Structure-Function Relationship and Role of Tumor Necrosis Factor-α-converting Enzyme in the Down-regulation of L-selectin by Non-steroidal Anti-inflammatory Drugs*

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It has been recently described that some non-steroidal anti-inflammatory drugs (NSAIDs) are able to induce the shedding of L-selectin in neutrophils, an adhesion molecule that plays an essential role in the inflammatory response. We have found that, according to this capability, NSAIDs could be grouped into three categories. A high releaser group (flufenamic, meclofenamic, and mafenamic acids, diclofenac and aceclofenac), a group of moderate releasers (aspirin, indomethacin, nimesulide, flurbiprofen, and ketoprofen), and a non-releaser group (phenylbutazone and the oxicsams, piroxicam and meloxicam). Only NSAIDs from the high releaser group shared diphenylamine in their chemical structure. The amine group of this chemical agent proved to be essential for the anti-L-selectin activity of diphenylamine-based NSAIDs. The presence of a carboxylic acid group in the diphenylamine (N-phenylanthranilic acid) highly increased its ability to reduce the L-selectin surface expression in neutrophils. Diphenylamine and N-phenylanthranilic acid neither affected COX activity in platelets nor modified the activation state of neutrophils. Diphenylamine-related compounds, which include the diphenylamine-based NSAIDs caused a variable reduction in the neutrophil intracellular ATP concentration, which correlated with the differential ability of such compounds to trigger L-selectin shedding (r = 0.97, p < 0.01). Diphenylamine-related compounds failed to down-regulate L-selectin in a tumor necrosis factor-α-converting enzyme (TACE)-deficient murine monocytic cell line. Our data indicate that diphenylamine seems to be the structural core of NSAIDs accounting for their down-regulatory activity of L-selectin leukocyte expression. Diphenylamine and its related compounds exert this action on L-selectin through a prostaglandin-independent, TACE-dependent mechanism that seems to be linked to the capability of these agents to uncouple the mitochondrial oxidative phosphorylation.

The extravasation of leukocytes is an essential event for a correct inflammatory response. Transmigration of flowing neutrophils through vascular endothelium and its accumulation into inflamed tissues requires that highly coordinated adhesive events take place between neutrophils and endothelial cells, a process commonly known as adhesion cascade. Members of three major families of adhesion receptors have been implicated in this cascade: selectins, integrins, and the immunoglobulin superfamily (1–3). The existence of congenital human diseases, in which a dysfunction in either integrin (4) or selectin (5) adhesion pathways (leukocyte adhesion deficiency types I and II, respectively) causes an abnormal inflammatory response, indicates that each one of the adhesive events of the adhesion cascade are interdependent. Many efforts in medicine are currently aimed to the development of antagonists of adhesion receptors, a therapeutic approach known as anti-adhesive therapy. The assumption of this theory is that, if any of sequential steps of the adhesion cascade is inhibited, the inflammatory response is suppressed or, at least, ameliorated because the cascade cannot be completed (6, 7). The anti-adhesive therapy that specifically blocks members of the selectin family has already proved beneficial in several animal models of inflammation (8–11).

Non-steroidal anti-inflammatory drugs (NSAIDs)1 are a heterogeneous group of chemical compounds clustered in different chemical families that show differences in both clinical response and pharmacokinetic profile (12–14). Used in clinics throughout this century, NSAIDs continue to be an important therapeutic intervention for patients with disorders that cause pain, fever, or moderate inflammation. The inhibition of the prostaglandin synthesis through the blockade of cyclooxygenase (COX) has been widely accepted as the mechanism of action of these compounds (15). However, during the last decade, many groups have described a number of non-prostaglandin-mediated anti-inflammatory effects of NSAIDs, suggesting that COX inhibition does not represent the only explanation for the anti-inflammatory action of this group of therapeutic agents (16–20). In this regard, it has been proposed that several NSAIDs are able to interfere with the function of adhesion molecules that participate in the adhesion cascade (21). In neutrophils, some of these agents induce the down-regulation

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1 The abbreviations used are: NSAID, non-steroidal anti-inflammatory drug; COX, cyclooxygenase; TNF, tumor necrosis factor; TACE, TNF-α-converting enzyme; mAb, monoclonal antibody; 6-MNA, 6-methoxy-2-naphthyl acetic acid; PMA, phorbol 12-myristate 13-acetate; IC-3, Immunex compound-3; PBS, phosphate-buffered saline; MFI, mean fluorescence intensity; rMFI, relative MFI; TxB2, thromboxane B2.
of L-selectin (22, 23), an adherence molecule constitutively expressed by most leukocytes that plays a key role in the inflammatory response mediating the initial rolling of flowing leukocytes over the endothelial cells (2). Remarkably, this effect of NSAIDs on L-selectin expression is not a common characteristic of this group of agents, because a number of them do not modify the basal expression of this adherence molecule (21, 22). These data strongly suggest that a chemical structure present in only a limited group of NSAIDs is responsible for the L-selectin shedding activity that these compounds exert in neutrophils.

Upon cell activation, L-selectin is enzymatically cleaved by the action of a surface metalloprotease that belongs to the ADAM (a disintegrin and metalloprotease domain) family, the TNF-α-converting enzyme (TACE, ADAM-17 or CD154b) (24). Although the effect of NSAIDs on L-selectin seems to be linked to the uncoupling capability of these agents (25), the molecular mechanisms, including the role of metalloproteases, involved in the down-regulation of L-selectin by NSAIDs remains to be clarified.

This work aims to: 1) identify the chemical structure responsible for the shedding of L-selectin induced by NSAIDs and 2) characterize the molecular mechanisms involved in this effect of NSAIDs in leukocytes. We report here that diphenylamine is the structural core responsible for such effect of NSAIDs. This chemical is able by itself to down-regulate in vitro the L-selectin expression in neutrophils without interfering with either cell activation or COX activity, and this effect on L-selectin expression seems to be linked to the capability of diphenylamine and related compounds to uncouple the oxidative phosphorylation. Finally, TACE is involved in the shedding of L-selectin induced by NSAIDs.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The following monoclonal antibodies (mAbs) were used: Bear-1 anti-CD11b, TPII/40 anti-CD11a, TPI/15 anti-CD31, D3/9 anti-CD45, Mo-2 anti-CD14, and P3X63 myeloma culture supernatant as a negative control (26, 27). The Leu-8 anti-L-selectin mAb was purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA).

Aceclofenac was provided by Almirall-Prodesfarma (Barcelona, Spain), nimesulide by Roche Molecular Biochemicals (Germany), and meloxicam by Roche Molecular Biochemicals (Ingelheim, Germany). The tabunemone-active compound 6-methoxy-2-naphthyl acetic acid (6-MeO-2-NAA) was provided by SKF (Medizin, Spain). Aspirin, diacerein, flufenamic acid, flurbiprofen, indomethacin, ketoprofen, mefenamic acid, meclofenamic acid, phenylbutazone, piroxicam, diphenylamine, or diphenylacetic acid, or diphenylamine for 5–90 min at 37°C. When dose-response experiments were performed, neutrophils were incubated with the different compounds at doses ranging from 3 to 50 μM for 15 min at 37°C. PMA was used at 20 ng/ml in neutrophils and at 100 ng/ml in experiments with DRM cells. In experiments with inhibitor, cells were pre-incubated with 50 μM of aceclofenac, flufenamic acid, N-phenylanthranilic acid, diphenylacetic acid, or diphenylamine for 5–90 min at 37°C. When dose-response experiments were performed, neutrophils were incubated with the different compounds at doses ranging from 3 to 50 μM for 15 min at 37°C. N-phenylanthranilic acid, diphenylacetic acid, diphenylamine, or meclofenamic acid at 50 μM were added, and neutrophils were incubated for an additional 15 min at 37°C. All compounds were solubilized in Me2SO (Panreac, Barcelona, Spain) and added to the cell solution in a final concentration below 0.2% of Me2SO.

**Flow Cytometry Analysis**—Cells were treated as described above and then incubated with the different mAbs at 4°C for 30 min. After washing in PBS, cells were labeled with fluorescein isothiocyanate-labeled goat anti-mouse Ig (Dako, Saltsrup, Denmark). At least 5 × 10^3 cells of each sample were analyzed in a FACScan flow cytometer (Becton Dickinson), and data were collected in both linear and logarithmic scales. In experiments with mononuclear cells, the L-selectin expression was analyzed in cells with side and forward scatter characteristics of either lymphocytes or monocytes separately. In these two populations, CD14-positive cells were below 5% and up to 90% in regions with the lowest fluorescence. In these populations, CD14-positive cells were below 5% and up to 90% in regions with the lowest fluorescence. In these populations, CD14-positive cells were below 5% and up to 90% in regions with the lowest fluorescence.

**Platelet Thromboxane B2 Release Induced by Arachidonic Acid**—Thromboxane B2, the stable end product of thromboxane A2 generation, was determined in gel-filtered platelets in the presence or absence of the different compounds. Briefly, platelet isolation was carried out at room temperature from normal human venous blood mixed with buffered sodium citrate (0.13% wt/vol) in the proportion 10:1. Platelet-rich plasma was obtained by centrifugation of the blood at 1000 rpm for 10 min and applied to a Sepharose 2B column equilibrated with saline. Gel-filtered platelets were incubated with the different NSAIDs (50 μM, except for aspirin 2 mM), N-phenylanthranilic acid or diphenylacetic acids (0.1 mM) for 15 min at 37°C and then activated with 1 μM arachidonic acid for 5 min at 37°C. The reaction was quenched by addition of ice-cold ethanol and the samples were centrifuged at 14,000 rpm for 5 min at room temperature. The ethanol of the supernatant was evaporated using a lyophilizer, and the pellet was resuspended in the immunossay buffer. Thromboxane B2 concentration ([TxB2]) in supernatants was measured with a quantitative TxB2 immunoassay according to the manufacturer’s protocol (R&D Systems Europe, Abingdon, UK). Data were normalized to express inhibition of TxB2 production as a percentage.

**ATP Quantification Assays**—Intracellular ATP concentration in neutrophils was measured with an ATP Bioluminescence Assay kit obtained from Roche Molecular Biochemicals (Mannheim, Germany). Neutrophils (7 × 10^6 cells/ml) were incubated in PBS alone and in the presence of the different compounds at doses indicated. After 15 min at 37°C, cells were pelleted and resuspended in the lysis buffer provided with the kit. ATP was assayed following the manufacturer’s instruc-
the intracellular ATP concentration ([ATP]) as follows,

\[
\% \text{ variation in [ATP]} = \left( \frac{[\text{ATP}_{\text{compound}}]}{[\text{ATP}_{\text{medium}}]} \right) \times 100
\]  

(Eq. 3)

Statistical Analysis—Results were expressed as arithmetic mean ± S.D. Student’s t test for paired samples was used to determine significant differences between means.

RESULTS

NSAIDs Can Be Classified in Three Categories According to Their Ability to Down-regulate L-selectin Expression in Human Neutrophils—L-selectin is constitutively expressed on essentially all leukocytes, and it is cleaved and released from cell surface in response to a variety of stimuli (31). We have previously described that several NSAIDs, although not all, are able to induce the shedding of L-selectin in neutrophils (22, 23, 25). We decided to expand these studies by testing, in addition to previously described agents, non-assayed NSAIDs such as nimesulide, nabumetone, flurbiprofen, and meclofenamic acid in an attempt to determine how common this effect on L-selectin expression was among the different families of NSAIDs.

NSAIDs derived from both arylcarboxylic acids (the fenamates: flufenamic, meclofenamic, and mefenamic acids) and arylalkanoic acids (aceclofenac and diclofenac) induced the strongest reduction in the basal expression of L-selectin in neutrophils (range, 90–60%) (Fig. 1, A and B). Aspirin, the proponic acid derivatives ketoprofen and flurbiprofen, nimesulide, as well as the indole-acetic acid derivative indomethacin, also promoted the down-regulation of L-selectin in neutrophils but to a lesser extent, (range, 30–15%) (Fig. 1, A and B). However, NSAIDs derived from enolic acids such as pyrazolones (phenylbutazone) and oxicams (piroxicam and meloxicam) as well as the active metabolite of the non-acidic NSAID nabumetone, the 6-MNA, not only reduced but instead seemed to slightly increase the basal expression of L-selectin in neutrophils (Fig. 1B). When the L-selectin expression was assessed in neutrophils maintained in medium at 4°C, it was significantly higher (about 25%) than that observed at 37°C in medium alone (Fig. 1B). Neutrophils treated with whatever NSAIDs, including the enolic acids, showed a lesser neutrophil L-selectin expression than the basal at 4°C. When NSAIDs were assayed at 4°C, no modification of the basal expression of L-selectin was observed in neutrophils (data not shown). Interestingly, none of the NSAIDs tested was able to modify the basal expression of other adhesion molecules that play a relevant role in the adhesion cascade such as CD11a or CD31 (data not shown).

These data indicate that currently available NSAIDs can be divided in three categories according to their ability to release L-selectin from the neutrophil surface: high, moderate, and non-releaser NSAIDs. In addition, the effector mechanism through which these compounds induce the shedding of L-selectin is temperature-sensitive.

The L-selectin Shedding Activity of NSAIDs Can Be Related to Their Chemical Structure—The foregoing experiments suggested that a chemical structure shared by all members of the high releaser group but absent in the non-releaser group of NSAIDs might be responsible for the L-selectin shedding activity of these agents. The comparative analysis between the strength of NSAIDs in the induction of L-selectin down-regulation (Fig. 1B) and their formulas (Fig. 2A) suggests that the diphenylamine, a compound formed by two benzene rings joined by an amine group, was the chemical structure core common to and exclusively present in the high releaser NSAIDs (Fig. 2A). Then, we decided to assay the effect of diphenylamine itself as well as diphenylacetic acid and N-phenylanthranilic acid (Fig. 2B), two chemical acides structurally related to diphenylamine, on neutrophil L-selectin expression.

The diphenylamine and the N-phenylanthranilic acid (diphenylamine structure with a carboxylic radical in position 2), two compounds without known anti-inflammatory properties, induced a clear-cut time- and dose-dependent L-selectin down-regulation in neutrophils. In contrast, the diphenylacetic acid, two benzene rings joined by the acetic acid, did not induce any significant change in the L-selectin expression in neutrophils, even at prolonged exposure time or at high doses (Fig. 3, A and B). N-Phenylanthranilic acid showed to be as potent as flunisalamic acid in the induction of L-selectin down-regulation in neutrophils after 40 min of incubation (Fig. 3A), whereas diphenylamine showed a moderate effect (about 50% of decrement). These data strongly suggest that diphenylamine represents the skeleton structure responsible for the L-selectin shedding activity of NSAIDs.

The Diphenylamine-based Compounds Induce the L-selectin Down-regulation by a Cycloxygenase- and Cell Activation-independent Mechanism—The fact that some NSAIDs but not others were able to shed L-selectin strongly suggested that the common characteristics of this family of therapeutic agents, the inhibition of COX activity, must not account for this phenomenon. However, to rule out this possibility, we tested the effect of different NSAIDs, N-phenylanthranilic acid and diphenylacetic acids, on a well-established experimental model to determine the COX-1 activity, the thromboxane B2 (TxB2) release induced by arachidonic acid in platelets. It was found that there was no relationship between the capability of NSAIDs to inhibit COX-1 activity and to shed L-selectin. Indomethacin and aspirin, agents included in the group of moderate releasers (Fig. 1B), caused almost a complete TxB2 release inhibition in platelets (Fig. 4A). Both aceclofenac and meloxicam showed a poor inhibition (about 30%) of TxB2 production (Fig. 4A). Finally, neither N-phenylanthranilic acid nor diphenylactic acid showed any effect on COX activity in platelets (Fig. 4A). These data exclude a role of COX-1 activity in the down-regulation of L-selectin by NSAIDs.

Upon activation, L-selectin is enzymatically cleaved from neutrophils cell surface both in vitro (32, 33) and in vivo (27, 34). Variations in the expression of CD11b and CD45, two glycoproteins contained in the membrane of secretory granules of neutrophils, can be considered as an indirect measurement of neutrophil activation (26, 35). As shown in Fig. 4B, neither N-phenylanthranilic acid, diphenylamine, nor diphenylactic acid caused any significant effect on CD11b basal expression on neutrophils. Similar results were obtained when the CD45 expression was studied (data not shown). These data demonstrate that the ability of diphenylamine-based NSAIDs to induce the down-regulation of L-selectin from the cell surface of neutrophils is neither a prostaglandin-dependent nor an unspecific cell activation effect.

Diphenylamine-based Compounds Induce the Down-regulation of L-selectin Expression Proportionally to Their Ability to Reduce the Energy Capacity of Neutrophils—It is well known that NSAIDs are able to uncouple the mitochondrial oxidative phosphorylation reducing the intracellular ATP synthesis (20, 36). Recently, our group has proposed that the maintenance of L-selectin on the neutrophil surface requires energy consumption and that the capability of NSAIDs to reduce the energy status of the cell might account for the ability of these agents to induce the shedding of L-selectin in human neutrophils (25). Because it has been demonstrated that both diphenylamine and N-phenylanthranilic acid are uncouplers of oxidative phos-
phorylation in mitochondria (37), we decided to test the potential relationship between variations in the intracellular ATP concentration and L-selectin expression in neutrophils induced by diphenylamine-based compounds. Meclofenamic acid, diphenylamine, and N-phenylanthranilic acid, but not diphenylacetic acid, decreased the intracellular ATP concentration (Fig. 5A), and a significant direct correlation was observed between variations in intracellular ATP and surface expression of L-selectin in neutrophils (Fig. 5B; \( r = 0.97 \), \( p < 0.01 \)).

These results suggest that the diphenylamine chemical structure plays an important role in the uncoupling effect of NSAIDs with diphenylamine skeleton. In addition, these data indicate that diphenylamine-based compounds might exert their action on L-selectin expression in neutrophils through a mechanism coupled to the reduction of oxidative phosphorylation in mitochondria.

**NSAIDs Are Able to Down-regulate L-selectin Surface Expression in Different Cell Types**—As stated previously, L-selectin is expressed by most leukocytes. To determine if the capability of NSAIDs to reduce L-selectin expression in neutrophils...
was also exerted in other cell types, we studied the effect of different anti-inflammatory agents upon the basal expression of L-selectin in human neutrophils, lymphocytes, and monocytes. As expected, when these cell types were incubated with piroxicam even at high concentration, no significant effect was observed on the L-selectin basal expression (data not shown). By contrast, aceclofenac and indomethacin induced the down-regulation of L-selectin in all cell types in a time- and a dose-dependent manner (Fig. 6, A and B, and data not shown). In lymphocytes and monocytes, the L-selectin was more resistant to the action of NSAIDs, because these cell types required both a higher concentration and a longer exposure time to aceclofenac than neutrophils, to achieve a significant reduction in L-selectin expression (Fig. 6, A and B).

**NSAIDs Require the Presence of TACE to Down-regulate L-selectin Expression**—The shedding of L-selectin targeted by stimuli such as TNF-α or PMA is the result of its proteolytic cleavage conducted, at least in certain cell types and under certain conditions by a membrane metalloprotease, shown to be identical with TACE (24). To ascertain the potential role of TACE in the down-regulatory activity of NSAIDs on L-selectin expression in neutrophils, we assayed the effect of IC-3, a hydroxamic acid-based inhibitor that has been shown to block TACE (38). This pharmacologic agent prevented the reduction of L-selectin surface expression in neutrophils treated with either PMA or flufenamic acid in a dose-dependent manner (Fig. 7A and data not shown). To further clarify the role of TACE in this effect of NSAIDs on L-selectin, we also assayed the effect of several diphenylamine-based compounds on both a TACE-deficient monocytic cell line, DRM−/− cells, and in the same cell line expressing TACE wild type, DRM+/+ cells. On DRM−/− cells neither PMA nor any of the diphenylamine-based compounds tested were able to modify the basal expression of L-selectin. However, on DRM+/+ cells, most of the compounds tested down-regulated the L-selectin expression (Fig. 7B) in a similar range to that obtained in human peripheral blood monocytes (Fig. 6, A and B). Only piroxicam did not show any effect on L-selectin expression, in accordance with previous data (22). These data demonstrate that NSAIDs cause L-selectin shedding by a TACE-dependent mechanism.

**DISCUSSION**

NSAIDs comprise a heterogeneous group of chemical agents with different chemicals and pharmacokinetics characteristics (14). Although it is commonly accepted that NSAIDs exert their properties through the inhibition of COX (15), several clinical reports (13, 16, 39) showed that it is very unlikely that this array of different compounds share a single mechanism of...
anti-inflammatory action. Our group has suggested that some leukocyte adhesion molecules might be therapeutic targets for NSAIDs (21). A number of these compounds, but not all, are able to induce the proteolytic shedding of L-selectin in neutrophils both in vitro and in vivo (40–42). L-selectin is the member of the selectin family, which seems to play the major role in the initial events of the inflammatory response that allow the entry of neutrophils into inflamed tissues (40–42). The importance of L-selectin shedding activity in neutrophils as a mechanism of action of NSAIDs in vivo has not been established yet. However, the relevant role that L-selectin plays in inflammation (38–40) in addition to the fact that indomethacin decreases in vivo the L-selectin expression in human neutrophils (22) give a potential biologic relevance to this effect of NSAIDs.

In this work we propose that, according to the capability to induce the shedding of L-selectin in neutrophils, NSAIDs could be divided in three groups. A group of NSAIDs formed by some arylcarboxylic and arylalkanoic acids derivatives (flufenamic, meclofenamic, and mefenamic acids and diclofenac and aceclofenac, respectively) showed a very potent L-selectin shedding activity, the high releaser group. Other NSAIDs such as aspirin, indomethacin, nimesulide, flurbiprofen, and ketoprofen exerted only a moderate but consistent activity on L-selectin expression in neutrophils. Finally, a third group of NSAIDs, including phenylbutazone and the oxicams (piroxicam and meloxicam) did not show any effect on L-selectin expression in neutrophils.

At 4 °C these NSAIDs and their precursors (diphenylamine and N-phenylanthranilic acid) did not show effect on the basal expression of L-selectin, demonstrating that the shedding of L-selectin induced by these agents is temperature-dependent. Interestingly, members of the non-releaser group, especially the phenylbutazone, appeared to slightly increase...
the basal L-selectin expression in neutrophils. This could be likely due to the prevention of the slow rate of L-selectin shedding that undergo neutrophils when maintained in vitro (17).

The differential effect of NSAIDs on L-selectin expression makes very feasible that a chemical structure common to the high releaser but absent in the non-releaser group might be responsible for the L-selectin down-regulation in neutrophils. All NSAIDs from the high releaser group shared a chemical structure containing two benzene rings joined by an amine (-NH-) group, the diphenylamine, which was not present in the non-releaser group. Notably, the diphenylamine was found to induce by itself a significant reduction in the L-selectin surface expression of neutrophils in vitro. The -NH- group of this chemical compound proved to have a remarkable role in the anti-L-selectin activity of diphenylamine-based NSAIDs, because, when this group was either absent (flurbiprofen) or replaced by radicals such as O (nimesulide), CO (ketoprofen), or CH2-COOH (diphenylacetic acid), there was an important reduction or even a complete loss in the L-selectin down-regulation activity. Most of the currently available NSAIDs have in common that they are weak organic acids. The N-phenylanthranilic acid is a compound obtained by the substitution of a carboxylic acid group in one of the aromatic rings of diphenylamine. This compound showed a stronger capability to induce the surface down-regulation of L-selectin in neutrophils compared with diphenylamine. The importance of the presence of an acid radical in the L-selectin down-regulation activity of NSAIDs

**FIG. 5.** Diphenylamine-related compounds induce a down-regulation in the surface expression of L-selectin proportionally to the reduction in intracellular ATP concentration. A, effect of diphenylamine-related compounds on intracellular ATP concentration. Neutrophils were incubated with different diphenylamine-related compounds under conditions described in Fig. 1. After centrifugation, cell pellets were lysed, and the ATP concentration was determined in duplicate by a commercial kit as described under “Experimental Procedures.” Values were obtained in duplicate determinations for each sample. Results are presented as the mean ± S.D. (μM/10⁶ cells) from four independent experiments. B, correlation between the reduction of ATP concentration and surface expression of L-selectin induced by NSAIDs in neutrophils. Neutrophils were incubated with the different compounds under the same conditions described in Fig. 1. After centrifugation, cell pellets were divided in two parts; one was used for ATP determination, for which results are depicted in A, and the other part was used for the quantification of the L-selectin surface expression by flow cytometry. Results represent the percentage of variation on intracellular ATP and L-selectin expression with respect to medium (considered 100%) expressed as mean ± S.D. of four independent experiments. There was a highly significant direct correlation between the reduction of intracellular ATP and surface expression of L-selectin (r = 0.97, p < 0.01, n = 4).

**FIG. 6.** NSAIDs induce the down-regulation of L-selectin expression in different cell types. A, kinetic of the effect of aceclofenac on neutrophils (○), lymphocytes (△), and monocytes (■). Cells were cultured at 37°C in the presence of 50 μM aceclofenac for 5–90 min. Data represent the mean ± S.D. of rMFI related to the expression by culture cells in medium alone in each time. B, dose response of the L-selectin down-regulation induced by aceclofenac in neutrophils (○), lymphocytes (△), and monocytes (■). Cells were incubated for 15 min at 37°C in doses of aceclofenac ranging from 1 to 100 μM. Data are presented as mean ± S.D. of rMFI from three independent experiments.
seems to be independent of the complexity of the carboxylic group (aceclofenac and diclofenac). Regarding the non-substituted benzene ring of N-phenylanthranilic acid, the presence of radicals in positions 2, 5, and 6 does not seem to play a significant role in the ability of NSAIDs to cause the neutrophil L-selectin down-regulation.

**Fig. 7.** NSAIDs induce the L-selectin shedding by a TACE-mediated mechanism. *A*, the TACE inhibitor IC-3 prevents the L-selectin down-regulation induced by PMA and flufenamic acid in neutrophils. Cells were preincubated with IC-3 (50 μM for 15 min at 37 °C) and then incubated with flufenamic acid (50 μM) and PMA (20 ng/ml) for 15 min at 37 °C. L-selectin expression was measured by flow cytometry. The shaded histograms represent the L-selectin expression. The dotted histograms represent the negative control (fluorescence produced by the supernatant of P3X63 myeloma). A representative experiment out of three independent ones is shown. *B*, cells expressing a mutated, non-functioning TACE (DRM−/− cells) lack the capability to down-regulate L-selectin expression in response to diphenylamine-based compounds. Incubation for 90 min at 37 °C in the presence of 50 μM of the different compounds and 100 ng/ml PMA did not cause any significant modification in the basal expression of L-selectin in DRM−/− cells. Under the same conditions, cells able to process TACE-specific substrates, DRM+/+ (◼) demonstrated shedding of L-selectin in response to PMA, diphenylamine-based NSAIDs, diphenylamine itself, and N-phenylanthranilic. Data represent the mean ± S.D. of rMFI from three independent experiments. *p < 0.05 versus medium and **p < 0.01, by Student paired t test.
The fact that some NSAIDs but not others were able to shed L-selectin strongly suggested that the common characteristics of this family of therapeutic agents, the inhibition of COX activity, must not be responsible for this phenomenon. The N-phenylanthranilic acid did not show any effect in the COX-1 activity in platelets. In addition, the ability of aceclofenac, meloxicam, aspirin, and indomethacin to block COX-1 activity was unrelated to their effects on L-selectin expression in neutrophils. These data strongly support the notion that the effect of diphenylamine-based NSAIDs on L-selectin expression in neutrophils is a prostaglandin-independent phenomenon. The potential implication of COX-2 inhibition in the L-selectin shedding has been previously ruled out (25). Diphenylamine and N-phenylanthranilic acid caused the shedding of L-selectin without an evident variation in the neutrophil activation state, as assessed by their failure to affect CD11b and CD45 basal expression.

Recently, TACE, a cell-surface metalloprotease from the ADAM family responsible of the cleavage of the surface form of TNF-α, also known as ADAM-17 or CD155b, has shown proteolytic activity on L-selectin (24). The TACE metalloprotease inhibitor prevented the down-regulation of L-selectin expression induced by flufenamic acid in neutrophils, suggesting the potential role of this metalloprotease in the effect of NSAIDs on L-selectin expression. Although this inhibitor has shown to block the activity of TACE (38), results obtained with inhibitors in living cells neither support nor preclude definitive conclusions. To further clarify this, we used a cellular system to block the activity of TACE (38), results obtained with inhibitors in living cells neither support nor preclude definitive conclusions. To further clarify this, we used a cellular system for reconstituting TACE activity to test the role of this protease in the L-selectin shedding mediated by NSAIDs. TACE-expressing DRM-/+ cells down-regulated L-selectin expression in response to both PMA and diphenylamine-based NSAIDs. However, TACE-deficient DRM-/-- cells lacked this capability, which clearly indicates that TACE is able to cleave L-selectin in response to NSAIDs. Nevertheless, these data do not exclude that other metalloproteases might participate in the shedding of L-selectin induced by NSAIDs in other cell types under other conditions.

Recent observations suggest that the maintenance of L-selectin on the membrane of neutrophils might be an energy-dependent process (25). NSAIDs are able to interfere, at different extent, with the mitochondrial oxidative phosphorylation reducing the intracellular ATP synthesis (20, 36). Interestingly, diphenylamine as well as N-phenylanthranilic acid have been also described as uncouplers of oxidative phosphorylation of rat liver mitochondria (37). The effect of diphenylamine and related compounds at the level of intracellular ATP strongly correlated with the ability of these agents to reduce the surface expression level of L-selectin in neutrophils. All these observations suggest that diphenylamine-based NSAIDs might induce the down-regulation of L-selectin in neutrophils through the reduction of energy status of the cell. How this lack of energy capability induces the L-selectin shedding is currently unknown. Nonetheless, it may be feasible that the metalloprotease, either TACE or another member of the ADAMs family, is active by default but, in resting cells, its ability to release L-selectin is blocked by a mechanism that requires ATP consumption.

Current hypotheses suggest that differences in the clinical response of NSAIDs might be explained by variations in their mechanism of action, including differences both in the potency to inhibit prostaglandin synthesis and in the effectiveness to interfere with other non-prostaglandin mediated biologic effects. Therefore, it is conceivable that the differential effect that NSAIDs on L-selectin surface expression might help to explain the variable clinical response of patients to these compounds (13). The development of new drugs based on the skeleton structure of diphenylamine might represent a breakthrough in the therapy against inflammation. It is feasible to expect that this new family of anti-inflammatory agents will show a better safety profile than the currently available NSAIDs, because its therapeutic action will be based on the induction of the L-selectin shedding in neutrophils, instead of on the prostaglandin synthesis inhibition. However, because TACE cleaves a variety of different substrates, including the pro-inflammatory cytokine TNF-α, the possibility that the activity of this metallopeptase by drugs results in unexpected biological effects must be investigated. Finally, the better understanding of the capability of NSAIDs to modulate the TACE activity might have possible medical application in the field of inflammation and cancer (43).

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Structure-Function Relationship and Role of Tumor Necrosis Factor-α-converting Enzyme in the Down-regulation of L-selectin by Non-steroidal Anti-inflammatory Drugs

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