very few positive cells; IHC = immunohistochemistry; WB = western blotting; NB = northern blotting; RPA = Rnase protection assay; np = not present.

processes of small- and medium-sized neuronal cell bodies (65, 71). In the spinal cord, COX-2 immunoreactivity is present in neurons of all lamina, particularly in the superficial layers. No COX-1 has been reported in neurons at the spinal level, but both COX-1 and COX-2 are found in astrocytes. COX-2 immunoreactivity is strong on the nuclear membrane but is also clearly present in neuronal and glial cytoplasm (68, 71, 73, but see 74), whereas COX-1 in glia cells is typically cytoplasmic (75).

Induction of Spinal COX Isozymes

COX-2 mRNA and protein in spinal cord increases after hind paw injection of carrageenan (66, 72, 76, 81), FCA (43, 67, 69, 70), and zymosan (75, 82). The changes

in expression produced by unilateral hind paw injection were found to occur both ipsi- and contralaterally in the lumbar cord and in the cervical cord. The increased COX-2 expression after peripheral injury occurs in both neuronal and nonneuronal elements (43, 67, 75, 82). Nonneuronal cells expressing COX-1 and COX-2 include astrocytes (83), microglia (33), endothelial (84), and leptomeningeal cells (85). Importantly, the increased expression of COX-2 in these cell systems is paralleled by increases in basal and evoked PG release. The release comes from astroglia by IL-1β and sP (86), from microglia by LPS and cytokines (83), and from rat spinal slices by capsaicin, sP, or NMDA (87). Intravenous injection of LPS (81) and IL-1β (43) resulted in increased COX-2 expression in the spinal cord over an interval of 4–24 h, whereas a more pronounced increase was seen after IT administration of LPS (81), IL-1α (88), and IL-1β (43). These data indicate that elevated COX-2 can be a major contributor to hypersensitivity after peripheral and central inflammation, although the surprising lack of effect of IT administration of NSAIDs and COX-2 inhibitors (43, 89) at later times in preclinical studies indicates that yet other systems may also be involved.

COX Pharmacology

Until recently, the common agents employed clinically were equally effective in blocking COX-1 and COX-2. In the past several years there has been a rapid proliferation of agents with a usefully high (>500–1000-fold) selectivity for COX-2, though only a few exist with a reported specificity for COX-1 (90) (Table 2).

Spinal COX in Regulating Hyperalgesic Behavior

Agents with a COX-1/2 nonselective inhibitory effect, given intrathecally, typically have no effect upon threshold models of acute stimulation (see Table 3). Such delivery will, however, produce a dose-dependent/stereospecific reduction in pain states that involve central facilitation. Such test models include the writhing test

TABLE 2 Summary of drug selectivity[a]

COX-1 = COX-2	COX-1 >COX-2	COX-2 > COX-1
Ketorolac		SC58125
Indomethacin	SC58560	SC384
Flurbiprofen		SC385 (inactive isomer)
Zomepirac		Celecoxib
S(+)ibuprofen		Rofecoxib
R (−)ibuprofen (inactive isomer)		NS398
Acetylsalicylic acid acetaminophen		DFU Meloxicam[b]

[a]See respective papers in Table 3 for references as to selectivity.

[b]Moderate selectivity.

TABLE 3 Intrathecal cyclooxygenase inhibitors in rat models of nociception[a]

Pain model	Intrathecal drug (ordering of activity)	Maximum efficacy	Refs
Acute			
Acute thermal escape	S(+)-ibuprofen = SC58125 >SC384 ≫	0	79
	SC560 = 0	0	96
	NS398 = indomethacin = 0	0	91
	Indomethacin = ketorolac, zomepirac,	0	97
	acetylsalicylate = 0	0	98
	Diclofenac = 0		
	Acetylsalicylate > indomethacin > 0		
Acute mechanical escape	Acetylsalicylate > indomethacin > 0		98
	Ketorolac = S(+)-ibuprofen = 0		99
PBQ evoked writhing	Zomepirac > acetylsalicylate > 0	++	16
	Diclofenac > 0	++	97
IPLT Formalin-Ph 1	ketorolac ≥ indomethacin = flurbiprofen = zomepirac = S(+)ibuprofen = ibuprofen = acetylsalicylic acid = acetaminophen = 0	+	91
Inflammation and tissue injury			
IPLT Formalin-Ph 2	Indomethacin ≥ flurbiprofen > ketorolac ≥	++	91
	zomepirac > S(+)ibuprofen ≥ ibuprofen >	+++	100
	acetylsalicylic acid > acetaminophen > R(−) ibuprofen = 0	++	96
	S(+)-Ibuprofen > SC58125 = 0		
	Indomethacin > 0	0	101
IPLT carrag-TH	S(+)-ibuprofen = SC58125 >SC384 ≫	+++	79
	SC560 = SC 385 = R(−) ibuprofen = 0	+++	102
	S(+) ibuprofen = SC58125 > 0	+++	93
	NS398 > 0		
IPLT Zymosan-TH	Indomethacin > 0	+++	103
IPLT Freunds-TA	NS398 > 0	+	43
NERVE INJURY			
Bennett-CCI-TH	Ketorolac > 0	+	104
Chung injury-TA	Indomethacin > 0	+	105
	Piroxicam = NS398 = ketorolac (R) and (S) = 0	0	106
Spinal activation			
IT NMDA-TH	S(+)-ibuprofen > R(−) ibuprofen = 0	+++	95
	SC58125 (2) > SC560(1) = 0	+++	79
	NS398 = indomethacin > 0	+++	94
IT NMDA-BSL	Diclofenac > S(+) ibuprofen > 0	++	97
	Acetaminophen > 0	++	107
IT NMDA-MH	DFU > 0	++	108

(*Continued*)

TABLE 3 (*Continued*)

Pain model	Intrathecal drug (ordering of activity)	Maximum efficacy	Refs
IT AMPA-TH	S(+)-ibuprofen > R(−) ibuprofen = 0	+++	95
IT-sP-TH		+++	95
		+++	79
IT-sP-BSL		++	107

[a]Abbreviations: BSL = biting, scratching, and licking; Carrag = carrageenan evoked; CCI = chronic compression nerve injury; IPLT = intraplantar (hind paw); IT = intrathecal (lumbar); MH = mechanical hyperalgesia; NMDA = n-methyl-d-aspartate; Ph = phase; sP = substance P; TA = tactile allodynia; TH = thermal hyperalgesia; Therm = thermal; PBQ = phenylbenzoquinone; maximum efficacy at highest usable intrathecal dose = 0 = no effect, + = <50%, ++ = 50< <100, +++ = 100%.

(16), phase 2 of the formalin test (91, 92), and thermal hyperalgesia induced by inflammation (89, 93), by IT-sP, or by IT-NMDA (94, 95).

By using agents with selectivity for the specific isozymes, it has been shown that (*a*) IT-COX-2- but not COX-1-preferring inhibitors dose-dependently and stereospecifically block the hyperalgesia induced by intraplantar carrageenan, IT-sP, and IT-NMDA; (*b*) the relative activity of the several COX-2 inhibitors in blocking the paw carrageenan and the IT-sP-evoked thermal hyperalgesia is the same as their in vitro potency in blocking COX-2; and (*c*) drugs given by a spinal route are 100–500 times more potent than when given systemically (79, 89). It is interesting that whereas phase 2 of the formalin test is blocked by COX-1/2 inhibitors, it is not blocked by COX-1 or COX-2 inhibitors (100; C.I. Svensson & T.Y Yaksh, unpublished observations). Whether this reflects a role for the combined actions of these two isozymes or perhaps experimental differences is not presently known.

Consistent with the behavioral pharmacology, in vivo release of PGE_2 evoked by IT-sP is reduced by nonspecific COX inhibitors and by COX-2- but not by COX-1-selective inhibitors (79, 87, 109). Importantly, the spinal release of PGE_2 evoked by IT-sP is blocked by systemic COX-1/2 and COX-2 but not COX-1 inhibitors, at COX-1/2 inhibitor doses that block the observed thermal hyperalgesia (79, 87, 109). Unexpectedly, although it is constitutively present in the cord, COX-1 appears to play little role in this spinal PG-mediated cascade as assessed in a variety of pain models and in terms of the ability of COX-1 inhibition to block evoked spinal PGE_2 release. This assertion is based on results from a single COX-1 inhibitor and from the observation that the maximum effect of COX-2 inhibitors does not differ from COX-1/2 inhibitors. The absence of other well-defined COX-1 inhibitors, however, precludes absolute dismissal of a role for COX-1 in hyperalgesia. Nevertheless, these observations emphasize that these isozymes do not play equivalent roles. Differential effects are noted in vitro in cells cotransfected with combinations of PLA_2 and COX isozymes (110). Based on electron microscopy (EM) studies, differences do not result from differential localization (108, 109).

Two possibilities may be that (*a*) COX-1 requires higher AA concentrations than COX-2 (110), or (*b*) there is differential coupling to cytosolic, secretory, and non-calcium-dependent phospholipases (110, 111). As noted above, these isozymes, constitutively present in spinal cells, are subject to induction, and increased expression by injury may contribute to changes in different models of post–tissue injury nociceptive processing. Nevertheless, it is clear that the antihyperalgesic actions of the spinal COX-2 inhibitors can be expressed under conditions and at times when enzyme induction has not likely occurred, stressing the importance of the constitutive COX-2 isozyme.

Blockade of COX Isozyme Expression

COX-1- AND COX-2-DEFICIENT MICE It has been reported that COX-1-deficient (homozygotes), COX-2-deficient (heterozygotes and homozygotes), and wild-type mice respond to the hot plate test, a finding consistent with the absence of an NSAID effect upon acute nociception. Surprisingly, COX-1 heterozygotes showed less nociception than control animals. In the writhing test, COX-1-deficient mice showed decreased hyperalgesia, whereas COX-2-deficient mice exhibited normal writhing responses to acetic acid. Analyses of mRNA levels in spinal cord of naïve animals demonstrated an increase of COX-1 mRNA in COX-2-deficient mice, which may compensate for the absence of COX-2 and explain the normal writhing response. No increase in COX-2 mRNA was detected in spinal cords of COX-1-deficient mice, indicating a role for COX-1-derived PGs in this model (112).

INTRATHECAL ANTISENSE IT delivery of a 20-mer phosphodiester-linked oligonucleotide antisense targeting a unique sequence within the COX-2 gene in rats produces a significant reduction in the expression of spinal COX-2, but not COX-1, protein. This treatment has no effect on acute thermal nociception but prevented thermal hyperalgesia induced by IT NMDA (C.I. Svensson & T.L. Yaksh, unpublished observations).

SPINAL PROSTAGLANDINS (PG)

Prostanoid Synthesis

Prostanoids are the products of cyclooxygenase metabolism of AA (113). Once AA is converted to PGH_2 by the action of COX, several downstream enzymes synthesize a variety of prostanoids including PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2. PGs are released into the extracellular space and enhance spinal excitability by binding to specific prostanoid receptors (see e.g., 114). Although PGs are fatty acids and assumed to diffuse freely across the lipid membrane, these lipidic acids exist primarily as charged molecules at physiological pH (115), so such free movement is not certain. Poor permeability for PGs across cell membranes has, in fact, been reported (115–117), and active transport systems for PGs have been suggested (118).

Prostaglandin Receptors

At the membrane level, prostanoids affect cells by binding to specific receptors in the plasma membrane. PG receptors (DP, EP, FP, and IP) are G-protein coupled molecules with seven transmembrane domains (118–121). Activation of PG receptors triggers intracellular signals that can be stimulatory [stimulation of adenylate cyclas (121, 122) and activation of phospholipase C (PLC) (123, 124)] or inhibitory [depressed cAMP production (125, 126)]. In situ hybridization and immunohistochemical studies have localized EP1, EP2, EP3, EP4 (68, 127, 128), and IP (129) receptors in the superficial layers of spinal cord, and DP, EP1, EP3, and IP receptors have been detected on DRG neurons (130, 131).

Release of Spinal Prostanoids

A variety of studies examining spinal cord slices in vitro have shown that PG concentrations are increased by local application of NK-1, vanilloid (VR1), and glutamate receptor agonists (87) and that this release is blocked by COX-2 inhibition (87).

A close correlation exists between manipulations that induce hyperalgesia and those that evoke the spinal release of prostanoids. In vivo spinal microdialysis and cerebrospinal fluid sampling show an increase in PGE_2 following (*a*) acute activation of small afferents (intraplantar formalin, heat) (102, 132, 133) that are capsaicin sensitive (102, 132–134); (*b*) persistent inflammation (carrageenan in knee joint, intraplantar Freunds adjuvant, intraplantar zymosan) (43, 72, 103, 135); (*c*) intrathecal (IT)-substance P (sP), IT-NMDA, or IT-kainate (136, 137); or (*d*) systemic cytokines (43). The functional relevance of this release is emphasized by observations that this spinal PG release is typically blocked by IT and systemic doses of COX inhibitors that reverse the respective hyperalgesia, and that IT-prostaglandin type E (EP) receptor antagonists reduce hyperalgesia produced by tissue injury and inflammation. These observations indicate the importance of spinal prostanoids and the upstream PLA_2-COX cascade in spinal systems mediating hyperalgesia.

Spinal Prostanoid–Mediated Effects on Hyperalgesic Processing

CELLULAR ACTIONS Sensitization of sensory neurons by prostaglandins is initiated in part by activation of adenylate cyclase and phospholipase C through prostanoid receptor binding, leading to enhanced protein kinase C (PKC) activity and increased levels of cAMP IP3 (138). This linkage modulates ion channel activity and thereby neuronal excitability. Thus, PGE_2 (*a*) increases calcium influx in cultured avian neurons (139), (*b*) decreases an outward potassium current (140), and (*c*) increases tetrodotoxin-resistant sodium influx in rat sensory neurons (141). PGE_2 and PGI_2, but not $PGF_{2\alpha}$, enhance depolarization-evoked increases in intracellular calcium and a resulting release of sP from capsaicin-sensitive DRGs (139, 142). IL-1β results in a calcium-dependent release of sP from ganglion cells, and this release is blocked by aspirin, indomethacin, and NS-398 (143). Glutamate

release in vivo evoked by IT capsaicin and NMDA is attenuated by IT delivery of COX inhibitors (102, 144), suggesting that spinal terminals from which glutamate is released are subject to facilitation by COX products. Aside from this presynaptic action, PGE_2 directly activates dorsal horn nociceptors (145), a finding consistent with the presence of PGE_2 receptor mRNA in dorsal horn neurons (146).

BEHAVIOR IT delivery of a variety of prostanoids, including PGE_1, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane B2, evokes thermal and mechanical hyperalgesia (147, 148, 149, 150) and sensitizes the animal to additional stimuli (151). Consistent with the ability of PGs to enhance glutamate release, the hyperalgesia resulting from the IT injection of PGE_2 is diminished by NMDA receptor antagonists (152). An interaction with nitric oxide synthase (NOS) systems is also evident. NOS inhibitors block allodynia induced by IT PGE_2 (153), whereas in iNOS-deficient mice, prostaglandins mediated thermal hyperalgesia, and PG production was diminished (103).

Of the many prostanoids, PGE_2 is the best-characterized mediator of hyperalgesia, though significant quantities of other lipidic acids are released by injury or inflammation. For example, the levels of spinal PGI_2 are greater than those of PGE_2 after hind paw injection of FCA (154), and PGI_2 is as effective in producing hyperalgesia and facilitation as PGE_2 both in vivo and in vitro (138). Of the few available prostanoid receptor antagonists, the majority are EP1 receptor antagonists. IT administration of the EP1 receptor antagonists SC51089 and SC51234A suppress phase 2 of formalin-induced flinching (155).

Caveats to Prostanoid Link in Cascade

The focus of this analysis is on the cascade leading to prostanoids. Other data suggest that arachidonic acid itself is released (156) and modulates cellular excitability (157). In addition, COX-independent mechanisms can form isoprostanes, which act on prostaglandin receptors (158).

REGULATION OF SPINAL PLA_2 AND COX ISOZYME EXPRESSION

It has been shown that after peripheral injury and/or inflammation, there are enhanced basal and evoked levels of prostanoids in the spinal extracellular fluid (43, 87). The increase may arise from several sources, including increased expression or activity of PLA_2 or COX isozymes.

Factors Regulating Spinal PLA_2-COX-2 Induction

Current evidence suggests that spinal COX-2 upregulation after tissue injury arises from persistent small afferent activity and/or an increase in circulating proinflammatory factors.

NEURAL ACTIVATION If a local afferent input initiates upregulation of protein expression in spinal cells it is believed that the effect will be (*a*) ipsilateral in the segments of afferent input (e.g., ipsilateral-lumbar for each hind paw), (*b*) prevented by spinal block of afferent transmitter receptor, and (*c*) mimicked by spinal injection of the putative transmitters. These properties have been observed for the immediate early gene cFos. Hind paw intraplantar formalin or carrageenan (159–162) increases spinal dorsal horn cFos ipsilaterally, an effect that is blocked by opiates, spinal anesthetics NK1 and NMDA antagonists (159–163). For COX-2, unilateral intraplantar hind paw, carrageenan (74), and Freund's complete adjuvant (43, 67) lead to an ipsilateral increase in COX-2 expression in the dorsal and ventral horn of the spinal cord, and a smaller, but nonetheless prominent, increase in contralateral spinal cord and cervical cord (e.g., extrasegmentally). This somatotopic specificity associated with unilateral hind paw intraplantar formalin is consistent with the local activation of small afferents (164) and the comparative lack of inflammation observed over several hours after formalin injection. This specificity contrasts with models that induce significant inflammation (such as injection of Freund's adjuvant). Local anesthetic blockade of the hind limb partially reduces the Freud's adjuvant–increased COX-2 expression (43). IT NMDA increases COX-2 protein but not COX-1 protein expression in the spinal cord, and this effect is blocked by spinal NMDA receptor antagonism (81). These results are consistent with studies in brain, in which PLA_2 and COX-2 are induced by NMDA-dependent synaptic activity (165, 166).

CIRCULATING FACTORS After tissue injury, a variety of circulating factors are increased, including TNF and IL-1 (29). Cells in close proximity to the microvasculature and leptomeninges (presumably microglia or astrocytes) increase their COX-2 mRNA levels within 2 h, and this subsides by 24 h after IV cytokines (IL1-β) (43, 85, 167, 168) or LPS (81). Importantly, codelivery of IL1-β and TNFα synergistically enhances (*a*) arachidonic acid release, (*b*) expression of COX-2 mRNA, and (*c*) PG release in fibroblasts (51). In spinal cord, recent studies have shown that COX-2 protein upregulation by carrageenan is largely in neurons, whereas following LPS, it is found in both neurons and in endothelial cells (T.L. Yaksh, J.R. Ghilardi, C.I. Svensson & P.W. Mantyh, unpublished observations).

Signal Transduction Pathways Linked to Transcriptional Activation

The signaling pathways that lead to transcriptional activation are complex and difficult to identify owing to the many activators and different conditions that are involved in induction of PLA_2 and COX-2 enzymes. However, for agents such as LPS, IL-1β, and TNF-α, common pathways are likely to be involved in the regulation of the enzymes. These include transcription factors such as NF-kappaB, AP-1, and CREB, as well as the mitogen-activated protein kinase (MAPK) cascade.

NF-KAPPAB Although the cPLA transcript and protein are constitutively expressed, they are also subject to induction by cytokines (36). Sites for NF-kappaB and AP-1 transcription factors have been identified in the $cPLA_2$ gene (169). Although the signaling pathways leading to induction of $sPLA_2$ by cytokines have not been elucidated, there is evidence that this process also involves DNA-NF-kappaB binding (170). Activation of NF-kappaB pathways are part of the transcriptional activation of COX-2 by IL-1 (171), TNFα (172), and LPS (173). There are at least two binding sites for NF-kappaB in the COX-2 5′-promoter region (174). NMDA receptor activation can also be linked to transcriptional regulation of COX-2 because rapid (<5 min) activation of NF-kappaB is evoked by NMDA (175, 176). Activation of NF-kappaB by seizures/ischemia is reduced by NMDA antagonists (176–178).

MAP KINASES The MAPK family consists of three major subgroups: p38, p42/44 (ERK), and c-jun NH2-terminal kinase/stress activated protein kinase (JNK/SAPK). Depending upon stimulus and cell phenotype, in vitro work shows that these kinases can regulate cPLA2 activity and COX-2 expression. The p38 and p42/44 pathways are activated by IL-1 (179–185), TNFα (186), and LPS (179–185, 187–191). Phosphorylation of $cPLA_2$ via p38 and/or p42/44 pathways increases its enzymatic activity (35, 192–197). Further, activation of p42/44 and/or p38 MAPK leads to phosphorylation of transcription factors with binding sites present in the COX-2 promotor, thereby eliciting upregulation of COX-2 mRNA and protein (198, 199). In addition, p38 MAPK not only activates transcription factors, but can stabilize COX-2 mRNA (185, 200). MAPK inhibitors prevent cytokine- and LPS-induced COX-2 elevation in cell cultures (187). These observations suggest a "cross-talk" between pathways at the level of $cPLA_2$ activation and $sPLA_2$ and COX-2 induction, making the MAPK cascade a potential target for reduction of PG synthesis and modulation of inflammatory-mediated pain processing.

STEROIDS Upregulation of COX-2 expression and PG release is suppressed by steroids (201), and this suppression is antagonized by a steroid antagonist (RU38486) (202). Current data suggest that suppression of COX-2 transcription rates by dexamethasone is mediated by direct inhibition of p38 kinases (203).

IN VIVO FACTORS The majority of the work on transcription factors outlined above has come from in vitro studies. Whether these mechanisms underlie spinal PLA_2-COX regulation in vivo seems plausible but is not certain. Following intraplantar Freund's adjuvant, IP-dexamethasone prevented induction of COX-2 mRNA and reduced elevated levels of prostaglandins in spinal cord (70). Whether this is a central or peripheral action of the steroid at a neuronal or nonneuronal site of COX expression is not known. IT-methylprednesolone reduces formalin phase 2 (204). Moreover, brain PGE-2 synthesis is exaggerated in adrenalectomized rats, suggesting an endogenous regulation (205). Seizure induced, p38-mediated phosphorylation (206) or NMDA-induced MAPK activation (207) is blocked by

the NMDA antagonist MK801 and P38 inhibition, respectively, suggesting that glutamatergic receptors in neuronal systems can evoke such MAPK activation as we anticipate at the spinal level. In that regard, IT P38 kinase inhibitors diminish thermal hyperalgesia induced by intraplantar carrageenan, IT NMDA, and formalin-mediated flinching and reduced the otherwise increased COX-2 expression evoked by IT NMDA (208). These data indicate a role for p38MAPK in synaptic plasticity and provide a plausible link between MAPK activation, upregulation of spinal COX-2 protein levels and hyperalgesia induced by peripheral inflammation or IT NMDA.

ROLE OF CONSTITUTIVE VERSUS INDUCIBLE SPINAL COX-2 IN NOCICEPTIVE PROCESSING

This review emphasizes that unlike in the periphery there is a constitutive expression of both COX-1 and COX-2 in spinal cord. Behavioral studies in preclinical models of nociceptive processing suggest that, as with systemic delivery, the spinal action of COX inhibitors occurs in models of facilitated processing initiated by tissue injury and inflammation. The pharmacology of these behavioral effects indicates that the effects are mediated in large part by COX-2 and not by COX-1. Whereas it is clear that spinal COX-2 protein expression and PGE_2 release can be increased by peripheral injury and inflammation (43, 87), the current data unequivocally emphasize the importance of constitutive COX-2. Thus, in the hyperalgesia induced by IT-sP and NMDA, the block of hyperalgesia by IT COX1/2 or COX-2 inhibitors is observed immediately (79, 95). In the carrageenan model hyperalgesia is observed within 30 min after the injection, and even at this time IT COX inhibition is fully effective (209). In contrast, measurement of COX-2 protein suggests that induction takes at least 2–4 h after the stimulus. Such observations argue that constitutive COX-2 is necessary and sufficient to explain the effects of even systemically delivered COX-2 inhibitors. The additional contribution of the induced spinal isozymes remains to be determined.

CENTRAL NERVOUS SYSTEM ACTIONS OF COX INHIBITORS IN MAN

Given the evidence supporting the central actions of the NSAIDs in general, and COX-2 inhibitors in particular, what observations exists to argue for a central or spinal action in humans? Several lines of evidence can be marshalled.

Noninflammatory-Induced Experimental Pain

Direct electrical activation of peripheral sensory nerves can initiate nociceptive response. Sural nerve stimulation in human observers will evoke the *biceps femoris*

flexion reflex. This response is attenuated by systemically delivered ketoprofen, difunisal, indomethacin, acetylsalicylic acid or paracetamol (210–212). Similarly, the electrically evoked *orbicularis oculi* muscle reflex is diminished by piroxicam (213). These results reflecting the direct activation of small afferent input support a nonperipheral action of these NSAIDs.

Spinal Drug Delivery

Bolus IT delivery of lysine acetylsalicylate produced reliably reported pain relief in late-stage cancer patients (214–216). Though intriguing, further IT administration of this family of agents in humans must be considered ill advised until appropriate safety studies are accomplished.

COX Isozyme Inhibition in Human Pain States

Highly selective COX-2 inhibitors have significant antihyperalgesic activity in a variety of arthritic (217) and postsurgical pain states (218, 219). Two points should be emphasized that relate to achievable efficacy and the time of onset. First, while there are no clinically useful COX-1 selective antagonists, the failure to report any marked difference in efficacy with COX-1/2- and COX-2-selective inhibitors suggests, as do the preclinical data, a minimal role for COX-1 in human post–tissue injury pain processing studied to date (217, 220, 221). Secondly, as regards the time of onset, COX-2 inhibitors have a rapid onset of activity after a brief surgical intervention (e.g., third molar extraction). Given that COX-2 is not constitutively expressed in the periphery, the antihyperalgesic actions of COX-2 inhibitors after surgery suggest that these agents must be exerting their action at a site wherein COX-2 is constitutively expressed and does not require any induction. Thus, though indirect, this observation argues for a site within the central nervous system wherein this isozyme is constitutively expressed. Regarding the ability of COX inhibitors to penetrate the brain, it should be stressed that in the absence of a likely peripheral mechanism of action, the actions of these systemic agents must be central and hence reflect a central bioavailability. As noted above, systemic delivery of COX-1/2 and COX-2 inhibitors diminish the spinal release of prostanoids by both peripheral and spinal stimuli at drug doses that are observed to have antihyperalgesic activity (91).

SIGNIFICANCE

The clinical importance of research on the spinal role of COX is emphasized by the fact that COX-1/2 and COX-2 inhibitors are potent agents in managing pain states ranging from postoperative to cancer pain. Their efficacy verifies the role played by the PLA_2-COX cascade in these human conditions. In bone cancer models, a prominent upregulation of astrocytes occurs in the ipsilateral lumbar cord (222, 223). It is highly likely that these activated astrocytes will display increased

PLA_2 and COX-2 expression. We believe that such observations along with the eponymous role of spinal PGs provide an explanation for the particular efficacy that NSAIDs have in managing bone cancer pain (224). Understanding the spinal PLA_2-COX-prostanoid cascade will provide insights into the organization of pain processing arising from tissue injury and inflammation and the reorganization that accompanies such states.

The circle is now complete. Flower and colleagues (225), in the sixth edition of Goodman & Gilman, noted that ". . . aspirin works peripherally . . . preventing the synthesis and release of prostaglandins in inflammation . . ." In the eighth edition it was emphasized that the aspirin-like drugs are particularly effective in "settings . . . in which inflammation has caused sensitization of pain receptors to normally painless mechanical or chemical stimuli." (226) Yet, earlier, Woodbury in the third edition of Goodman & Gilman (9) noted that ". . . the salicylates are capable of alleviating certain types of pain by virtue of a selective depressant effect on the CNS . . . . A subcortical site is suggested by the fact that analgesic doses do not cause mental disturbances, hypnosis, or changes in the modality of sensations other than pain."

ACKNOWLEDGMENT

The authors' work was supported by NIH grant NIH-NS16541.

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