Published in final edited form as:
Curr Mol Pharmacol. 2009 January 1; 2(1): 1–14.

Mechanisms of Non-Opioid Analgesics Beyond Cyclooxygenase Enzyme Inhibition

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Abstract

Non-opioid analgesics including both selective and non-selective cyclooxygenase (COX) inhibitors and acetaminophen are the most widely used treatments for pain. Inhibition of COX is thought to be largely responsible for both the therapeutic and adverse effects of this class of drugs. Accumulating evidence over the past two decades has demonstrated effects of non-opioids beyond the inhibition of COX and prostaglandin synthesis that might also explain their therapeutic and adverse effects. These include their interaction with endocannabinoids, nitric oxide, monoaminergic, and cholinergic systems. Moreover, the recent development of microarray technology that allows the study of human gene expression suggests multiple pathways that may be related to the analgesic and anti-inflammatory effects of non-opioids. The present review will discuss the multiple actions of non-opioids and their interactions with these systems during inflammation and pain, suggesting that COX inhibition is an incomplete explanation for the actions of non-opioids and proposes the involvement of multiple selective targets for their analgesic, as well as, their adverse effects.

Keywords

NSAIDs; endocannabinoids; monoaminergic systems; cholinergic system; nitric oxide; interleukin-6; matrix metalloproteinases; inflammatory pain

INTRODUCTION

Non-opioid analgesics are among the most widely used medications due to their efficacy for a wide range of pain and inflammatory conditions. In the United States alone, over 172 million prescriptions for cyclooxygenase (COX) inhibitors (both selective and non-selective) were dispensed in the year 2004 [1]. Inhibition of prostaglandin (PG) synthesis has been widely accepted since the 1970s as the mechanism underlying the pharmacological actions of both the therapeutic and adverse effects of this group of drugs. The application of new molecular-genetic technologies to classic analgesic paradigms has resulted in compelling evidence to question the unitary COX-inhibition hypothesis of non-opioids. We review here evidence that suggests alternative mechanisms of non-opioids actions that may hold promise for new strategies for analgesic drug development.

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IS INHIBITION OF PROSTAGLANDIN SYNTHESIS THE SOLE MECHANISM UNDERLYING THE PHARMACOLOGICAL ACTIONS OF NON-OPIOID ANALGESICS?

The inhibition of PG synthesis has been reported in almost every study that has used a non-opioid. We have shown in several studies a temporal association between decreased PGE_2 levels at the site of extraction in the oral surgery model of acute inflammatory pain and the analgesic effects of NSAIDs and coxibs [2–4]. However, observations dissociating the analgesic and the anti-inflammatory activities of non-opioids have also been reported. At analgesic doses, sodium salicylate does not inhibit urinary excretion of PGE_2 or PGI_2 metabolites in human volunteers [5]. Furthermore, though the COX inhibitory activities of salicylate and acetylsalicylic acid (ASA; aspirin) differs markedly [6], they show similar anti-inflammatory potencies [7]. The same discrepancy applies to R- and S-enantiomers of flurbiprofen, without possible explanation based on pharmacokinetics or chiral inversion. While S-flurbiprofen has anti-inflammatory activity and inhibits PGE_2 synthesis, R-flurbiprofen at equianalgesic doses is devoid of both properties [8]. Furthermore, a meta-analysis reported that the analgesic efficacy of different non-opioids in the clinical dental pain model, does not correspond to the degree of inhibition of PG synthesis *in vitro* [9], which suggests that other molecular mechanisms contribute to the analgesic effects of NSAIDs.

The introduction and eventual recognition of an increased risk of cardiovascular adverse effects attributed to selective COX-2 inhibitors [10] also challenges the COX-inhibition hypothesis. It was proposed that selective COX-2 inhibitors, in contrast to non-selective ones, affect the balance between prothrombotic and anti-thrombotic eicosanoids, thereby shifting the balance to a prothrombotic state [11]. However, to date, this proposed mechanism of cardiovascular risk has not been confirmed [12]. In fact, several findings argue against this assumption. In healthy volunteers, therapeutic doses of rofecoxib that are known to inhibit vascular PGI_2 production, do not result in significant changes in endothelial vasodilator responses [13]. In another clinical study, rofecoxib neither affected the levels of both PGI_2 and thromboxane A_2 metabolites, nor did it have an effect on bleeding time, platelet aggregation or thrombin generation after 7 days of treatment in an ex-vivo model of microvascular injury [14]. Further, internal mammary and radial arteries and saphenous veins donated by individuals with ongoing cardiovascular disease (during standard coronary artery bypass surgery) showed detectable levels of COX-1 but not COX-2 [15]. Interestingly, in bovine aortic endothelial cells, prostacyclin synthase and COX-1 were colocalized to the nuclear envelope and endoplasmic reticulum. However, there was a lack of colocalization of COX-2 with prostacyclin synthase [16]. Taken together, these studies argue against the role of vascular COX-2 in PGI_2 mediated vasodilation. Recent epidemiologic reports suggest that cardiovascular risk associated with COX-2 inhibitors extends to some non-selective NSAIDs and to acetaminophen, particularly with higher doses or higher frequency of use [17], further lack of evidence for COX-2 mediated imbalance resulting in a prothrombotic state.

The recent development of microarray technology allows study of gene expression across the whole human genome, adding further possibilities to explore drug effects. We have recently shown in two different studies [18,19] the effect of both ibuprofen and rofecoxib on gene expression in the oral surgery model of acute inflammatory pain. Both drugs induce over 3-fold up-regulation of more transcripts than did the placebo 48 hours after surgery. Among the upregulated genes identified in these studies are interleukin-6 (IL-6), suppressor of cytokine signaling 3 (SOCS3), and matrix metalloproteinases (MMPs), which we will discuss in detail here. Taken together, it appears plausible that multiple mechanisms contribute to non-opioids' therapeutic and adverse effects. Indeed COX-independent mechanisms have been reported for the antiproliferative and antineoplastic effects of NSAIDs and coxibs [20,21]. The present

review will focus on the interaction of non-opioids with endocannabinoids, nitric oxide (NO) and monoaminergic and cholinergic pathways in relation to their analgesic effect, and their effect on IL-6 and MMPs regulation.

ENDOCANNABINOID SYSTEM AND ITS RELATION TO NON-OPIOIDS

Cannabinoids, including endogenous ones, have been implicated in the modulation of a large number of behavioral processes, including pain and inflammation [22,23]. Anandamide (AEA), the amide of arachidonic acid (AA) with ethanolamine and 2-arachidonyl glycerol (2-AG) are the most widely studied endocannabinoids, even though several other have been identified. The synthesis, release and metabolism of endocannabinoids have been discussed in detail elsewhere [24,25]. Important targets in the metabolism of endocannabinoids are AEA membrane transporter (AMT), which facilitates the transport of AEA into the cells [26] to be hydrolyzed by fatty acid amidohydrolase enzyme (FAAH) [27,28]. AEA may also be metabolized by COX-2 and to a less extent COX-1 into PGE_2-ethanolamide. However, AEA is a significantly poorer substrate than AA for COX-2. 2-AG is selectively metabolized by COX-2 at a much higher rate than AEA and the products of its oxygenation closely parallel those for AA oxygenation [29,30].

The involvement of endocannabinoids in the analgesic anti-inflammatory effects of non-opioids is suggested by both *in vitro* and *in vivo* evidence. In a macrophage cell line, indomethacin induced AEA synthesis in the presence of a calcium ionophore [31]. *In vivo*, ibuprofen and rofecoxib injected with AEA increased the levels of the endocannabinoids AEA, oleoylethanolamide and palmitoylethanolamide in inflamed paw tissues. Interestingly, higher levels were produced by rofecoxib. Paw level of AEA was also elevated non-significantly after injection of either ibuprofen or rofecoxib alone. [32].

As shown in Fig. (1), the increase in endocannabinoids levels following non-opioids treatment could be explained based on either: 1) the inhibition of their metabolism by FAAH; several non-opioids, including indomethacin, ibuprofen and flurbiprofen, inhibit the activity of FAAH [33–35], particularly at low pH [35–37], often a characteristic of the site of inflammation. 2) Inhibition of their oxidative metabolism by COX-2; at least *in vitro* COX-2 can metabolize AEA [30]. 3) Increase endocannabinoid synthesis as a result of shunting of free AA away from PG synthesis [38,39]. 4) In case of acetaminophen after being metabolized into N-acylphenolamine (AM-404) in the brain and spinal cord, inhibition of the cellular uptake of AEA thus preventing its inactivation and enhancing its potency [40]. 5) Inhibition of NO synthesis and thus inactivating the endocannabinoid transporter [41,42].

In vivo studies support the involvement of endocannabinoids in the analgesic and anti-inflammatory effects of non-opioids. The selective cannabinoid CB_1 receptor (CB_1) antagonist AM-251 antagonizes the antinociceptive activity of indomethacin in the mouse formalin test and zymosan-induced heat hyperalgesia [38] and that of flurbiprofen in the rat formalin test [39]. Other observations supporting the role of endocannabinoids include: 1) failure of intrathecal indomethacin to induce an antinociceptive effect in CB_1-receptor knockout mice [38]. 2) Both ibuprofen and rofecoxib induce a synergistic antinociceptive effect when injected with AEA into the rat paw before the formalin test and CB_1 and CB_2 antagonists completely antagonize their effects [32,43]. 3) The 6-methyl-pyridin-2-yl analogue of ibuprofen, which is equipotent as a COX inhibitor yet more potent as FAAH inhibitor [44] was more efficacious than ibuprofen in the acetic acid writhing test [45]. 4) The CB_1 receptor antagonists (AM-281 and SR141716A) prevent the analgesic effects of acetaminophen in the hot plate test [46], and the CB_1 receptor antagonist AM-251 blocks the antinociceptive effect of acetaminophen in the mouse formalin test*. Where not all studies show reversal of non-opioids antinociceptive effects after CB_1 receptor blockade [47,48], the discrepancy might be due to the differences

in pain models used. The preponderance of evidence is strongly suggestive that endocannabinoids contribute to the analgesic effects of non-opioids.

NON-OPIOIDS AND MONOAMINERGIC PATHWAYS

The antinociceptive effects of non-opioids might also be related to their effects on the monoaminergic pathways, namely the noradrenergic and the serotonergic systems.

The Serotonergic System and its Relation to Non-Opioids

The regulation of spinal nociceptive processing by serotonin (5-HT) may induce facilitation or inhibition of nociception due to the different classes of 5-HT receptors and their location on facilitating (primary afferent fibers, projection neurons, excitatory interneurons) and attenuating (inhibitory interneurons) neurons in the superficial laminae of the spinal cord [49].

The involvement of the serotonergic system in the antinociceptive effects of non-opioids has been extensively studied. In rats, ASA increases 5-HT content in the cerebral cortex and pons [50,51], as does acetaminophen in the striatum, posterior cortex, hypothalamus, hippocampus and brain stem but not the spinal cord of rats [52]; while rofecoxib increases 5-HT in the frontal cortex [53]. Lysine ASA increases concentrations of 5-hydroxyindole acetic acid, in several areas of the brain in rats [54]. This increase in 5-HT levels is accompanied by down-regulation of 5-HT_2 receptors expression in several studies [50,51,53]. Furthermore, administration of acetaminophen for 15 days results in a dose-dependent downregulation of the 5-HT_{2A} receptor in the frontal cortex of rats. These effects were accompanied by an increase in 5-HT levels in platelets [55], which might reflect a parallel change in 5-HT level in the central nervous system [56].

The increase in 5-HT in acetaminophen-treated rats is not due to increased synthesis since quantitative determination of 5-hydroxytryptophan accumulation after aromatic L-amino acid decarboxylase blockade showed no changes, nor does the increase in 5-HT is due to blockade of its catabolizing enzyme, MAO A, as 5-hydroxyindoleacetic acid levels do not decrease concomitantly with the increases in 5-HT levels [52]. *In vitro*, acetaminophen exerts no direct effect on MAO A activity. It increases K^+-evoked [^{3}H]5-HT overflow from slices of the posterior cortex, but not the striatum, the brain stem or the hypothalamus, eliminating the possibility that changes in 5-HT release/reuptake might account for the increased levels of 5-HT in these areas [52].

In vivo studies support the involvement of a central serotonergic mechanism in the antinociceptive activity of non-opioids. Depletion of central 5-HT antagonizes the antinociceptive activity of ASA, acetaminophen, diclofenac, ketoprofen, metamizol, acetaminophen, piroxicam, meloxicam and rofecoxib in several pain models [53,57–59]. Furthermore, the analgesic effect of ASA is enhanced by central administration of either 5-HT or its precursor 5-hydroxytryptophan [57]. There is considerable controversy on identifying the role of different 5-HT receptor subtypes in the mechanism of action of acetaminophen, e.g. [60–62]. Recently, Pickering *et al*. [63] reported the reversal of the analgesic effect of acetaminophen by the 5-HT_3 antagonists, tropisetron or granisetron in a pain self-evaluation test based on the electrical stimulation of the median nerve in man. At least in case of acetaminophen, central serotonergic effects appears to be primarily supraspinal [64]. Since acetaminophen possesses no binding affinity to any type of 5-HT receptor or transporter [65], a direct effect on 5-HT receptors or transporters is unlikely and the exact mechanism of

*Hamza, M. Role of transient receptor potential vanilloid-1 (TRPV1) and cannabinoid CB1 receptors in paracetamol induced antinociception and hypothermia in mice. *FASEB J.* **2008**, *22*, 1125.11.

acetaminophen effects on 5-HT signaling is yet to be determined. Based on behavioral studies using naloxone, activation of opiate receptors has been suggested as a mechanism for increasing 5-HT levels, at least in the cerebral cortex and pons [66]. However, acetaminophen has little affinity for opioid receptors [66]. The involvement of opiate receptors has been suggested also for ASA [50], diclofenac, indomethacin and sodium salicylates [67].

The molecular mechanism underlying acetaminophen–induced analgesia *via* the serotonergic system was recently studied [68]. Acetaminophen modulated the expression of four genes in the lumbar enlargement of the rat spinal cord following the formalin test, but not in naive rats. The gene and protein expression of the low-affinity neurotrophin receptor ($p75^{NTR}$), insulin-like growth factor-1 receptor alpha subunit (IGF-1Rα) and growth hormone receptor (GHR) were upregulated, while gene expression of the somatostatin 3 receptor (sst3R) was down-regulated. The changes in the gene expression of these four transcripts were dependent on spinal 5- receptor stimulation, since they were completely blocked by HT_{1A} WAY-100635. While a GHR antagonist partially reversed the anti-nociceptive effect of acetaminophen in the second phase, an IGF-1R antagonist completely antagonized its effect in both phases implying that these cellular events are important for the antinociceptive activity of acetaminophen. Acetaminophen also increases the activity of both extracellular signal-regulated kinases 1 and 2 (ERK1/2), an effect that was again blocked by the 5-HT_{1A} receptor antagonist WAY-100635. The down-regulation of sst3R mRNA depends on an acetaminophen-induced 5-HT_{1A} receptor dependent increase in neuronal ERK1/2 activities that mediate antinociception. U0126, a specific inhibitor of mitogen-activated protein kinases 1/2 (MAPK1/2), which are kinases upstream of ERKs, totally prevented the augmentation of phosphorylation and activities of ERK1/2 elicited by acetaminophen, and the down-regulation of sst3R mRNA. U0126 only partially blocks acetaminophen-induced antinociception in the formalin test. While further studies are needed to test the effect of other non-opioids on these targets, collectively these observations suggest that the antinociceptive activity of non-opioids is mediated at least in part through interaction with the serotonergic system.

Noradrenergic System and its Relation to Non-Opioids

The noradrenergic system is involved in nociception at spinal and supraspinal levels. Its effects are mediated through activation of α-adrenoceptors and descending inhibitory pathways. At the spinal level, norepinephrine produces potent analgesia through activation of α_2 adrenoceptors. It is likely that norepinephrine modulates nociception *via* indirect control of the activity of other descending pathways including histaminergic and serotonergic pathways [49,69]. The role of α adrenoceptors subtypes in nociception is reviewed in detail elsewhere [49].

PGE_2 causes a significant reduction of the stimulation-induced overflow from peripheral noradrenergic nerve terminals and a small yet significant reduction from central noradrenergic nerve terminals *in vitro* [70]. This action may contribute to the pronociceptive effects of PGE_2, and consequently, to the antinociceptive effect of non-opioids. In-vivo studies support this hypothesis; both destruction of bulbospinal noradrenergic projection neurons by intracerebroventricular injection of 6-hydroxy dopamine and intrathecal injection of phentolamine (a non selective α-adrenoceptor antagonist) prevents the pronociceptive effect of PGE_2 and the antinociceptive effect of indomethacin in the rat tail-flick and mechanical Randall-Selitto paw-withdrawal tests [71]. Similarly, the α_2 adrenoceptor antagonist yohimbine, but not the α_1 adrenoceptor antagonist prazosin antagonizes the antinociceptive effects of the non-opioids ketoprofen, diclofenac and piroxicam in the mouse tail flick test [72]. In addition, the α_2 adrenoceptor antagonist atipamezole prevents the analgesic effects of systemic ketoprofen on mechanical noxious stimulation in sheep [73]. Yohimbine, however, did not antagonize the antinociceptive effect of diclofenac in the mouse writhing test [74],

which might be due to the different nature of the inflammatory stimulus and the predominant role of COX inhibition as an anti-inflammatory mechanism in this model. In the same model, systemic combination of α_1- and α_2-adrenoceptor agonists (phenylephrine and clonidine, respectively) with diclofenac or ketoprofen showed a synergistic antinociceptive effect, suggesting that they induce antinociception by activating different mechanisms (COX inhibition and α_2-adrenoceptor activation). On the other hand, intrathecal administration of the same drug combinations resulted in an additive rather than synergistic interaction [74,75]. Similarly, the concurrent intraperitoneal administration of clonidine with metamizol, nimesulide, acetaminophen, piroxicam or naproxen results in synergistic interactions. The intrathecal administration of these combinations, however, resulted in an additive interaction [76]. Taken together, these data clearly point to an interaction between non-opioids and the adrenergic system. Non-opioids may activate supraspinal mechanisms that indirectly cause descending inhibitory influences on the spinal transmission of nociceptive inputs [71–73], beside their COX inhibitory action.

CHOLINERGIC SYSTEM AND ITS RELATION TO NON-OPIOIDS

Acetylcholine in the dorsal horn of the spinal cord is released from both intrinsic neurons and supraspinal structures. Possible cholinergic mechanisms of antinociception may include the activation of non-cholinergic descending inhibitory pathways, mediation of descending inhibition following its own release from descending pathways and the induction of antinociception following release from inhibitory interneurons in the dorsal horn. Pharmacological studies have suggested that antinociceptive effects of acetylcholine in the dorsal horn are mediated through both nicotinic and muscarinic receptors. The mechanism of this analgesia, however, is not well defined as nicotinic receptor activation at the spinal level may affect several modulatory transmitters including inhibitory amino acids, norepinephrine and serotonin [49,69].

ASA administered subcutaneously, but not spinally, increases intraspinal acetylcholine release in anesthetized rats [77], which might contribute to its analgesic activity. In line with that, cholinergic depletion by intracerebroventricular hemicholinium-3 or systemic administration of the muscarinic antagonist atropine antagonizes the antinociceptive effect of both intraperitoneally and intrathecally administered clonixin, diclofenac, piroxicam, ketoprofen and meloxicam in the mouse tail flick test [78]. In support of the above theory, the cholinergic agonist carbachol shows a synergistic antinociceptive effect in the acetic acid writhing test when co-injected intraperitoneally with meloxicam, diclofenac, piroxicam or ketoprofen. Intrathecal administration, however, shows only an additive effect [79]. These studies point to a possible role for central cholinergic modulation of the antinociceptive effects of non-opioids, yet further investigations are needed to identify and localize the exact mechanism of this modulation.

NON-OPIOIDS AND NITRIC OXIDE

NO has been recognized as an important intra- and intercellular messenger molecule in the central nervous system [80]. Its release depends on its synthetic enzyme, nitric oxide synthase (NOS), which exist in three isoenzymes termed NOSs [81] and many of its effects are mediated by cyclic guanosine monophosphate (cGMP) [82]. NO is implicated in many physiological and pathological processes including nociception, inflammation and regulating the contractile activity of vascular smooth muscle cells. At the spinal level NO plays an important role in the development and maintenance of inflammatory hyperalgesia. Its role in the periphery is not as well studied [83,84].

Non-opioids inhibit NO production in different clinical and experimental studies. Ibuprofen (2400 mg p.o.) decreases alveolar NO flow rates and urinary excretion of nitrite and nitrate, in

both endotoxemic and normal subjects [85]. Similarly, ibuprofen-arginine (400 mg) reduces NO metabolites in serum twenty minutes after oral intake [86]. In spinally microdialyzed mice, indomethacin reduces NO metabolites in dialysate [38]. The inhibitory effect of indomethacin on NO production and or iNOS induction was reported in several other studies [87–89]. Acetaminophen also inhibits NO synthesis in murine spinal cord slices [90]. In RAW 264.7 macrophages, acetaminophen, ASA and sodium salicylate inhibits NO production and iNOS protein expression in a dose dependent manner. Further, acetaminophen inhibits iNOS mRNA expression [91]. Although the main body of evidence supports the inhibitory effect of ASA on NO synthesis, sporadic studies suggest a stimulatory role e.g. [92,93]. The discrepancies could be explained based on the difference of cell types and/or inflammatory model.

PG inhibition does not seem to contribute to this inhibitory process, since the effect of different non-opioids varies under the same experimental setting. For example, therapeutic concentrations of ASA, but not indomethacin inhibits the protein expression of iNOS and the production of nitrite in lipopolysaccharide (LPS) activated RAW 264.7 murine macrophages, while only ASA inhibits the catalytic activity of iNOS in cell free extracts [94]. Likewise, ASA, but not indomethacin or acetaminophen inhibits cytokine-induced nitrite production in cardiac fibroblasts [95]. Furthermore, there was no significant difference between the S- and R- pure enantiomers of flurbiprofen and ketoprofen as regards the reduction of NO release from IL-1β stimulated human chondrocytes [96], and exogenous PGE_2 did not reverse the inhibitory effects of celecoxib on NO production by activated human articular chondrocytes [97].

Ryu *et al*. [91] suggests that acetaminophen inhibits iNOS expression at the transcriptional level by suppression of nuclear factor kappa B (NF-κB) binding activity, whereas salicylates exerts their effects by inhibiting iNOS expression at the translational or post-translational level [94]. NF-κB expression is one of the integral contributors to iNOS transcription and expression [98]. LPS or cytokines were shown to activate the phosphatidyinositol 3-kinase/Akt (PI3K-Akt) pathway, which in turn activates the NF-κB pathway, and results in upregulation of iNOS expression in vascular smooth muscle cells [99]. In human articular chondrocytes, NO production is mediated *via* NF-κB, Jun NH_2-terminal kinase (JNK) and p38, with celecoxib inactivating NF-κB and JNK [97]. Similarly, acetaminophen inhibits NF-κB binding to the promoter region of the iNOS gene [91]. Since non-opioids regulate NF-κB, JNK, p38 and Akt [100], this might represent the molecular mechanism by which they regulate iNOS expression.

In agreement with the pronociceptive role of NO at the spinal level and the inhibitory effect of acetaminophen on its production, L-arginine, but not D-arginine, antagonizes the antinociceptive effect of acetaminophen in NMDA and substance P-induced nociception, suggesting that the analgesic effect of acetaminophen is related to inhibition of NO generation [101]. Further, intrathecal treatment with L^G-nitro-L-arginine, a non-selective NOS inhibitor, or with 7-nitroindazole, a selective nNOS inhibitor, potentiates the antinociceptive activity of submaximal doses of acetaminophen in Randall-Selitto and writhing tests [102]. In the periphery, however, the picture is not as clear. Several studies show inhibition of the antinociceptive effects of non-opioids by local administration of L-NAME in inflammatory pain models, including ketorolac, dipyrone [103], indomethacin [104], rofecoxib [105], nimesulide [106], meloxicam [107] and lumiracoxib [108]. These effects range from partial inhibition to complete reversal of the analgesic activity of the non-opioids used. However, in all these studies the dose of L-NAME used did not have any effect on the nociceptive threshold. The controversy might be due to type and intensity of the noxious stimuli, rat strain and the dose or concentration reached at the active site [108]. The contradictory roles NO plays in nociception [109,110] might contribute to this complexity.

In vascular endothelium, ASA elicits NO release by direct acetylation of the eNOS protein. This effect is independent of COX inhibition [111]. Further studies are needed to elucidate the

effect of different non-opioids on the NO/cGMP pathway in vessels, as this may be one of the mechanisms of cardiovascular adverse effects recently associated with the use of this group of drugs.

NON-OPIOIDS AND IL-6

IL-6 is a pleiotropic cytokine that modulates a variety of physiological functions including cell proliferation, differentiation, survival, inflammation and apoptosis. IL-6 gene transcripts are expressed in human atherosclerotic lesions [112] and circulating IL-6 levels may predict the risk of future cardiovascular events [113,114]. With the recent recognition of the cardiovascular adverse events of non-opioids, their relation to IL-6 becomes of special interest.

We recently reported an increase in gene and protein expression of IL-6 in response to both rofecoxib and ibuprofen in the oral mucosa, after tissue injury and 48 hours of acute inflammation in the oral surgery model [18]. As seen in Table (1), the effect of non-opioids vary remarkably in a variety of inflammatory models and at different time points; some non-opioids even induce variable effects on IL-6 production in the same experimental setting [115,116]. Not only the drug used, but the sample tested could contribute to these discrepancies. In a clinically relevant rat model of polymicrobial peritonitis and sepsis, induced by cecal ligation and puncture, the levels of IL-6 were significantly higher in ascetic fluid than in circulating blood both after 6 and 24 hours, a finding that may indicate more activity at the local site of inflammation and infection than systemically [116]. The same finding was reported in a clinical setting [117].

The effect of non-opioids on IL-6 production could be due to inhibition of PGs synthesis. The regulatory effect of PGE_2 on IL-6 has been reported in many studies e.g. [119,120,126,127]. Furthermore, exogenous PGE_2 reverses the stimulatory effect of indomethacin on IL-6 gene expression in human dental pulp cells [127] and in IL-1β-stimulated human gingival fibroblasts [120]. This could explain the variable effects of non-opioids in different inflammatory models, since IL-6 production is differentially modulated by PG receptor agonists: in IL-1β stimulated human gingival fibroblasts, a selective EP_2 agonist, butaprost, inhibited IL-6 production in a concentration dependent manner, while 17-phenyl-ω-trinor PGE_2, a selective EP_1 agonist, upregulated IL-6 production [120]. Different roles of PGE_2 receptor subtypes was also seen in human periodontal ligament cells [119], murine bone marrow denderitic cells [126] and in RAW 264.7 macrophages [128]. It is therefore suggested that PGE_2 induces variable regulatory effects on IL-6 production through different subtypes of EP receptors, the selectivity of which depends on expression of EP subtypes of PGE_2 receptors [119,120].

EP_2 and EP_4 are G-protein-coupled receptors that activate adenylyl cyclase upon ligand binding and result in increased cyclic adenosine monophosphate (cAMP) levels, while EP_1 receptor activation results in an increase in intracellular calcium levels [129]. In cloned osteoblast-like MC3T3-E1 cells, PGE_2 stimulates IL-6 synthesis through Ca^{2+} mobilization from the extracellular space *via* EP_1 receptors [130]. The effect of cAMP on IL-6 production varies between studies [119,120,131]. Many non-opioids also affect cAMP level, but different studies show varying results [132–134]. We have recently reported downregulation of gene and protein expression of phosphodiesterase type IV (PDE4D) enzyme by rofecoxib and ketorolac in oral mucosal biopsies, 3 hours after third molar tooth extraction [135]. Fig. (2) summarizes the possible sites of interactions between non-opioids and IL-6 regulatory pathways.

NF-κB, also plays an important role in the upregulation of IL-6 in response to several inflammatory mediators [136,137]. It is known that different non-opioids produce variable effects on the activation of NF-κB; for review see [100]. A binding site for transcription factor NF-κB is present in the 5′ promoter region of the IL-6 gene [138]. NF-κB inhibition lowers peptidoglycan- and PGE_2-induced IL-6 production in RAW 264.7 macrophages and IKKαβ-

dependent NF-κB activation occurs downstream of the signaling pathway of COX-2-generated PGE_2 and PKA activation stimulated by peptidoglycan [128]. In the same study, the selective COX-2 inhibitor, NS398, inhibited the peptidoglycan-induced NF-κB-specific DNA protein complex formation from 2–12 h of treatment, but not in the first 60 min, suggesting that NF-κB activation may be PGE_2/cAMP dependent [128]. Furthermore, activation of NF-κB blocks IL-6-induced late phase STAT3 activation in Mock-transfected HepG2 cells [139].

We also reported an increase in gene and protein expression of SOCS3 in response to both rofecoxib and ibuprofen in the oral mucosa, 48 hours after tissue injury and acute inflammation in the oral surgery model [18]. To our knowledge, the effect of other non-opioids on the expression of SOCS3 has not been reported. Over expression of SOCS3 blocks the proinflammatory effects of IL-6 signaling through gp130 [140,141]. Thus, even if non-opioids under certain conditions might upregulate IL-6 production, an accompanying over expression of SOCS3 might in fact block its proinflammatory effects.

NON-OPIOIDS AND MATRIX METALLOPROTEINASES

The matrix metalloproteinases (MMPs) are a family of enzymes that cleave the various components of the extracellular matrix. MMPs are activated by tissue plasminogen activator (tPA)/plasmin, and are inactivated by their endogenous protein inhibitors, tissue inhibitors of metalloproteinases (TIMPs). The dynamic interaction between MMPs and their endogenous inhibitors, the TIMPs, determine their overall activity (for review, see [142,143]). MMPs can be both pro-inflammatory and anti-inflammatory and the same MMP might have opposite roles in different conditions. They contribute to the vulnerability of atherosclerotic plaques [144], which on rupture could be a predisposing factor to acute coronary syndrome. This adds to the importance of their relation to non-opioids.

As seen in Table (2) non-opioids have different effects on MMPs and TIMPs in different inflammatory models. Both COX-1 and COX-2 seem to be involved in MMP-9 induction [155], though COX-2 seems to have a key role in the signaling pathway leading to increased proteinase expression [155].

Possible Factors Contributing to Non-Opioids Effects on MMPs

Inhibition of PGs Synthesis—Accumulating data have revealed that PGs are involved in the regulation of MMP pathways in various cell types [156,157]. The use of selective EP receptors subtypes agonists and antagonists, however, do not show a consistent pattern for the role of each sub-type [158–160]. How much inhibition of PGE_2 production contributes to the final effect of non-opioid analgesics on MMP production is far from clear, particularly considering the differential effects of non-opioid analgesics on MMPs. The variable response to different non-opioid analgesics on MMP-1 and MMP-3 production in bovine chondrocytes cultured in alginate gel beads [161] argues against the possibility of a common mode of action. The use of the pure enantiomers of flurbiprofen and ketoprofen can help answer this question, since the S-enantiomer inhibits PGE_2 synthesis, while the R-enantiomer is devoid of this property. Panico *et al*. [96] showed in human chondrocytes that S-flurbiprofen and S-ketoprofen inhibits IL-1β induced MMP-3 production to a greater extent than R-flurbiprofen and R-ketoprofen. However, R-flurbiprofen and R-ketoprofen significantly inhibited IL-1β induced MMP-3 production, suggesting that inhibition of PGE_2 production, though participating in this process, is not the sole player. The ability of exogenous PGE_2 to reverse the effect of COX-inhibitors on MMPs shows variable results [146,162–165]. Thus it is likely that the effects of non-opioid analgesics on MMPs are both PG dependent and independent. It is suggested that the PGE_2 requirement in MMP synthesis may vary with different cell types as well as duration of exposure [163].

Transcriptional Regulation of MMPs—The molecular mechanisms of MMP regulation have been extensively studied (for review see [166,167]). Cytokines are key regulators of MMP expression, and the concentrations and combinations of cytokines may determine the extent of matrix degradation [168]. Different cytokines may lead to the activation of at least one of the MAPK pathways (ERK1/2, JNK, and p38 MAPK), which may result in upregulation of secondary mediators such as IL-6 or PGE_2 that contribute to the upregulation of MMP-1 and MMP-3 expression [169]. Translocation of activated MAPKs to the nucleus results in phosphorylation of the components of activator protein 1 (AP-1) [170]. The interplay between different transcription factors contributes to the control of MMP expression. The NF-κB pathway also contributes to the regulation of MMP-1, -3, -9 and -11 expression [167,171].

The interaction of non-opioids with the MMP regulatory pathway is expected at different levels (Fig. 3). (1) Non-opioids are known to differentially affect cytokine expression, including TNF-α, IL-1 and IL-6, all of which are major regulators of MMP expression [18,172–175]. (2) They regulate different MAPKs as well as NF-κB [100]. (3) Non-opioids inhibit AP-1 activation by different stimuli [100]. The inhibition of AP-1 activation together with inhibition of NF-κB by ASA and sodium salicylate results in reduction of MMP-9 levels [176].

MMP Activators and Inhibitors—As mentioned earlier MMPs are activated by tPA/plasmin, and are inactivated by TIMPs. Therefore, affecting any of these activators or inhibitors would alter the activity of MMPs (Fig. 4). In bovine articular chondrocytes, ASA, diclofenac, indomethacin, meloxicam, naproxen, and tiaprofenic acid dose dependently inhibited the gne expression of tPA. However, only indomethacin and tiaprofenic acid reduced the expression of uPA [161]. The effect of non-opioids on plasminogen activators was reported in other studies including [149,177,178]. TIMPs, on the other hand, have been extensively studied; a few examples of the effect of non-opioids on TIMPs are shown in Table (2).

IL-8 and Monocyte Chemoattractant Protein-1 (MCP-1)—IL-8 is another target for non-opioids that may affect the overall activity of MMPs. IL-8 downregulates TIMP-1 expression in cholesterol-loaded human macrophages [179], and induces the gene expression of MMP-2 and MMP-9 in cultured neurons [180] and in tumor cells [181]. Again, IL-8 is differentially regulated by non-opioids [19,182].

MCP-1 also causes an increase in MMP-1 in cytokine stimulated monocytes [183], and MMP-9 secretion by primary isolated rat brain microglia *in vitro* [184] and non-opioids differentially modulate the expression of MCP-1 [18,185,186].

Nitric Oxide—The modulatory role of NO on MMPs and TIMP expression and/or activity has been shown in rat aortic smooth muscle cells [187], rat primary astrocytes [188] and murine macrophages [189]. Since non-opioids modulate NO synthesis (as discussed earlier), this may represent another mechanism by which non-opioids regulate MMP production and activity.

Mechanical Regulation of MMPs—MMPs are regulated by changes in mechanical forces applied to tissues (for review see [190]). NSAIDs are known to increase blood pressure [191], and acetaminophen was reported to have the same effect [192,193]. Thus, non-opioids might upregulate vascular production of MMPs by elevating blood pressure.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Inhibition of eicosanoids synthesis represents an important aspect of non-opioid action, yet accumulating data points to several other targets (reviewed here) that contribute either to their analgesic effects, anti-inflammatory actions or to their adverse effects (Fig. 5). The interaction between non-opioids and these targets can be prostanoid-dependent or independent, and in

many cases these mechanisms are interactive. The studies cited in this review demonstrate the wide variability in response to non-opioids in a variety of cells and tissues under different experimental conditions. These observations suggest that except for a common action as COX inhibitors, these drugs have diverse pharmacological actions making it problematic to consider them as a single group. While these discrepant observations prevent generalization about which mechanisms predominate in the action of non-opioids, these recently appreciated alternatives to a unitary COX-inhibition hypothesis may form the basis for the development of new analgesics and anti-inflammatory medications with more favorable safety profiles.

Acknowledgments

This work was supported by Division of Intramural Research, NINR/NIH.

ABBREVIATIONS

2-AG
2-Arachidonyl glycerol

5-HT
5-Hydroxy treptamine (serotonin)

AA
Arachidonic acid

AEA
Arachidonoylethanolamide (anandamide)

AP-1
Activator protein 1

ASA
Acetylsalicylic acid (aspirin)

cAMP
Cyclic adenosine monophosphate

CB_1
Cannabinoid CB_1 receptor

cGMP
Cyclic guanosine monophosphate

COX
Cyclooxygenase enzyme

EP
Prostaglandin E receptor

ERK1/2
Extracellular signal-regulated kinases 1 and 2

FAAH
Fatty acid amidohydrolase enzyme

GHR
Growth hormone receptor

IL

Interleukin

IL-6R
Interleukin-6 receptor

JNK
Jun NH_2-terminal kinase

MAPK
Mitogen-activated protein kinase

MCP-1
Monocyte chemoattractant protein-1

MMPs
Matrix metalloproteinases

NF-κB
Nuclear factor kappa B

NO
Nitric oxide

NOS
Nitric oxide synthase

NSAIDs
Non-steroidal anti-inflammatory drugs

PG
Prostaglandin

PI3K-Akt
Phosphatidylinositol 3-kinase/Akt

PKA
Protein kinase A

SOCS3
Suppressor of cytokine signaling 3

sst3R
Somatostatin 3 receptor

STAT
Signal transducers and activators of transcription

TIMPs
Tissue inhibitors of metalloproteinases

TNFα
Tumor necrosis factor α

t-PA
Tissue plasminogen activator

u-PA
Urokinase

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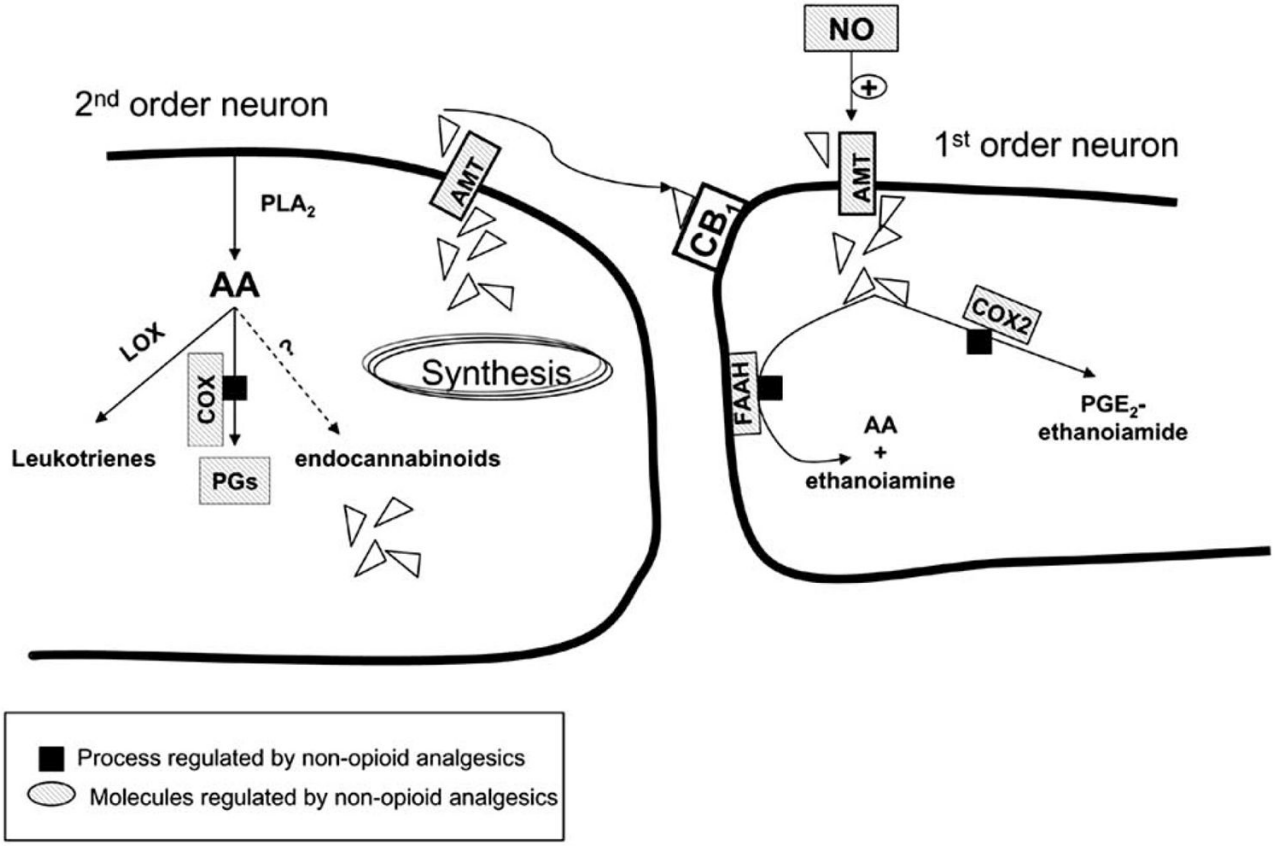

Fig 1.

A hypothetical diagram of possible sites of interaction between non-opioids and the endocannabinoid system. Endocannabinoids including anandamide are proposed to be released from the post-synaptic neurons upon depolarization, diffuse or actively transported *via* AMT (anandamide membrane transporter) to the presynaptic neuron and activate cannabinoid CB_1-receptors. AMT, which is activated by nitric oxide (NO), is also responsible for the cellular uptake of AEA where it is hydrolyzed by either FAAH (fatty acid amidohydrolase) into arachidonic acid (AA) and ethanolamine, or by cyclooxygenase 2 (COX2) into PGE_2 ethanolamide. Non opioids are known to inhibit COX2 and some of them can inhibit FAAH. Non-opioids also modulate NO synthesis and AMT might be inhibited by acetaminophen. Finally, inhibition of COX might result in a shift of AA metabolism towards the synthesis of endocannabinoids.

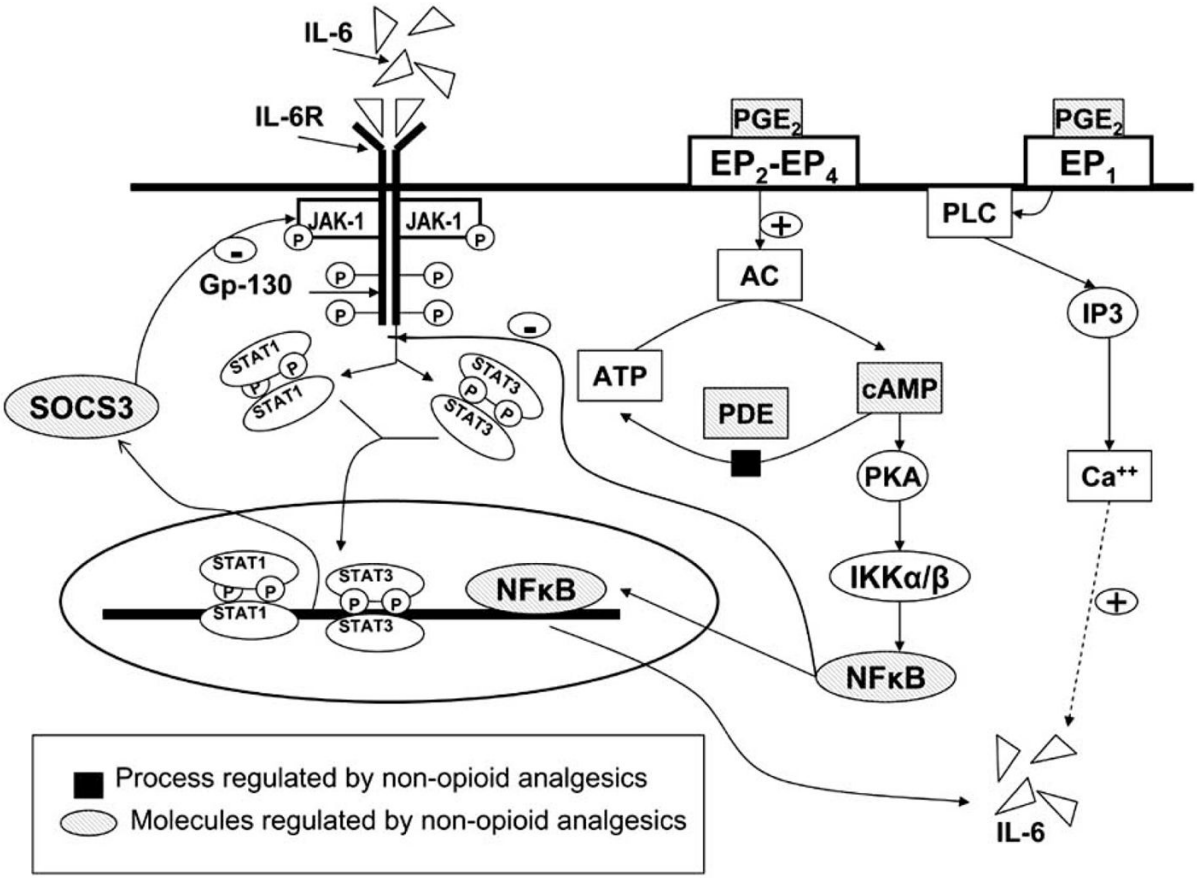

Fig 2.

A hypothetical diagram of possible sites of action of non-opioids in the regulatory pathway of IL-6. IL-6 binds to IL-6 receptor forming a hexadimer with intracellular gp-130 molecule. This activates (phosphorylates) JAK-1 (Janus kinase 1), which leads to the phosphorylation of gp-130 and subsequently the activation of STAT1 and STAT 3 (signal transducers and activators of transcription). The activation of this signaling cascade results in the induction of SOCS3 formation that ultimately inhibits the signaling transduction of IL-6. PGE_2 *via* EP_2 and EP_4 receptors activates adenylyl cyclase leading to the formation of cAMP that activates (protein kinase A) PKA and subsequently NFkB (nuclear factor kappa B). NFκB activation results in further IL-6 expression and it also interferes with activation of STAT. Non-opioids interfere with synthesis of PGE_2, the degradation of c-AMP and also regulates SOCS 3and NFκB. It should be noted that non-opioids have different effects on these targets in different cells and under different experimental conditions.

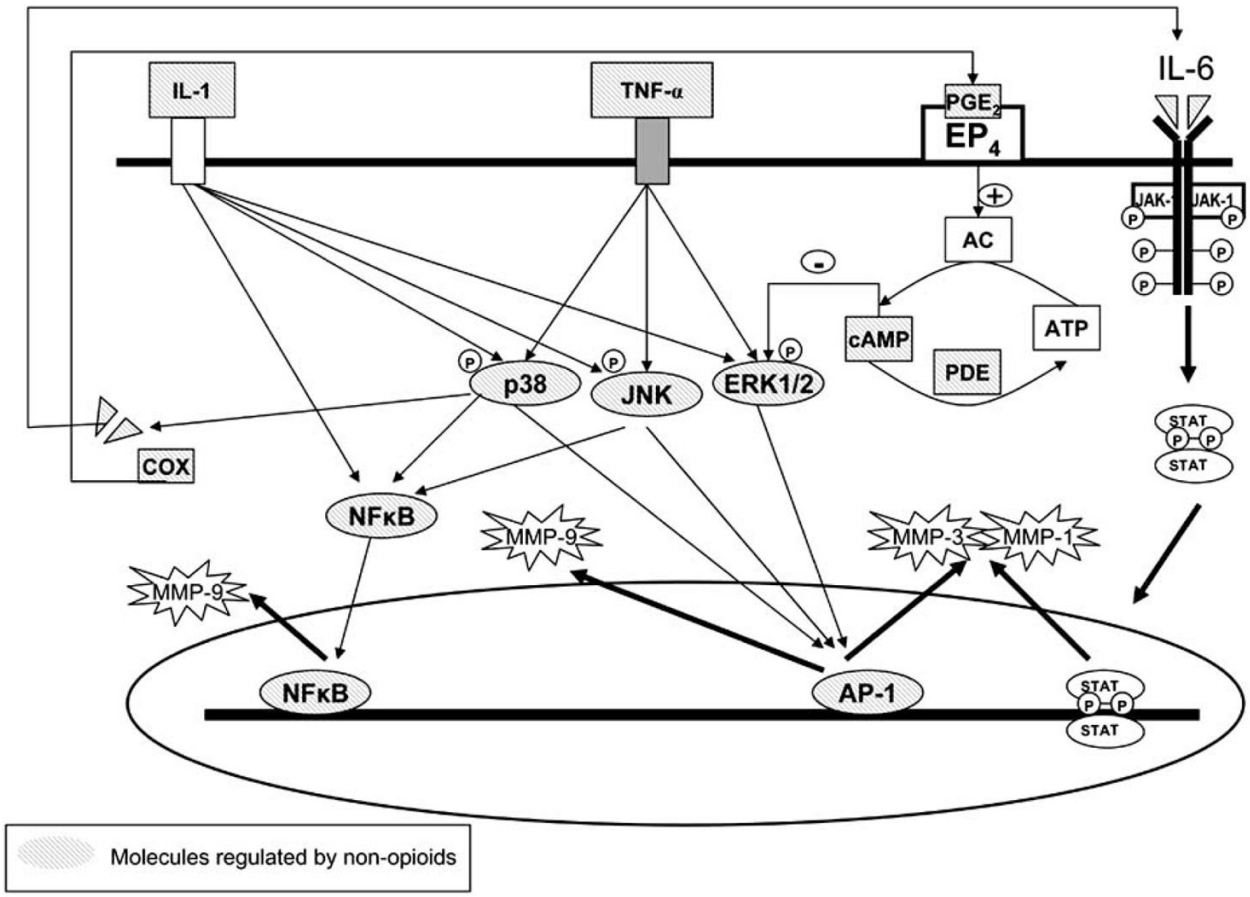

Fig 3.

A hypothetical diagram of possible sites of action of non-opioids in the regulatory pathway of MMPs. TNF-α and IL-1 activates the MAPKs ERK1/2, p38, and JNK, which results in phosphorylation of the components of AP-1, and the upregulation of MMPs. Activation of MAPKs also may result in upregulation of secondary mediators such as, IL-6 or PGE_2. IL-6 *via* the JAK/STAT pathway may upregulate MMPs. PGE_2 *via* EP_4 receptors activates adenylyl cyclase leading to the formation of cAMP that interfere with the activation of ERK. NFκB is also involved in the regulation of MMPs expression. Non-opioids interfere with synthesis of PGE_2, the degradation of c-AMP and also regulate various cytokines. They regulate AP-1, NFκB and MAPKs. It should be noted that non-opioids have different effects on these targets in different cells and under different experimental conditions

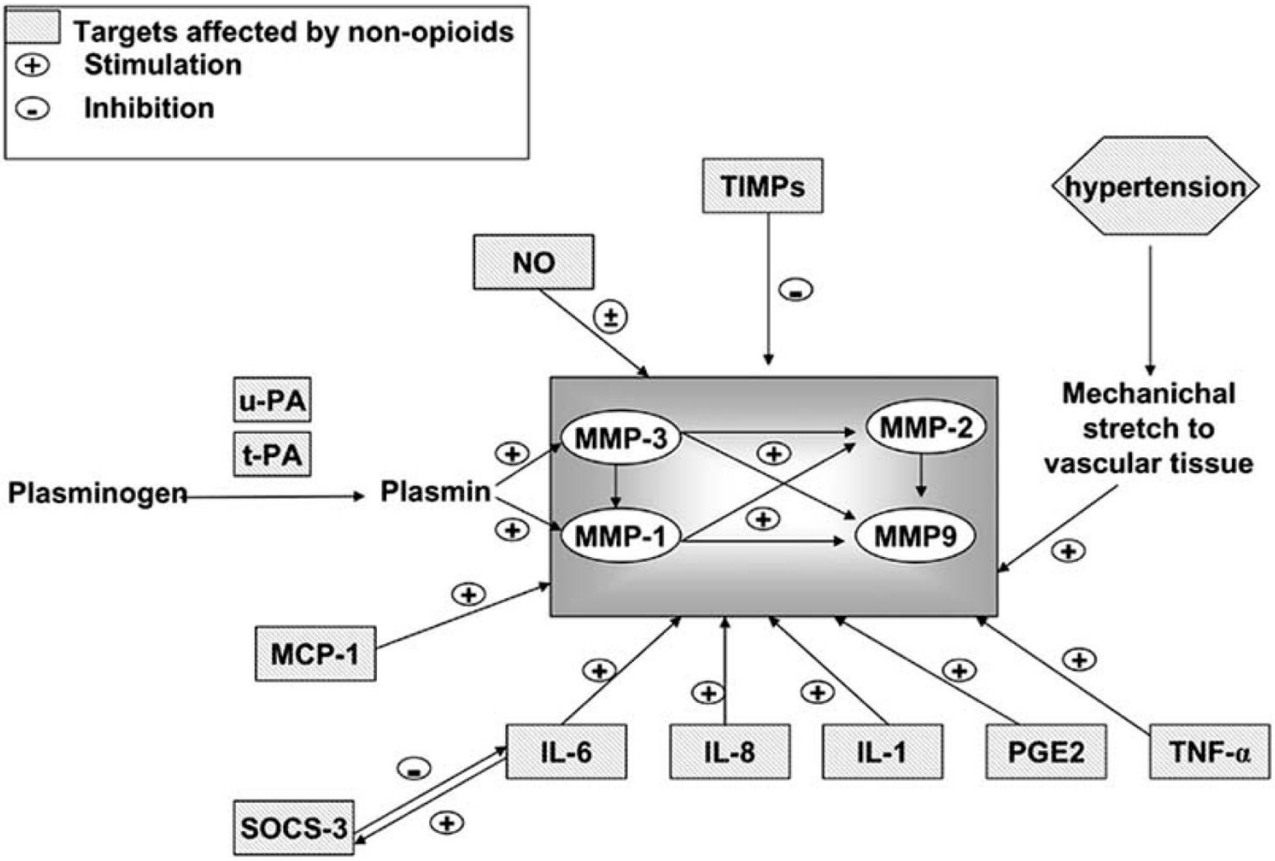

Fig 4.

A hypothetical diagram of possible regulatory mechanisms of non-opioids on MMPs. It should be noted that non-opioids have different effects on these targets in different cells and under different experimental conditions. Abbreviations: NO, nitric oxide; IL-6, Interleukin-6; MCP-1, Monocyte Chemoat-tractant Protein-1; MMPs, matrix metalloproteinases; SOCS3, suppressor of cytokine signaling 3; IL-8, Interleukin-8; IL-1, Interleukin-1; PGE_2, prostaglandin E_2; TIMPs, tissue inhibitors of metalloproteinases; t-PA, tissue plasminogen activator; u-PA, urokinase; TNFα, tumor necrosis factor α.

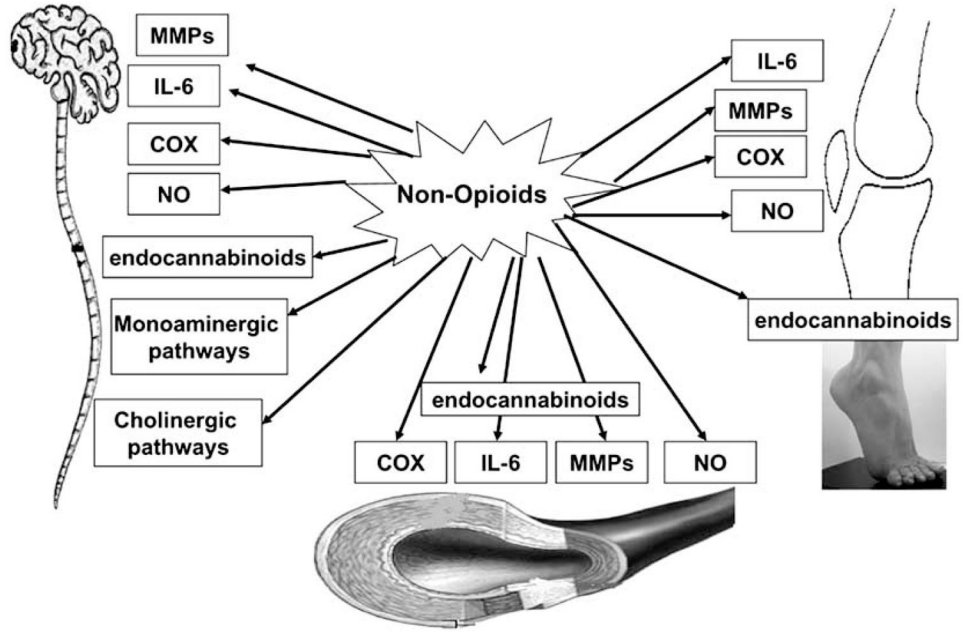

Fig 5.

Possible targets that contribute to the analgesic effects, anti-inflammatory action or adverse effects of non-opioids in the central nervous system, the peripheral sites of inflammation or the blood vessels.

Table 1

Effect of Different Non-Opioids on IL-6 Expression

Drug	Duration	Effect	Biomarker	Model	Ref.
Rofecoxib (50 mg)	48 hours	Elevated	Gene & protein expression in oral mucosal biopsies	Acute inflammation following oral surgery	[18]
Ibuprofen (1600 mg)					
Ibuprofen (600 &1200 mg)	48 hours	Elevated	Protein level in serum	Athletes competing in a 160-km race	[118]
Indomethacin (1 μM)	25 hours	Elevated	Protein level in the supernatant	Stimulated human gingival fibroblast and human periodontal ligament cells	[119,120]
Rofecoxib & piroxicam	12 days	No change	Protein level in the supernatant and cellular matrix	IL-1β stimulated human chondrocytes cultured in alginate beads	[115]
Indomethacin (75 mg/d)	5 days	Lowered	Protein level in serum	2-h of experimental exercise	[121]
Rofecoxib (25 mg)	1 week	Lowered	Protein level in serum	Patients with acute coronary event, taking aspirin	[122]
	1 month, but not at 3 months				[123]
	6 months				[124]
Indomethacin (1 μM)	18 hours	Lowered	Gene expression level	Oncostatin and IL-1α stimulated human lung or synovial fibroblasts	[125]
Aceclofenac, sodium diclofenac, indomethacin, nimesulide, celecoxib and ibuprofen	12 days	Lowered	Protein levels in the supernatant, and cellular matrix	IL-1β stimulated human chondrocytes cultured in alginate beads	[115]

Table 2

Effect of Different Non Opioids on MMPs 1,2,3,9 and TIMPs 1,3

Non-opioids	Time Point	Effect	Clinical Condition or Inflammatory Model	Biomarker	Ref.
MMP-1					
Rofecoxib, ibuprofen	48 hrs	↑	Acute inflammation following oral surgery	Gene expression	[19]
Celecoxib	48 hrs	NC	IL-1 stimulated chondrocytes or unstimulated chondrocytes from arthritic patients	Protein in supernatant	[145]
Indomethacin (20 μM)	48 hrs	↓	LPS-stimulated or LPS- plus H_2O_2-stimulated human primary monocytes	Protein expression in supernatant	[146]
MMP-2					
ASA (500 mg)	1 hr	NC	Activated platelets in vivo in humans	Activity and protein expression in plasma from blood of a skin wound	[147]
ASA (0.1 mM)	1–4 days	↑	Human umbilical vein endothelial cells	Protein expression in culture media and activity	[148]
Diclofenac sodium, nimesulide, celecoxib, valdecoxib, rofecoxib and etoricoxib	4 days	↓	Articular cartilage, meniscus and synovium of osteoarthritis patients	Activity	[149]
MMP3					
Rofecoxib, ibuprofen	48 hrs	↑	Acute inflammation following oral surgery	Gene expression	[19]
Nimesulide (200 mg)	28 days	↓	Patients with osteoarthritis	Protein expression in serum	[150]
Ibuprofen (1200 mg)		↑			
Celecoxib	48 hs	NC	IL-1 stimulated chondrocytes or unstimulated chondrocytes from arthritic patients	Protein in supernatant	[145]
MMP-9					
Indomethacin (10 mg/kg)	24 hrs	↓	TNF-α-induced neuroinflammation in rat	Protein expression and activity	[151]
ASA (0.1 mM)	1–4 days	↑	Human umbilical vein endothelial cells	Protein expression in culture media and activity	[148]
Indomethacin (1 μM)	24 hrs	NC	Human cultured monocytes	Protein expression	[152]
TIMP1					
Nimesulide (200 mg), ibuprofen (1200 mg)	28 days	NC	Patients with osteoarthritis	Protein expression in serum	[150]
Indomethacin (10 μM)	24 hours	↓	Cytokine stimulated pulp fibroblast	Gene expression	[153]
Indomethacin & diclofenac (0.1–10 μM)	72 hours	↑	Synovial fibroblasts from rheumatoid arthritis patients	Protein expression	[154]
TIMP3					
Rofecoxib (50 mg)	48 hrs	↓	Acute inflammation following oral surgery	Gene expression	[19]