Title
NS-398: cyclooxygenase-2 independent inhibition of leukocyte priming for lipid body formation and enhanced leukotriene generation

Journal
Prostaglandins Leukotrienes and Essential Fatty Acids, 67(4)

ISSN
0952-3278

Authors
Bozza, PT
Pacheco, P
Yu, W
et al.

Publication Date
2002-10-01

DOI
10.1054/plef.2002.0425

Peer reviewed

Prostaglandins, Leukotrienes and Essential Fatty Acids (2002) **67**(4), 237–244

doi:10.1054/plef.425, available online at http://www.idealibrary.com on IDEAL®

NS-398: cyclooxygenase-2 independent inhibition of leukocyte priming for lipid body formation and enhanced leukotriene generation

P. T. Bozza,[1,2] P. Pacheco,[1] W. Yu,[1] P. F. Weller[1]

[1]Harvard Thorndike Laboratory and Charles A. Dana Research Institute, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA
[2]Immunopharmacology Laboratory, Department of Physiology and Pharmacodynamics, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil

Summary Because the induction of new lipid body formation in leukocytes correlates with and likely contributes to their enhanced 'primed' prostaglandin and leukotriene formation, we evaluated two selective cyclooxygenase (COX)-2 inhibitors. Three types of stimuli, *cis*-unsaturated fatty acids, platelet activating factor and protein kinase C activators, stimulate lipid body formation. NS-398 (0.1–10 μM), but not another COX-2 inhibitor, SC58125 (0.1– 10 μM), blocked leukocyte lipid body formation elicited by all three types of stimuli and also blocked priming for enhanced LTB_4 production and PGE_2 production. The effect of NS-398 on lipid body formation was independent of its inhibitory effects on COX-2 since arachidonate-induced lipid body formation in COX-2-deficient mouse leukocytes was also inhibited by NS-398. By means of its ability to inhibit leukocyte lipid body formation, NS-398 may exert actions independent of its COX-2 inhibition and more broadly contribute to the suppression of formation of COX-1 and lipoxygenase-derived eicosanoids.

Leukocytes associated with inflammatory reactions are known to exhibit, as yet poorly understood, 'priming' responses, by which these leukocytes respond more prominently to agonists and may, for instance, generate greater quantities of eicosanoid mediators of inflammation. One mechanism that can contribute to the heightened capacity of leukocytes to form both lipoxygenase (LO) and cyclooxygenase (COX) pathway-derived eicosanoids involves the regulated synthesis of eicosanoids at intracellular organelles, termed lipid bodies. If their defining lipid content is preserved during staining, lipid bodies have long been recognized to be characteristically increased in numbers within leukocytes in vivo at sites of experimental inflammatory exudates[1] and at sites of human inflammatory reactions, such as in synovial fluid leukocytes from patients with inflammatory arthritis.[2–5] Roles for lipid bodies in the formation of eicosanoid mediators are supported by the localization of enzymes, such as cytosolic phospholipase A_2, COX, 5-LO and leukotriene (LT) C_4 synthase, pertinent to eicosanoid formation to lipid bodies in leukocytes and other cells.[6–11] More directly, lipid bodies have been shown by immunofluorescent localization to be the intracellular sites of formation a newly synthesized eicosanoid, LTC_4, in eotaxin- and RANTES-stimulated eosinophils.[12]

Lipid body formation in leukocytes can be rapidly induced by several agonist-elicited signaling pathways. In human eosinophils, both platelet activating factor (PAF)[9,13] and eotaxin and RANTES[12] act via their specific G-protein-linked receptors to activate intracellular signal transducing pathways that lead to new lipid body formation. In human neutrophils, PAF likewise elicits lipid body formation[14] as do *cis*-unsaturated fatty acids (e.g., arachidonic and oleic acids)[5,15] and agents that activate protein kinase C (e.g., phorbol esters and oleoyl-acetyl-glycerol).[15] The induction of new lipid body

Received 31 January 2002
Accepted 11 June 2002

Correspondence to: Peter F. Weller, M.D., Beth Israel Deaconess Medical Center, 330 Brookline Ave. DA-617, Boston, MA 02215, USA.
Tel.: +1-617-667-3307; Fax: +1-617-277-6061;
E-mail: pweller@caregroup.harvard.edu

This work was supported by National Institutes of Health grant AI 22571 (to PF Weller) and a grant from FAPERJ and CNPq-Brazil (to PT Bozza).

formation with each of these agents in neutrophils and eosinophils correlates quantitatively with their enhanced 'priming' capacity to form prostaglandins (PGE_2) and 5-LO derived leukotrienes (LTB_4 and LTC_4, respectively).[5,9,12,14] Because the induction of lipid body formation contributes to enhanced formation of eicosanoid mediators, inhibition of lipid body formation represents an attractive target for anti-inflammatory therapeutics.

The anti-inflammatory activities of non-steroidal anti-inflammatory drugs (NSAIDs) are principally attributed to their ability to inhibit prostaglandin production by inhibiting COX, the rate-limiting initial enzyme for prostaglandin synthesis.[16] There are two isoforms of COX, COX-1, a constitutive isoform believed to be involved in physiologic production of prostanoids, and COX-2, a cytokine- and mitogen-inducible form thought to be involved in the heightened prostanoid production during inflammatory and other pathological conditions.[17] However, some anti-inflammatory effects of NSAIDs may not be explained by inhibition of COX. There is evidence suggesting that aspirin-like drugs exert their anti-inflammatory effects both by COX inhibition-dependent and -independent ways.[18,19] For instance, aspirin-like drugs inhibit neutrophil activation including aggregation, degranulation and leukotriene synthesis by as yet ill-defined mechanisms other than COX inhibition.[20–22] We have demonstrated that some conventional NSAIDs (aspirin, sodium salicylate, piroxicam and indomethacin) in low micromolar concentrations, acting independently of COX-1 or COX-2 inhibition, potently inhibited both *cis*-fatty acid-induced lipid body formation and the concomitant priming for enhanced LTB_4 and LTC_4 generation in human neutrophils and eosinophils, respectively.[5] In contrast, these NSAIDs did not inhibit PAF-induced lipid body formation or priming for enhanced LTB_4 formation in neutrophils.[14]

Because inhibition of leukocyte lipid body formation and priming for enhanced eicosanoid synthesis represent a candidate anti-inflammatory target, we have evaluated whether two COX-2 inhibitors might inhibit these responses in leukocytes. NS-398[23,24] and SC5812[25,26] are structurally distinct prototypical COX-2 selective inhibitors that have been evaluated for their COX inhibitory actions in vitro and their anti-inflammatory effects in vivo in animal models.[27] NS-398 is a methanesulfonanilide type of inhibitor, whereas SC58125 is a tricyclic-based inhibitor and is closely related to SC58635 (celecoxib), recently introduced for clinical use.[28] Our findings demonstrate that NS-398, unlike SC58125, effectively inhibits lipid body formation elicited by a range of stimulating agents and concomitantly inhibits priming for increased prostaglandin and LT production in leukocytes.

METHODS

Materials

Aspirin, acetaminophen, oleic acid (OA), 1-oleoyl-2-acetyl-glycerol (OAG), phorbol myristate acetate (PMA), arachidonic acid (AA) (Sigma Chemical Co., St. Louis, MO, USA), PAF and A23187 (Calbiochem, La Jolla, CA, USA), ^{3}H-arachidonic acid (specific activity 200 Ci/mmol, New England Nuclear, Boston MA, USA), U937 human monocytic leukemia cells (American Type Culture Collection, Rockville, MD, USA) and NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl) methane sulfonamide) (BIOMOL, Plymouth Meeting, PA) were obtained as indicated. SC58125 was kindly donated by Dr. Peter Isakson, GD Searle, St. Louis, MO, USA.

Human neutrophil purification

Neutrophils were purified from fresh human blood obtained by venipuncture from healthy adult volunteers and collected into acidified citrate. After addition of 6% dextran 70 (McGaw, Irvine, CA, USA), RBCs were allowed to sediment for 1 h at room temperature. The leukocyte-rich supernatant was overlaid onto an equal volume of Ficoll-Paque gradient (Pharmacia, Piscataway, NJ, USA), and centrifuged at 400*g* for 20 min. PMNs (>95% pure, rest being eosinophils) were recovered from the pellet and washed in Ca^{2+}/Mg^{2+}-free HBSS. Residual RBCs were lysed with hypotonic saline.

Investigations with mouse macrophages

COX-2 knockout mice were generated as described.[29] All experiments were carried out with male 8–12-week-old mice. Peritoneal macrophages from COX-2 genetically deficient mice and wild-type litter mates were obtained by lavaging the peritoneal cavity with 3 ml of cold HBSS containing heparin (20 IU/ml).

Lipid body induction and treatments

Neutrophils or macrophages (10^6 cells/ml) were incubated with 5 μM AA, 10 μM OA, 1 μM PAF, 20 nM PMA or vehicle (ethanol or DMSO at 0.1%) at 37°C in a 5% CO_2 atmosphere, and after 1 h leukocytes (10^5 cells per slide) were cytocentrifuged (550 rpm, 5 min) onto glass slides. Stock solutions for A23187, NS-398, acetaminophen, SC58125 and aspirin were prepared in DMSO. Aliquots were diluted in Ca^{2+}/Mg^{2+}-free HBSS to the indicated concentration immediately prior to use. The final DMSO concentration was always lower than 0.1% and had no effect on lipid body numbers. During inhibitor studies, leukocytes were pretreated for 1 h with varying concentrations of inhibitors. Cell viability, determined by trypan

blue dye exclusion at the end of each experiment, was always greater than 90%. Moreover, with NS-398 evidence of neutrophil apoptosis was sought by evaluating nuclear fragmentation and by flow cytometry using the annexin V-FITC apoptosis detection kit (R&D System). Neutrophils (10^6) were treated with 0.1% DMSO vehicle or concentrations of NS-398 for 1 h. Thereafter, cells were washed in cold PBS, resuspended in 100 μl of a solution containing 0.25 μg/ml annexin V-FITC and 5 μg/ml propidium iodide, incubated in the dark for 15 min and analyzed by flow cytometry. Annexin V binding cells were not increased by NS-398 (vehicle, 5.5%; 0.1 μM NS-398, 5.9%; 1 μM NS-398, 5.8%; 10 μM NS-398, 8.5%) and NS-398-treated cells displyed to alternations in nuclear integrity.

Lipid body staining and enumeration

Slides, while still moist, were fixed in 3.7% formaldehyde in Ca^{2+}/Mg^{2+}-free HBSS, pH 7.4, rinsed in 0.1 M cacodylate buffer pH 7.4, stained in 1.5% OsO_4 (30 min), rinsed in dH_2O, immersed in 1.0% thiocarbohydrazide (5 min), rinsed in 0.1 M cacodylate buffer, restained in 1.5% OsO_4 (3 min), rinsed in dH_2O, and then dried and mounted.[14] The morphology of fixed cells was observed, and lipid bodies were enumerated with phase contrast microscopy and a 100 × objective lens in 50 consecutively scanned leukocytes.[14]

LTB_4 measurement

Neutrophils (10^6 cells/ml) were pretreated for 1 h with 10 μM NS-398, 10 μM SC58125 or DMSO vehicle and then were stimulated with 5 μM AA, 1 μM PAF, 20 nM PMA or vehicle at 37°C for 1 h to induce lipid body formation. After incubations, samples were taken for lipid body enumeration and the cells were washed in Ca^{2+}/Mg^{2+}-free HBSS. Leukocytes were resuspended in 1 ml of HBSS containing Ca^{2+}/Mg^{2+} and then stimulated with 0.5 μM A23187 for 15 min. Reactions were stopped on ice, and samples were centrifuged at 1500 rpm for 10 min at 4°C. Concentrations of LTB_4 in each supernatant were assayed in duplicate by ELISA according to the manufacturer's instructions (Cayman, Ann Arbor, MI). Five replicate experiments were performed each with neutrophils from different donors.

Arachidonic acid uptake

U937 cells (10^6/ml) were washed and resuspended in Ca^{2+}/Mg^{2+} HBSS. Cells were pretreated with either vehicle, NS-398 (10 μM), acetaminophen (66 μM) or aspirin (55 μM) for 1 h. After the incubation period, cells were incubated with 250 pM (0.5 μCi) ^{3}H-AA for 15, 30, 60 and 240 min. Cells were washed 3 times in Ca^{2+}/Mg^{2+}-free HBSS and analyses of incorporated ^{3}H-AA in cell pellets were performed by liquid scintillation counting.

Statistical analysis

Results were expressed as mean ± SEM and were analyzed statistically by means of analysis of variance followed by the Newman–Keuls–Student test with the level of significance set at $p<0.05$.

RESULTS

Effects of NS-398, SC58215 and other NSAIDs on *cis*-unsaturated fatty acid-induced lipid body formation

cis-Unsaturated, but not saturated, fatty acids, rapidly stimulate de novo formation of lipid bodies in leukocytes; and aspirin, sodium salicylate and some conventional NSAIDs (piroxicam and indomethacin) inhibit *cis*-fatty acid-elicited lipid body formation.[5,15,30] With two *cis*-unsaturated fatty acids, AA and OA, as stimuli, acetaminophen (6.6–660 μM) failed to inhibit lipid body formation in human neutrophils (not shown). In contrast, one of two COX-2 inhibitors inhibited AA- and OA-induced lipid body formation in neutrophils. One hour pretreatments with NS-398, but not SC58215, dose-dependently inhibited lipid body formation induced by both AA and OA in neutrophils (Fig. 1) with IC_{50}s of ~1 μM.

Mechanisms of NS-398 inhibition of lipid body formation

The mechanism whereby NS-398 inhibited *cis*-fatty acid-induced lipid body formation was evaluated including assessing whether NS-398 inhibition was limited to lipid body formation elicited by *cis*-fatty acids. A trivial possibility was that NS-398 acted by blocking *cis*-fatty acid uptake by cells. As recently demonstrated with a non-esterifiable analog of AA, *cis*-fatty acid signaling for de novo lipid body formation is independent of the capacity of the eliciting fatty acids to become incorporated with glycerolipids.[31] The effect of NS-398 on lipid body formation was not attributable to inhibition of fatty acid uptake, since neither NS-398, aspirin nor acetaminophen inhibited ^{3}H-AA uptake at any time point from 15 to 240 min (data not shown). Moreover, cell viability was not diminished by NS-398. Conventional NSAIDs, including aspirin, inhibit leukocyte lipid body formation elicted by *cis*-fatty acids, but not by PAF.[5,14] We, therefore, evaluated whether NS-398 and SC58215 inhibited leukocyte lipid body formation elicited by PAF and by protein kinase C activators. NS-398, unlike SC58215 and

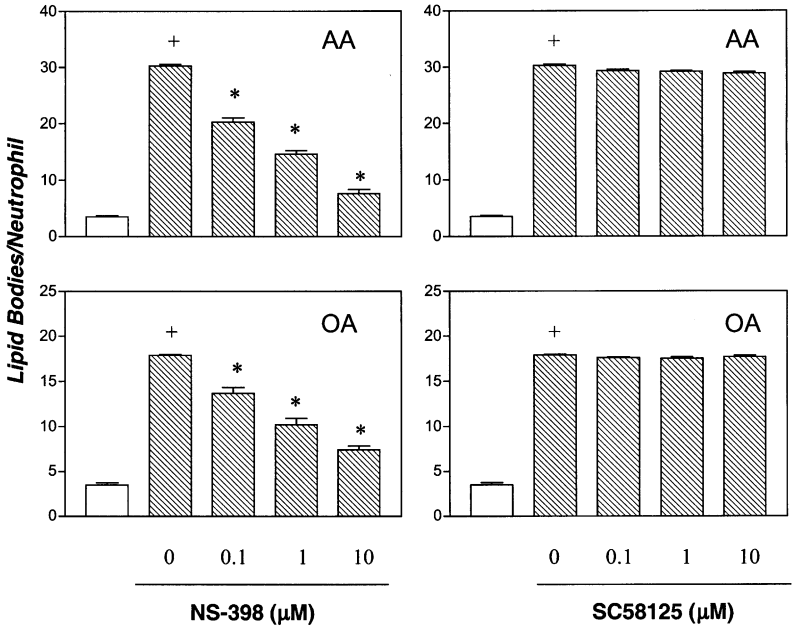

Fig. 1 Effects of COX-2 inhibitors, NS-398 and SC58125, on neutrophil lipid body formation induced by *cis*-unsaturated fatty acids. Human neutrophils were pretreated with vehicle or concentrations of NS-398 and SC58125 for 1 h before being stimulated with 5 μM AA or 10 μM OA for 1 h. Lipid bodies were enumerated microscopically following osmium staining in 50 consecutively counted cells. Values represent the mean ± SEM lipid bodies/neutrophil from 3 to 5 independent experiments each with different neutrophil donors. Plus(+) indicates statistically significant increases in lipid body numbers elicited by the fatty acids and asterisk(*) indicates statistically significant inhibition of lipid body formation by pretreatment with the COX-2 inhibitor.

aspirin, inhibited lipid body formation stimulated by PAF and by PMA (Fig. 2) and by OAG (not shown). Thus, NS-398's inhibitory actions were not restricted to *cis*-fatty acids as stimuli of leukocyte lipid body formation.

To investigate if the inhibitory effect of NS-398 on *cis*-fatty acid-induced lipid body formation was dependent upon COX-2 inhibition, peritoneal macrophages from wild-type and COX-2 knockout mice were studied. As with human cells, wild-type mouse macrophages contained only a few lipid bodies under normal conditions and could be stimulated in vitro with AA to form lipid bodies (Fig. 3). AA and OA (not shown) induced lipid body formation in macrophages from COX-2 deficient animals and no differences in lipid body induction by *cis*-fatty acids were noted between wild-type and COX-2 knockout cells (Fig. 3). Furthermore, NS-398, but not SC58215, significantly inhibited AA-induced lipid body formation in macrophages from both wild-type and COX-2 knockout mice (Fig. 3).

Effect of NS-398 on *cis*-fatty acid-induced leukocyte priming for LTB_4 and PGE_2 production

The stimulation of new lipid body formation correlates with, and likely contributes to, enhanced PGE_2 and LTB_4 production by human neutrophils[5,14] and PGE_2 and LTC_4 production by eosinophils.[9,12] Since NS-398 was effective in blocking lipid body formation induced by *cis*-fatty acids, we tested whether NS-398 would inhibit LO pathway-derived eicosanoid production. Neutrophils were pretreated with either vehicle, NS-398, SC58215 or aspirin for 1 h and then stimulated with 5 μM AA to induce lipid bodies. After the incubation time, lipid bodies were enumerated and replicate leukocytes were stimulated with a sub-maximal dose of calcium ionophore (A23187, 0.5 μM). Pretreatment of leukocytes with NS-398, but not SC58215 or aspirin, inhibited both AA-induced lipid body formation and AA-induced 'priming' for enhanced LTB_4 production by neutrophils (Fig. 4, bottom). A similar inhibitory effect of NS-398 was also observed for OA-induced 'priming' for LTB_4 production (not shown). NS-398 was not acting to block pathways of arachidonate release or metabolism that are activated by calcium ionophore, since NS-398 did not inhibit calcium ionophore-induced LTB_4 production in cells not prestimulated with *cis*-fatty acids (Fig. 4, top). With PAF and PMA as other stimuli for lipid body formation and eicosanoid priming, NS-398, and not SC58215 or aspirin, likewise inhibited the priming for enhanced LTB_4 production by neutrophils (Fig. 2). NS-398, and not SC58125, also inhibited the AA-induced priming for enhanced PGE_2 production by neutrophils (Table 1).

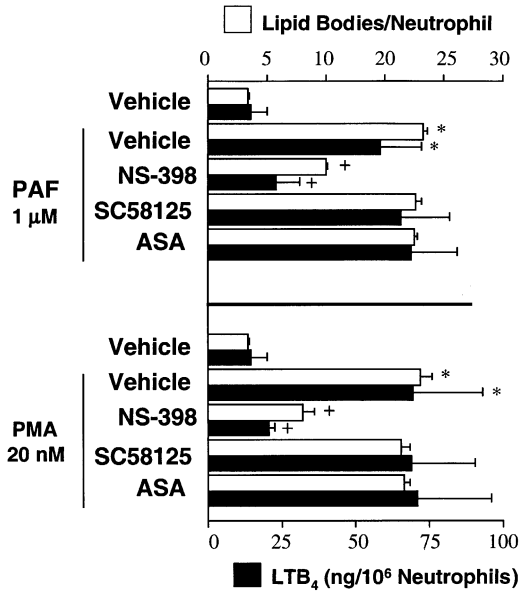

Fig. 2 Effects of NS-398 and SC58125 on neutrophil lipid body numbers and LTB_4 production by neutrophils stimulated with 1 μM PAF, 20 nM PMA or vehicle. Neutrophils were pretreated for 1 h with NS-398 (10 μM), SC58125 (10 μM) or DMSO vehicle and then were stimulated with PAF, PMA or vehicle at 37°C for 1 h to induce lipid body formation. Lipid body numbers were enumerated microscopically following osmium staining in 50 cells, and the cells were stimulated with A23187 (0.5 μM) for 15 min. Concentrations of LTB_4 in the supernatants, assayed by ELISA, are the means of duplicate assays. Values represent the mean ± SEM from 3 to 5 independent experiments each using different neutrophil donors. asterisk(*) indicates statistically significant increases in LTB_4 formation elicited by PAF and PMA and Plus(+) indicates statistically significant inhibition of LTB_4 formation by pretreatment with COX inhibitors.

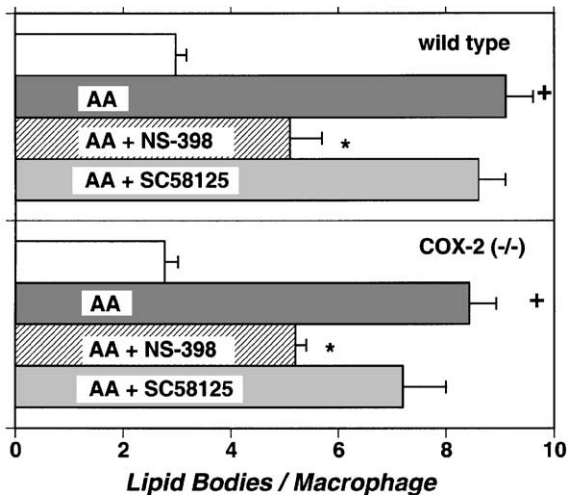

Fig. 3 Effects of NS-398 and SC58125 on lipid body numbers in peritoneal macrophages from COX-2 genetically deficient mice and wild-type litter mates. Macrophages were treated for 1 h with 10 μM NS-398,10 μM SC58125 or vehicle prior to being incubated for 1 h with 5 μM AA to form lipid bodies. Lipid bodies were enumerated microscopically following osmium staining. Values represent the mean ± SEM lipid bodies from 50 consecutively counted cells from four mice each counted blindly without knowledge of the genotypes of the mice. Plus(+) indicates statistically significant increases in lipid body numbers elicited by the fatty acids and asterisk(*) indicates statistically significant inhibition of lipid body formation by pretreatment with COX-2 inhibitors.

DISCUSSION

The induction of lipid body formation in leukocytes, including neutrophils and eosinophils, correlates with an enhanced 'priming' response to form both COX and LO pathway-derived eicosanoids.[5,9,12,14,32] Lipid body formation in leukocytes is elicitable by *cis*-unsaturated fatty acids,[15] that activate as yet undefined signaling mechanisms that are independent of the capacity of these fatty acids to be incorporated in glycerolipids.[31] Aspirin and certain other NSAIDs (e.g., indomethacin, sodium salicylate, piroxicam) not only block the *cis*-fatty acid-elicited lipid body formation, but also block the priming response for increased prostanoid and leukotriene formation.[5] In contrast, these conventional NSAIDs do not block lipid body formation elicited by PMA and OAG, which are acting at least in part by stimulating protein kinase C,[15] or by PAF, which is acting in a receptor-mediated signaling cascade that is obligately dependent on activation of 5-LO,[14] nor do these specific NSAIDS block the priming response of PAF for increased LTB_4 formation.[14]

We evaluated the capacity of two agents, known to have selective inhibitory effects on COX-2, NS-398[23,24] and SC58125,[25,26] to inhibit leukocyte lipid body formation and priming for enhanced eicosanoid generation. NS-398 inhibited lipid body formation induced by two *cis*-unsaturated fatty acids, whereas SC58125 did not (Fig. 1). NS-398 differed from aspirin and some other NSAIDs[14] in that NS-398 also inhibited lipid body induction elicited by both PAF and PMA (Fig. 2). Thus, NS-398 had a broader range of activity in blocking lipid body formation induced not only by *cis*-fatty acids, but also by PMA and PAF. This suggests that NS-398 was acting more distally in the signaling pathways that lead to lipid body formation at site(s) common to the intracellular cascades activated by *cis*-fatty acids, PAF and PMA.

The inhibitory effect of NS-398 on lipid body induction elicited by *cis*-fatty acids was independent of the action of NS-398 as an inhibitor of COX-2. NS-398 inhibited *cis*-fatty acid-induced lipid body formation even in macrophages from COX-2 genetically deficient mice (Fig. 3). Further, NS-398 inhibition of lipid body formation and eicosanoid priming was not found with SC58125, an established COX-2 inhibitor. Moreover, fresh resting human neutrophils contain little or no COX-2 protein.[33,34] Confirmation of the lack of COX-2 (and the presence of COX-1) activity in freshly isolated neutrophils in our studies was found in the inability of SC58125 to block AA-induced priming for increased PGE_2 production (Table 1). NS-398, like other NSAIDs, can have COX-

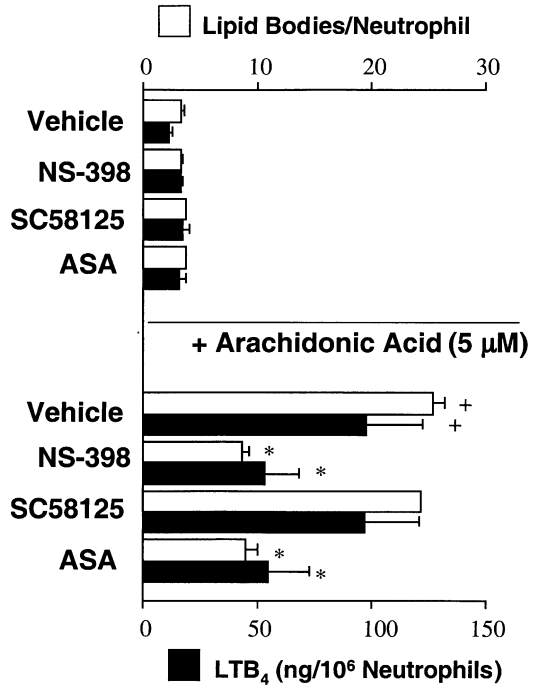

Fig. 4 Effects of NS-398 and SC58125 on neutrophil lipid body numbers and LTB_4 production in neutrophils stimulated with and without 5 μM AA. Neutrophils were pretreated for 1 h with NS-398 (10 μM), SC58125 (10 μM) or DMSO vehicle and then were stimulated with AA or vehicle at 37°C for 1 h to induce lipid body formation. Lipid body numbers were enumerated microscopically in 50 cells, and the cells were stimulated with A23187 (0.5 μM) for 15 min. Concentrations of LTB_4 in the supernatants were assayed in duplicate by ELISA. Values represent the mean ± SEM from 3 to 5 independent experiments each with different neutrophil donors. Plus(+) indicates statistically significant increases in LTB_4 formation elicited by AA and asterisk(*) indicates statistically significant inhibition of LTB_4 formation by pretreatment with COX inhibitors.

Table 1 Effects of NS-398 and SC58125 on AA-induced priming for PGE_2 production

Stimulus	Treatment	PGE_2 (ng/10^6)
Vehicle	Vehicle	0.4 ± 0.1
AA	Vehicle	4.5 ± 1.8[a]
AA	NS-398	0.4 ± 0.1[b]
AA	SC58125	4.1 ± 0.1

Neutrophils (10^6/ml) were pretreated with NS-398 (10 μM), SC58125 (10 μM) or vehicle for 1 h, then stimulated with AA (5 μM) for 1 h, and then activated with A23187 (0.5 μM) for 15 min. Concentration of PGE_2 in the supernatants were assayed in duplicate by ELISA. Values represent the mean ± SEM from 3 to 5 independent experiments each using different neutrophil donors.
[a]Denotes significantly increased PGE_2 production with AA priming.
[b]Denotes significant inhibition of PGE_2 production with NS-398.

dependent and -independent effects. In contrast to the low concentrations of NS-398 that inhibited lipid body formation (IC_{50}'s ~ 1 μM), some of the proapoptotic effects of NS-398 require very much higher concentrations over several days, e.g., IC_{50}'s of 64–244 μM for growth inhibition of colon adenoma and carcinoma cell lines.[35] Moreover, the apoptotic effects of NSAIDs, not yet clearly attributable to their COX inhibitory actions, are found with many NSAIDs.[36] NS-398 (0.1–10 μM) elicited no signs of apoptosis or cytotoxicty in neutrophils studied over 1 h. Our findings that NS-398, unlike other NSAIDs, effectively blocks lipid body formation and eicosanoid priming elicited by several different neutrophil agonists is distinct.

Interest in agents that inhibit lipid body formation is engendered by the roles that lipid bodies may play in the formation of eicosanoid mediators of inflammation. Lipid bodies are sites at which many of the enzymes involved in arachidonate mobilization and metabolism are localized.[6–12] In addition, as noted above, induction of lipid body formation correlates quantitatively with the capacity of leukocytes to elaborate greater quantities of eicosanoids; and conversely inhibition of lipid body formation correlates with suppression of the capacity for enhanced eicosanoid synthesis. NS-398 not only inhibited lipid body formation, but also suppressed the *cis*-fatty acid-, PAF- and PMA-elicited priming responses for increased production of LTB_4 by neutrophils (Fig. 4). NS-398 also inhibited AA-induced priming for enhanced PGE_2 production by neutrophil COX-1 (Table 1). These findings provide additional evidence that inhibition of lipid body formation is correlated with inhibition of the 'primed' or enhanced synthesis of eicosanoids. The capacity of NS-398 to inhibit lipid body formation correlated with its suppression of formation of LTB_4, a 5-LO-derived eicosanoid, and of PGE_2, a COX-1-derived eicosanoid. NS-398 may exert potential anti-inflammatory activities that are independent of its actions as a COX-2 specific inhibitor and may more broadly contribute to the suppression of formation of both COX-1- and LO-derived eicosanoids.

ACKNOWLEDGEMENTS

The authors thank Scott Morham, Ph.D. (University of North Carolina, Chapel Hill, NC) for COX-2 genetically deficient mice, Peter C. Isakson, Ph.D., GD Searle, St. Louis MO for the gift of SC58215, and Jessica Cassara and Jennifer P. Gray for excellent technical assistance.

REFERENCES

1. Robinson J. M., Karnovsky M. L., Karnovsky M. J. Glycogen accumulation in polymorphonuclear leukocytes, and other intracellular alterations that occur during inflammation. *J Cell Biol* 1982; **95**: 933–942.

2. Coimbra A., Lopes-Vaz A. The presence of lipid droplets and the absence of stable sudanophilia in osmium-fixed human leukocytes. *J Histochem Cytochem* 1971; **19**: 551–557.
3. Schlesinger P. A., Stillman M. T., Peterson L. Polyarthritis with birefringent lipid within synovial fluid macrophages: case report and ultrastructural study. *Arthritis Rheum* 1982; **25**: 1365–1368.
4. Weinstein J. Synovial fluid leukocytosis associated with intracellular lipid inclusions. *Arch Intern Med* 1980; **140**: 560–561.
5. Bozza P. T., Payne J. L., Morham S. G., Langenbach R., Smithies O., Weller P. F. Leukocyte lipid body formation and eicosanoid generation: cyclooxygenase-independent inhibition by aspirin. *Proc Natl Acad Sci USA* 1996; **93**: 11 091–11 096.
6. Dvorak A. M., Morgan E., Schleimer R. P., Ryeom S. W., Lichtenstein L. M., Weller P. F. Ultrastructural immunogold localization of prostaglandin endoperoxide synthase (cyclooxygenase) to nonmembrane-bound cytoplasmic lipid bodies in human lung mast cells, alveolar macrophages, type II pneumocytes and neutrophils. *J Histochem Cytochem* 1992; **40**: 759–769.
7. Dvorak A. M., Schleimer R. P., Dvorak A. M., Lichtenstein L. M., Weller P. F. Human lung mast cell and alveolar macrophage cytoplasmic lipid bodies contain arachidonic acid and prostaglandin endoperoxide synthase (cyclooxygenase), the substrate and enzyme necessary for prostaglandin production. *Int Arch Allergy Immunol* 1992; **99**: 208–217.
8. Dvorak A. M., Morgan E., Tzizik D. M., Weller P. F. Prostaglandin endoperoxide synthase (cyclooxygenase): ultrastructural localization to nonmembrane-bound cytoplasmic lipid bodies in human eosinophils and murine 3T3 fibroblasts. *Int Arch Allergy Immunol* 1994; **105**: 245–250.
9. Bozza P. T., Yu W., Penrose J. F., Morgan E. S., Dvorak A. M., Weller P. F. Eosinophil lipid bodies: specific, inducible intracellular sites for enhanced eicosanoid formation. *J Exp Med* 1997; **186**: 909–920.
10. Bozza P. T., Yu W., Cassara J., Weller P. F. Pathways for eosinophil lipid body induction: differing signal transduction in cells from normal and hypereosinophilic subjects. *J Leukocyte Biol* 1998; **64**: 563–569.
11. Yu W., Bozza P. T., Tzizik D. M., Gray J. P., Cassara J., Dvorak A. M., Weller P. F. Co-compartmentalization of MAP kinases and cytosolic PLA_2 at cytoplasmic lipid bodies of U937 cells. *Am J Pathol* 1998; **152**: 759–769.
12. Bandeira-Melo C., Phoofolo M., Weller P. F. Extranuclear lipid bodies, elicited by CCR3-mediated signaling pathways, are the sites of chemokine-enhanced leukotriene C_4 production in eosinophils and basophils. *J Biol Chem* 2001; **276**: 22 779–22 787.
13. Bartemes K. R., McKinney S., Gleich G. J., Kita H. Endogenous platelet-activating factor is critically involved in effector functions of eosinophils stimulated with IL-5 or IgG. *J Immunol* 1999; **162**: 2982–2989.
14. Bozza P. T., Payne J. L., Goulet J. L., Weller P. F. Mechanisms of PAF-induced lipid body formation: a central role for 5-lipoxygenase in the compartmentalization of leukocyte lipids. *J Exp Med* 1996; **183**: 1515–1525.
15. Weller P. F., Ryeom S. W., Picard S. T., Ackerman S. J., Dvorak A. M. Cytoplasmic lipid bodies of neutrophils: formation induced by *cis*-unsaturated fatty acids and mediated by protein kinase C. *J Cell Biol* 1991; **113**: 137–146.
16. Vane J. Aspirin and other anti-inflammatory drugs. *Thorax* 2000; **55** (Suppl 2):S3–S9.
17. Smith W. L., Langenbach R. Why there are two cyclooxygenase isozymes. *J Clin Invest* 2001; **107**: 1491–1495.
18. Serhan C. N., Clish C. B. Aspirin triggered lipid mediators: novel inhibitors of leucocyte trafficking. *Thorax* 2000; **55** (Suppl 2): S10–12.
19. Yin M. J., Yamamoto Y., Gaynor R. B. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* 1998; **396**: 77–80.
20. Abramson S., Korchak H., Ludewig R., Edelson H., Haines K., Levin R. I., Herman R., Rider L., Kimmel S., Weissmann G. Modes of action of aspirin-like drugs. *Proc Natl Acad Sci USA* 1985; **82**: 7227–7231.
21. Abramson S. Therapy with and mechanisms of nonsteroidal anti-inflammatory drugs. *Curr Opin Rheumatol* 1991; **3**: 336–340.
22. Villanueva M., Heckenberger R., Strobach H., Palmer M., Schror K. Equipotent inhibition by R(−)-, S(+)- and racemic ibuprofen of human polymorphonuclear cell function *in vitro*. *Br J Clin Pharmacol* 1993; **35**: 235–242.
23. Futaki N., Takahashi S., Yokoyama M., Arai I., Higuchi S., Otomo S. NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (Cox-2) activity *in vitro*. *Prostaglandins* 1994; **47**: 55–59.
24. Futaki N., Yoshikawa K., Hamasaka Y., Arai I., Higuchi S., Iizuka H., Otomo S. NS-398, a novel non-steroidal anti-inflammatory drug with potent analgesic and antipyretic effects, which causes minimal stomach lesions. *Gen Pharmacol* 1993; **24**: 105–110.
25. Masferrer J. L., Zweifel B. S., Manning P. T., Hauser S. D., Leahy K. M., Smith W. G., Isakson P. C., Seibert K. Selective inhibition of inducible cyclooxygenase 2 *in vivo* is antiinflammatory and nonulcerogenic. *Proc Natl Acad Sci USA* 1994; **91**: 3228–3232.
26. Seibert K., Zhang Y., Leahy K., Hauser S., Masferrer J., Perkins W., Lee L., Isakson P. Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc Natl Acad Sci USA* 1994; **91**: 12 013–12 017.
27. DeWitt D. L. Cox-2-selective inhibitors: the new super aspirins. *Mol Pharmacol* 1999; **55**: 625–631.
28. Penning, T. D., Talley J. J., Bertenshaw S. R., Carter J. S., Collins P. W., Docter S., Graneto M. J., Lee L. F., Malecha J. W., Miyashiro J. M., Rogers R. S., Rogier D. J., Yu S. S., Anderson G. D., Burton E. G., Cogburn J. N., Gregory S. A., Koboldt C. M., Perkins W. E., Seibert K., Veenhuizen A. W., Zhang Y. Y., Isakson P. C. Synthesis and biological evaluation of the 1,5-diarylpyrazole class of cyclooxygenase-2 inhibitors: identification of 4-[5-(4-methylphenyl)-3- (trifluoromethyl)-1H-pyrazol-1-yl]benze nesulfonamide (SC-58635, celecoxib). *J Med Chem* 1997; **40**: 1347–1365.
29. Morham S. G., Langenbach R., Loftin C. D., Tiano H. F., Vouloumanos N., Jennette J. C., Mahler J. F., Kluckman K. D., Ledford A., Lee C. A., Smithies O. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* 1995; **83**: 473–482.
30. Weller P. F., Ackerman S. J., Nicholson-Weller A., Dvorak A. M. Cytoplasmic lipid bodies of human neutrophilic leukocytes. *Am J Pathol* 1989; **135**: 947–959.
31. Bozza P. T., Weller P. F. Arachidonyl trifluoromethyl ketone induces lipid body formation in leukocytes. Prostaglandins, Leukot Essent Fatty Acids 2001; **64**: 227–230.
32. Bandeira-Melo C., Herbst A., Weller P. F. Eotaxins. Contributing to the diversity of eosinophil recruitment and activation. *Am J Respir Cell Mol Biol* 2001; **24**: 653–657.
33. Maloney C. G., Kutchera W. A., Albertine K. H., McIntyre T. M., Prescott S. M., Zimmerman G. A. Inflammatory agonists induce cyclooxygenase type 2 expression by human neutrophils. *J Immunol* 1998; **160**: 1402–1410.

34. Pouliot M., Gilbert C., Borgeat P., Poubelle P. E., Bourgoin S., Creminon C., Maclouf J., McColl S. R., Naccache P. H. Expression and activity of prostaglandin endoperoxide synthase-2 in agonist-activated human neutrophils. *FASEB J* 1998; **12**: 1109–1123.
35. Elder D. J., Halton D. E., Crew T. E., Paraskeva C. Apoptosis induction and cyclooxygenase-2 regulation in human colorectal adenoma and carcinoma cell lines by the cyclooxygenase-2-selective non-steroidal anti-inflammatory drug NS-398. *Int J Cancer* 2000; **86**: 553–560.
36. Marx J. Cancer research. Anti-inflammatories inhibit cancer growth—but how? *Science* 2001; **291**: 581–582.

