# Effect of Non-Steroidal Anti-Inflammatory Drugs on Amyloid-β Formation and Macrophage Activation after Platelet Phagocytosis

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**Abstract:** Recently, we showed that platelet phagocytosis occurs in human atherosclerotic plaques and leads to foam cell formation. Platelet phagocytosis, resulting in macrophage activation and iNOS induction, was associated with the formation of amyloid-β peptide (Aβ) via proteolytic cleavage of platelet-derived amyloid precursor protein (APP), possibly by secretases. To test the involvement of γ-secretase in this process, we used indomethacin, ibuprofen, and sulindac sulfide, non-steroidal anti-inflammatory drugs (NSAIDs) known to alter the γ-secretase cleaving site of APP, on their ability to inhibit macrophage activation evoked by platelet phagocytosis. J774 macrophages were incubated with human platelets or lipopolysaccharide (LPS) with or without NSAIDs. Nitrite was quantified as a measure for inducible nitric oxide synthase (iNOS) activity. Indomethacin, ibuprofen, sulindac sulfide, and meloxicam concentration-dependently reduced nitrite production by macrophages incubated with platelets, but did not alter LPS-induced iNOS activity or platelet uptake. However, acetylsalicylic acid and naproxen, two NSAIDs without effect on the γ-secretase cleaving site of APP, did not affect nitrite production in either platelet- or LPS-stimulated macrophages. Surface-enhanced laser desorption/ionization time-of-flight mass-spectrometry demonstrated time-dependent formation of Aβ-containing peptides after platelet phagocytosis, which could be inhibited by indomethacin. In conclusion, these results point to the involvement of γ-secretase in macrophage activation following platelet phagocytosis.

**Key Words:** amyloid precursor protein, β-amyloid peptide, inducible nitric oxide synthase, macrophage activation, NSAID

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Recently, we demonstrated the presence of amyloid-β (Aβ), which until then had only been studied in Alzheimer's disease, in macrophages around microvessels in advanced human atherosclerotic plaques.[1] In these macrophages, the formation of Aβ was associated with the expression of inducible nitric oxide synthase (iNOS). A source for Aβ in atherosclerotic plaques is blood platelets, which contain amyloid precursor protein (APP) in the α-granules. The platelets can enter atherosclerotic plaques via leaky microvessels and are then phagocytosed by macrophages,[2] which subsequently transform to foam cells.[1] We proposed that during this process Aβ-like peptides are formed, which then activate the macrophage as indicated by iNOS expression.[1] This novel mechanism of macrophage activation in atherosclerotic plaques may favor plaque expansion and/or rupture. Indeed, the massive nitric oxide (NO) release produced by iNOS has been reported to induce apoptotic cell death of smooth muscle cells by enhancing Fas-L/Fas interactions.[3] In the atherosclerotic plaque, smooth muscle cells are the only cells that are able to produce collagen isoforms that contribute to the strength of the plaque.[4] Moreover, activated macrophages can trigger the activation of matrix metalloproteinases in the vascular interstitium,[5] resulting in degradation of the interstitial collagen fibers and decreased strength of the fibrous cap of an atherosclerotic plaque.

The proteolytic enzymes responsible for Aβ formation in macrophages following platelet phagocytosis are not known yet. In Alzheimer's disease the insoluble $A\beta_{1\text{-}42}$ peptide is generated by the cleavage of its precursor APP, by two enzymes designated β- and γ-secretase. Recently, it was shown that indomethacin, ibuprofen, and sulindac sulfide directly affect the

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γ-secretase cleaving site of APP, thereby altering the amyloid pathology in the brain.[6] The latter effect results in a reduction of the highly amyloidogenic $A\beta_{1-42}$ levels and an increase in the less harmful $A\beta_{1-38}$ isoform. The interference with the γ-secretase cleaving site is not seen with all NSAIDs and was not dependent on cyclooxygenase (COX) inhibition. Indeed, acetylsalicylic acid and naproxen do not affect the γ-secretase cleaving site of APP. Since specific γ-secretase inhibitors are not available, we used a series of NSAIDs to investigate the participation of γ-secretase in macrophage activation and the formation of Aβ-like peptides following platelet phagocytosis.

## METHODS

### Cell Culture

The murine macrophage cell line J774A.1 (American Type Culture Collection, Rockville, MD) was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 150 U/ml polymyxin B, and 50 μg/ml gentamycin in a humidified 5% carbon dioxide/95% air incubator at 37°C. All cell culture media and supplements were from Invitrogen (Paisley, UK). For cell culture experiments 48 well plates (Costar, Cambridge, MA) or culture slides (Beckton Dickinson Labware, Sunnyvale, CA) were used. Cells ($0.5 \times 10^6$/800 μL) were allowed to adhere at 37°C for 2 hours. Thereafter, cells were washed with warm medium and the medium was replaced by Dulbecco Modified Eagles Medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin.

Human blood platelet concentrates were kindly provided by the blood transfusion center of the University Hospital of Antwerp (Belgium). These platelet concentrates had been deleukocytized by filtration and contained only 1 to 2 white blood cells per 300,000 platelets. Platelets were washed in warm PBS and resuspended in serum free Dulbecco Modified Eagles Medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin.

J774 macrophages, primed with recombinant mouse IFNγ (100 U/ml, Sigma), were incubated with washed human platelets ($10^8$ per $0.5 \times 10^6$ macrophages)[1] in the presence of an NSAID or its solvent. After an incubation period of 18 hours, the culture medium was removed from the macrophages, centrifuged, and stored at −20°C for nitrite determination. To study the specificity of NSAID treatment on the nitrite production evoked by platelet phagocytosis, experiments using lipopolysaccharide (LPS) from *Salmonella typhosa* as an alternative stimulus for iNOS induction were performed as well.

### Drugs

The following NSAIDs were examined (highest final solvent concentration is indicated): indomethacin (Federa, Brussels, Belgium, 0.5 mM TRIS pH 8.3), ibuprofen (Federa, Brussels, Belgium, 0.5% ethanol), acetylsalicylic acid (Federa, Brussels, Belgium, 0.5% ethanol), naproxen (Federa, Brussels, Belgium, 1% ethanol), meloxicam (Calbiochem, San Diego, CA, 0.2% DMSO), and sulindac sulfide (Sigma, St Louis, MO, 0.2% ethanol), the active metabolite of the prodrug sulindac. They were dissolved at the start of every experiment and the solutions were sterilized by filtration.

### Nitrite Measurement

Nitric oxide synthase activity was assessed by measuring nitrite, a stable NO metabolite, using the Griess reaction.[7] Briefly, 200 μL culture medium was transferred to a 96-well microtiter plate and mixed with 40 μL of 6.5 M HCl and 37.5 mM sulphanilic acid (1:1). After 10 minutes, 20 μL 12.5 mM N-(1-naphtyl)-ethylene diamine dihydrochloride was added. After 30 minutes at 37°C, the absorbance was read at 540 nm using a Titertek Multiscan MCC/340 plate reader (Labsystems). Nitrite concentration was calculated from a standard curve of sodium nitrite in culture medium.

### Oil Red O Stain

To examine whether platelet engulfment by macrophages was altered by NSAIDs, co-incubations of macrophages and platelets were stained with oil red O[8] after fixation of the cells with 4% formaldehyde (10 minutes). Nuclei were stained with hematoxylin.

### Flow Cytometry

Phagocytosis of platelets by macrophages was also evaluated by flow cytometry. J774 macrophages and washed human platelets were incubated for 45 minutes with DMEM supplemented with 5 μM of 5- and 6-([(4-chloromethyl) benzoyl] amino) tetramethylrhodamine (Cell Tracker Orange, Molecular probes) or 2.5 μM 5- chloromethylfluorescein diacetate (Cell Tracker Green, Molecular Probes), respectively.[9] Thereafter, the media were refreshed, and the macrophages were incubated with the platelets for 3.5 hours in the presence or absence of the highest concentrations of these NSAIDs that inhibited macrophage activation. This time point was based on previous experiments on the evaluation of platelet phagocytosis using confocal and electron microscopy.[1] Flow cytometry was performed using a FACSort (BD Biosciences, San Diego, CA). Forward and side scatters were set to include cells but to exclude debris. Green fluorescent-free platelets were monitored in the FL 1 channel, whereas orange fluorescent macrophages were measured in the FL 3 channel. Data were analyzed using CellQuestPro software (BD Biosciences). Phagocytosis (per cent) was calculated as the (number of cells containing cell tracker green and orange / total number of cells containing cell tracker orange) × 100.

### Cyclooxygenase Inhibition and Determination of Prostaglandin $E_2$

To document COX inhibition, we tested all NSAIDs on their ability to inhibit arachidonic acid-induced blood platelet

aggregation. Blood samples (9 mL) were obtained from a cannulated carotid artery of pentobarbitone (30 mg/kg)-anesthetized New Zealand White rabbits and collected on trisodiumcitrate (1 mL; 3.8%). Platelet-rich plasma was prepared by centrifugation (10 minutes, 200 g). The samples were exposed to 330 μM arachidonic acid after 3 minutes incubation in the presence or absence of the NSAIDs in a Chronolog dual channel aggregometer (37°C, 900 rpm).[9] The maximum increase in light transmission after the addition of arachidonic acid was measured.

$PGE_2$ was measured in the culture medium by radioimmunoassay. Dose interpolation was done with a four-parameter logistic function using the IBM-PC RIA data reduction package provided by M.L. Jaffe (Silver Spring, MD).[10]

### Surface-Enhanced Laser Desorption/Ionization Time-of-Flight (SELDI-TOF) Mass Spectrometry

Analysis of APP fragments in cell lysates of macrophages, platelets, and macrophages co-incubated with platelets for 3, 6, or 18 hours with or without indomethacin was performed by SELDI-TOF mass spectrometry using the ProteinChip system (PS20, protein chip array, Ciphergen Biosystems Inc., Fremont, CA). Cells were lysed for 30 minutes in 50 mM TRIS buffer containing 250 mM NaCl, 5 mM EDTA, 0.1% (vol/vol) Triton X 100, 1 mM dithiothreitol, 1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM $Na_3VO_4$, and 0.1 mM paramethylsulphonylfluoride.

The chip arrays were incubated overnight at 4°C with the antibody (6E10 or IgG) to allow coupling. 6E10 is an antibody specific for the 17 N-terminal amino acids of β-amyloid peptides (672–688 of the full-length APP sequence). IgG antibody coating was used as a control to discriminate between specific and aspecific peptide binding to the protein chip. The samples were diluted and 20 μg total protein was spotted on the 6E10 or IgG antibody-coated chips. After 4 hours, non-bound peptides were removed by extensive washing with PBS and PBS containing 0.5% Triton X 100. The arrays were rinsed several times with a 1.0-mM HEPES buffer, and allowed to air dry. Thereafter, 1 μL of a 20% saturated solution of α-cyano-4-hydroxycinnamic acid in 0.5% (vol/vol) trifluoroacetic acid: 50% (vol/vol) acetonitrile was applied to each spot. Mass spectrometric analysis was performed by averaging a minimum of 100 laser shots in a Ciphergen SELDI Protein Biology system II, with a linear time-of-flight mass analyzer (mass accuracy: 0.1%).[11–14]

### Statistical Analysis

Nitrite values were compared by using analysis of variance (ANOVA). A *P* value less than 0.05 was considered to be significant. All data are represented as mean ± SEM.

## RESULTS

### Effect of NSAIDs on Nitrite Production

Incubation of IFNγ-primed J774 macrophages with washed human platelets or LPS resulted in nitrite production by the macrophages. The extent of the nitrite production was comparable for both stimuli. Addition of indomethacin, ibuprofen, meloxicam, or sulindac sulfide to the culture medium concentration-dependently reduced nitrite production by macrophages evoked by platelet phagocytosis (Fig. 1, A–D), whereas acetylsalicylic acid or naproxen were without such an inhibitory effect (Fig. 1, E and F). As compared with indomethacin, sulindac sulfide, and meloxicam, the effect of ibuprofen was less pronounced. When macrophages were stimulated with LPS, nitrite production was not affected by any NSAID (Fig. 1, A–F).

### Effect of NSAIDs on Platelet Aggregation and $PGE_2$ Production

The production of $PGE_2$ by macrophages stimulated with either platelets or LPS was significantly reduced in the presence of all NSAIDs (results not shown). Moreover, all NSAIDs inhibited arachidonic acid-induced platelet aggregation. After 3 minutes of exposure the percentage inhibition was: indomethacin (10, 50, 100 μM), 97% for all concentrations, ibuprofen (100, 200, 300 μM), 9%, 92%, and 96%, meloxicam (10, 30, 50 μM), 50%, 97%, and 97%, acetylsalicylic acid (50, 250, 500 μM), 13%, 96%, and 96% and naproxen (75, 150 μM), 12% and 97%. Sulindac sulfide was less effective as platelet inhibitor: 1 μM reduced the platelet aggregation with 3% and 10 μM with 18%.

### Platelet Phagocytosis and Foam Cell Formation

Flow cytometry revealed that after 3.5 hours platelet phagocytosis was not altered in the presence of 100 μM indomethacin, 500 μM ibuprofen, 10 μM sulindac sulfide, and 50 μM meloxicam (Fig. 2).

Lipid accumulation in the macrophages as a result of platelet phagocytosis was not changed in the presence of 100 μM indomethacin, indicating that platelet phagocytosis was not altered by this NSAID (Fig. 2). Similarly, the other NSAIDs did not alter platelet phagocytosis (not shown).

### Detection of APP-Fragments by SELDI-TOF Mass Spectrometry

The mass spectrum of the lysate of macrophages incubated for 3 hours with human blood platelets did not show peaks corresponding to $A\beta_{1\text{-}40}$ or $A\beta_{1\text{-}42}$ fragments (Fig. 3). However, in the lysate of macrophages incubated with blood platelets for 6 or 18 hours, two peaks at m/z 6277.5 and m/z 6541.5 were detected, which correspond to the $[M + H]^+$ peaks of proteins with a molecular mass of 6276.5 Da and 6540.5 Da, respectively. The intensity of these peaks increased with an

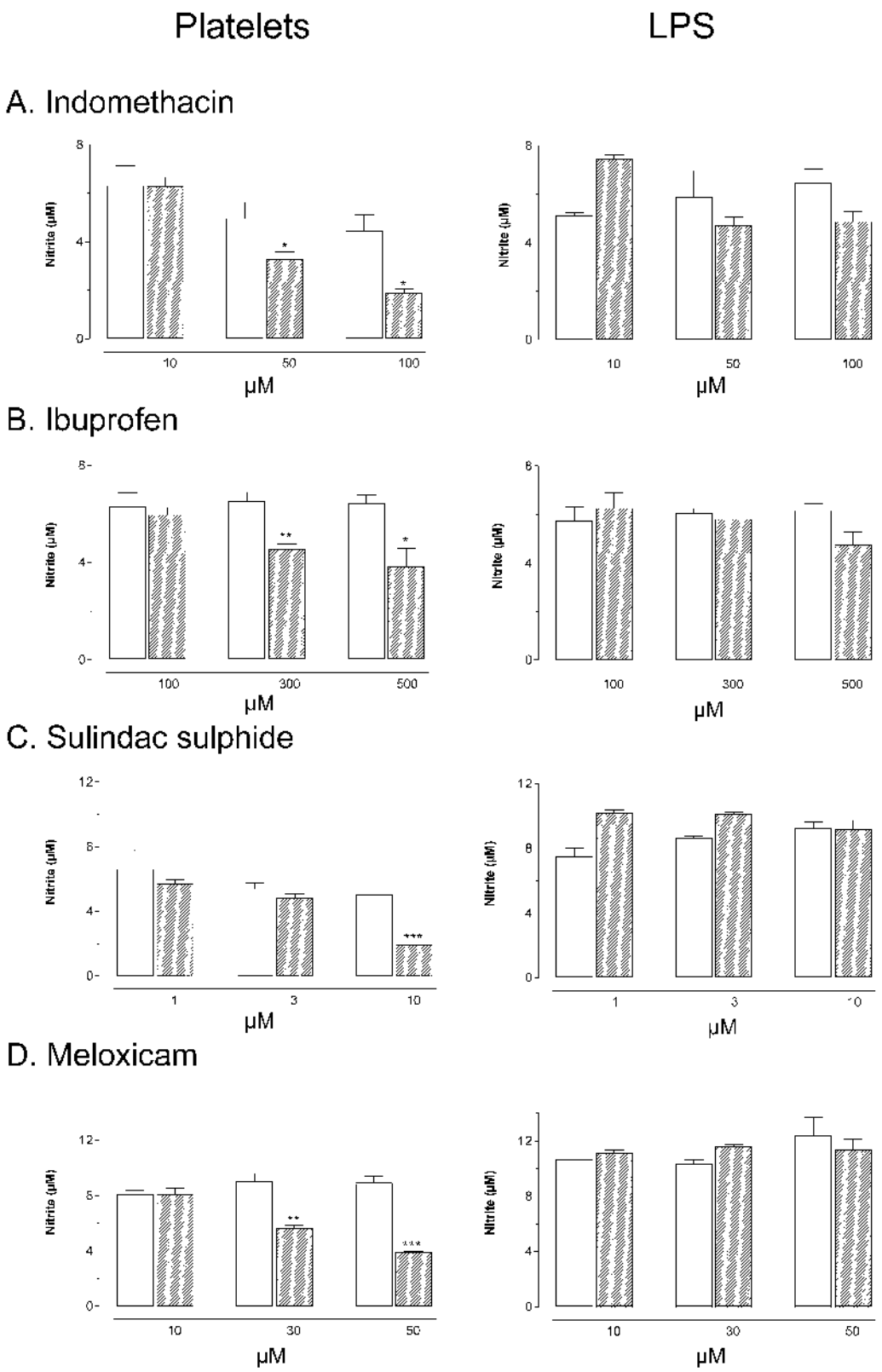


**FIGURE 1.** Legend on facing page.

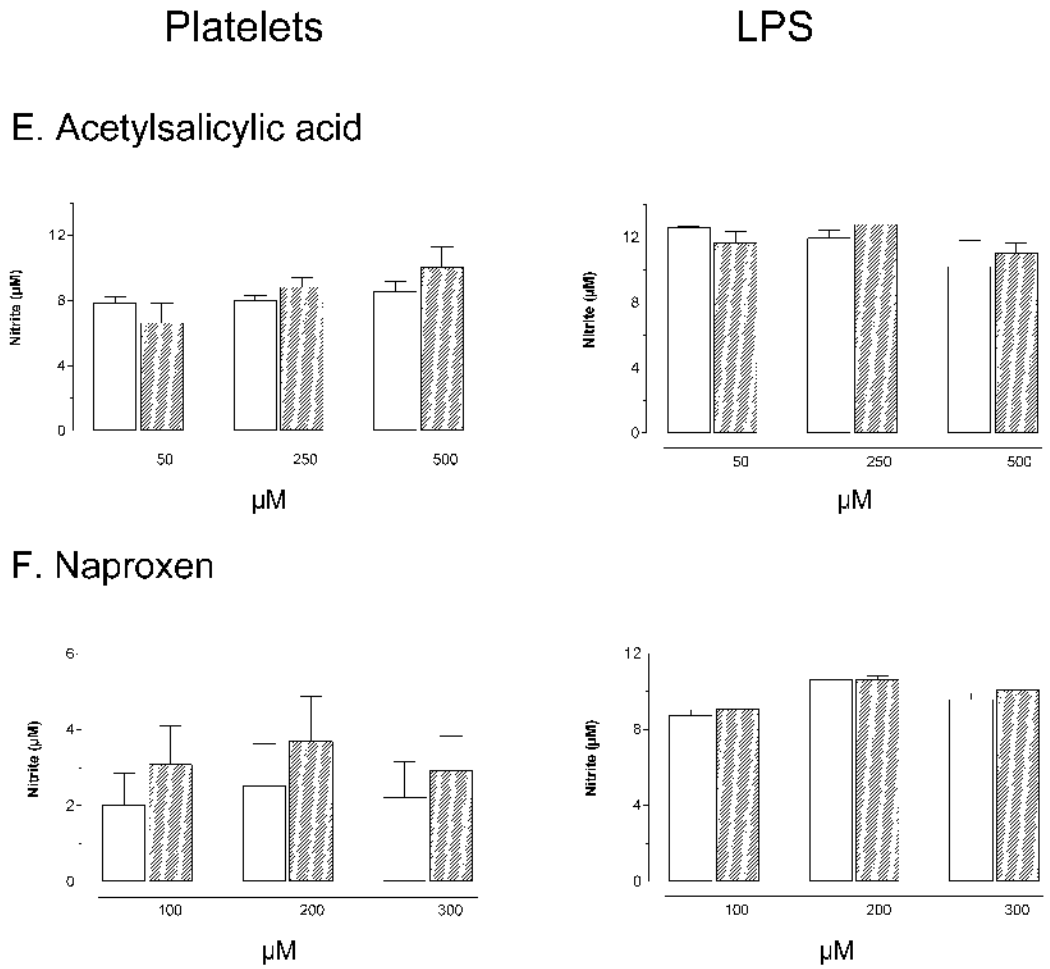


**FIGURE 1.** Effect of indomethacin (A, Platelets: n = 6, LPS: n = 2–4), ibuprofen (B, n = 4), sulindac sulfide (C, n = 3), meloxicam (D, n = 3), acetylsalicylic acid (E, PLT: n = 6, LPS: n = 2), and naproxen (F, PLT: n = 9, LPS: n = 3) on nitrite production by macrophages after incubation with platelets or LPS for 18 hours. Indomethacin, ibuprofen, sulindac sulfide, and meloxicam reduced platelet-induced nitrite production by macrophages concentration-dependently but did not affect LPS-induced nitrite production. Acetylsalicylic acid and naproxen did not alter nitrite production by macrophages after incubation with either blood platelets or LPS. Open bars: solvent control, hatched bars: NSAID-treated; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus solvent control.

increasing incubation time of platelets and macrophages (Fig. 3), which paralleled nitrite production (0.49 ± 0.03 µM after 3 hours, 0.80 ± 0.07 µM after 6 hours and 6.8 ± 0.2 µM after 18 hours). For the 6276.5 Da protein, six different APP-derived peptides are theoretically possible within a mass range of ± 5 Da (Table 1). The peak at m/z 6541.5 was not detected in every experiment and will not be considered in the present study.

When macrophages were incubated with platelets in the presence of indomethacin for 18 hours, $[M + H]^+$ peaks at m/z 6277.5 and 6541.5 disappeared completely. This was accompanied by peaks in the mass ranges m/z 5000–6000 and m/z 7000–8000 (Fig. 4). In all cases, a standard mixture gave the expected profile with $[M + H]^+$ peaks at m/z 1953.5 ($A\beta_{1-16}$, not shown), 4343.5 ($A\beta_{1-40}$), and 4527.6 ($A\beta_{1-42}$).

## DISCUSSION

Recently, it was demonstrated that in neuronal cells a subset of NSAIDs including indomethacin, ibuprofen, and sulindac sulfide alter the γ-secretase cleaving site of APP.[6] As a result, the formation of $A\beta_{1-42}$, the most toxic form of Aβ, was reduced in favor for the less toxic $A\beta_{1-38}$.[6] Indeed, it has been suggested[6] that this interference with the γ-secretase cleaving site of APP may explain the benefit of some NSAIDs seen in epidemiological[15–17] and clinical studies[18–20] of Alzheimer's disease. Since specific inhibitors of γ-secretase are currently not available or cytotoxic (unpublished observations), we investigated a series of NSAIDs to test whether γ-secretase is involved in platelet-induced macrophage activation. These NSAIDs were merely selected as pharmacological tools to un-

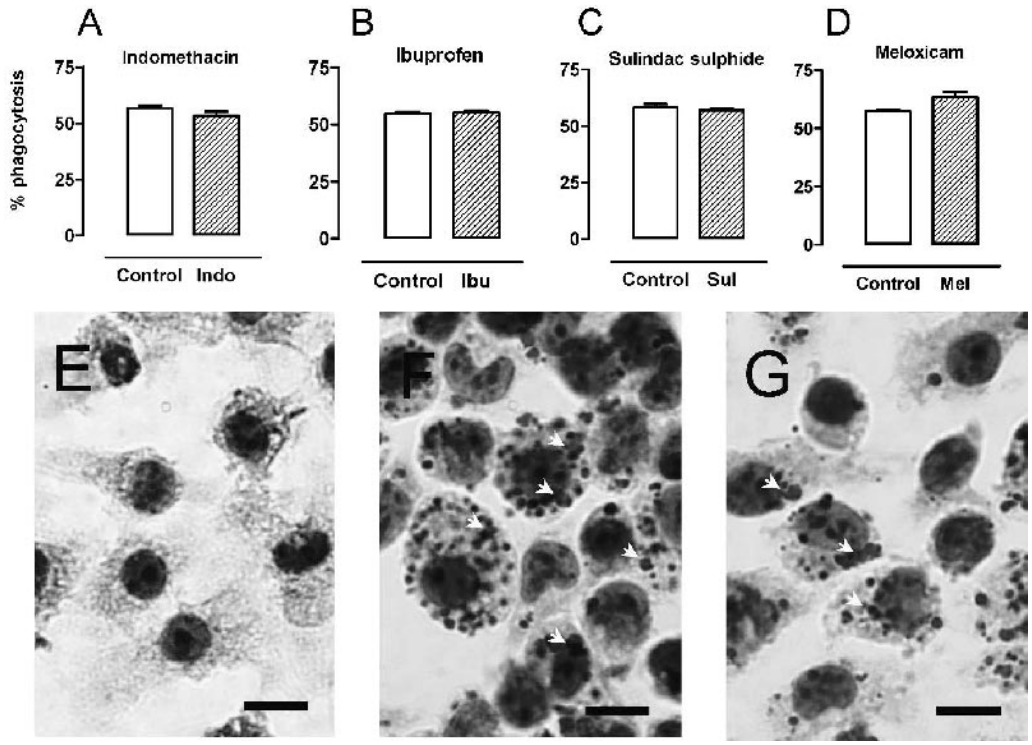


**FIGURE 2.** Effect of NSAIDs on platelet phagocytosis. Flow cytometric evaluation of platelet phagocytosis by macrophages with or without indomethacin 100 μM (Indo, A), ibuprofen 500 μM (Ibu, B), sulindac sulfide 10 μM (Sul, C), and meloxicam 50 μM (Mel, D). Results are presented as percentage of the population that has engulfed platelets. Oil red O stain of control macrophages (E) and macrophages incubated with human platelets in the presence (F) or absence (G) of 100 μM indomethacin. Lipid accumulation in the macrophages (arrowheads) was not changed in the presence of indomethacin further indicating that platelet phagocytosis was not altered by this NSAID. Bar = 10 μm.

ravel the mechanisms of macrophage activation after platelet phagocytosis.

The present data confirm that platelets are phagocytosed by macrophages (flow cytometry), that the macrophages become activated (nitrite production) and transform to foam cells (oil red O). Foam cell formation can result from other mechanisms than platelet phagocytosis. Since we used serum-free medium, endocytosis of lipid droplets can be excluded. Engulfment of LDL or modified LDL bound to the platelets is possible but seems to be less important because we used washed human platelets. With flow cytometry, we could demonstrate that platelets are indeed phagocytosed by macrophages, confirming previous results.[1]

Subsequently, our results clearly showed a reduced nitrite production when macrophages were incubated with platelets in the presence of indomethacin, ibuprofen, sulindac sulfide, and meloxicam as compared with the controls. Importantly, the concentrations of the NSAIDs used in the present study did not affect LPS-induced nitrite production, confirming previous reports.[21–23] The latter finding indicates that the inhibitory activity of NSAIDs on macrophage activation following platelet phagocytosis was not due to interference with the cell signaling pathways essential for iNOS induction or with the activity of iNOS. Indeed, Aeberhard et al[22] showed that only extremely high concentrations of NSAIDs, exceeding those of the present study, inhibit iNOS activity in LPS-stimulated macrophages. Moreover, the inhibitory activity of indomethacin, ibuprofen, sulindac sulfide, and meloxicam on macrophage activation evoked by platelet ingestion was not due to reduced platelet phagocytosis or processing. The different compounds did not inhibit the early platelet uptake as studied by flow cytometry, nor did they suppress the accumulation of platelet-derived lipid droplets inasmuch as oil red O staining of NSAID-treated macrophages did not differ from that of untreated macrophages. Furthermore, these experiments revealed no differences in platelet uptake between NSAID-treated and control macrophages.

Both $PGE_2$ measurements in the supernatant and arachidonic acid-induced blood platelet aggregation assays showed that all NSAIDs inhibited COX, confirming their activity and excluding possible degradation of the drugs. However, inhibition of macrophage activation by the NSAIDs was not due to COX-inhibition. Except for ibuprofen, the concentrations of the NSAIDs required to affect the macrophage activation after

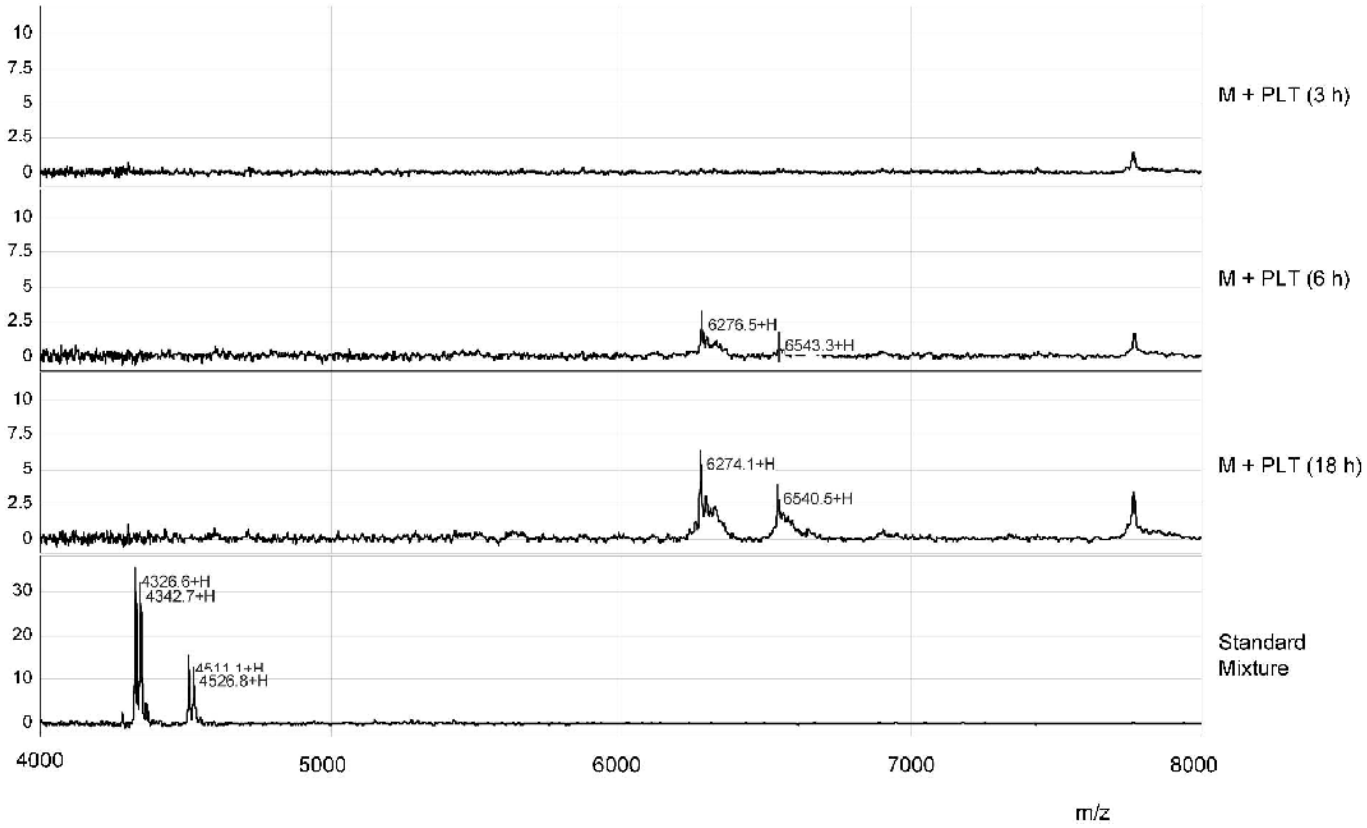


**FIGURE 3.** SELDI-TOF mass spectra of the lysate of co-incubations of macrophages and blood platelets (M + PLT) (3 hours, 6 hours, and 18 hours). When macrophages were incubated with blood platelets, an Aβ-like fragment was time-dependently formed with a molecular mass of 6276.5 Da. A standard mixture of $A\beta_{1\text{-}16}$, $A\beta_{1\text{-}40}$, and $A\beta_{1\text{-}42}$ gave the expected profile with $[M + H]^+$ peaks at m/z 1953.5 (not shown), 4343.5, and 4527.6, respectively.

platelet ingestion were higher than those necessary for COX inhibition. Secondly, sulindac sulfide, the least effective COX inhibitor, was even the most active inhibitor of macrophage activation following platelet phagocytosis. More importantly, although acetylsalicylic acid and naproxen inhibited COX, they did not alter nitrite production of macrophages induced by platelet phagocytosis. Indeed, the latter NSAIDs do not affect the γ-secretase cleaving site of APP.[6] Taken together, these findings suggest that the effect of some NSAIDs on nitrite formation could be due to interference with the γ-secretase cleaving site of APP, thereby reducing the formation of Aβ-like peptides.

Previously, Ogawa et al[24] reported that $A\beta_{1\text{-}40}$ could stimulate nitrite production in IFNγ-primed J774 macrophages. They further showed that indomethacin and ibuprofen inhibited the Aβ-effect and proposed that the inhibitory effects of these NSAIDs on iNOS expression were mediated via peroxisome proliferator-activated receptor-γ (PPAR-γ). Unfortunately, we were unable to reproduce these experiments. We encountered a great batch-to-batch variability in the response to $A\beta_{1\text{-}40}$ as well as $A\beta_{1\text{-}42}$ and the nitrite production of IFNγ-

**TABLE 1.** Different Theoretically Possible APP-Derived Peptides With a Molecular Mass of 6276.5 ± 5 Da That Contain the Epitope of the 6E10 Antibody

| Measured Mass (Da) | Theoretical Mass (Da) | Δ Mass (Da) | Number of Amino Acids | Amino Acids in APP Sequence |
|---|---|---|---|---|
| 6276.5 | 6278.9 | 2.4 | 56 | 635–690 |
| | 6271.9 | −4.6 | 56 | 640–695 |
| | 6273.9 | −2.6 | 57 | 644–700 |
| | 6273.9 | −2.6 | 57 | 648–704 |
| | 6275.1 | −1.4 | 58 | 656–**713** |
| | 6273.3 | −3.2 | 57 | **672**–728 |

All peptides contain the complete epitope of the 6E10 antibody (17 N-terminal amino acids of Aβ (ie amino-acids 672–688 of full length APP)). Peptides are ranked according to their sequence in full-length APP, with their theoretical mass, the difference between the theoretical mass and the measured mass and the number of amino acids. Cleaving sites of β- or γ-secretase are marked in bold.

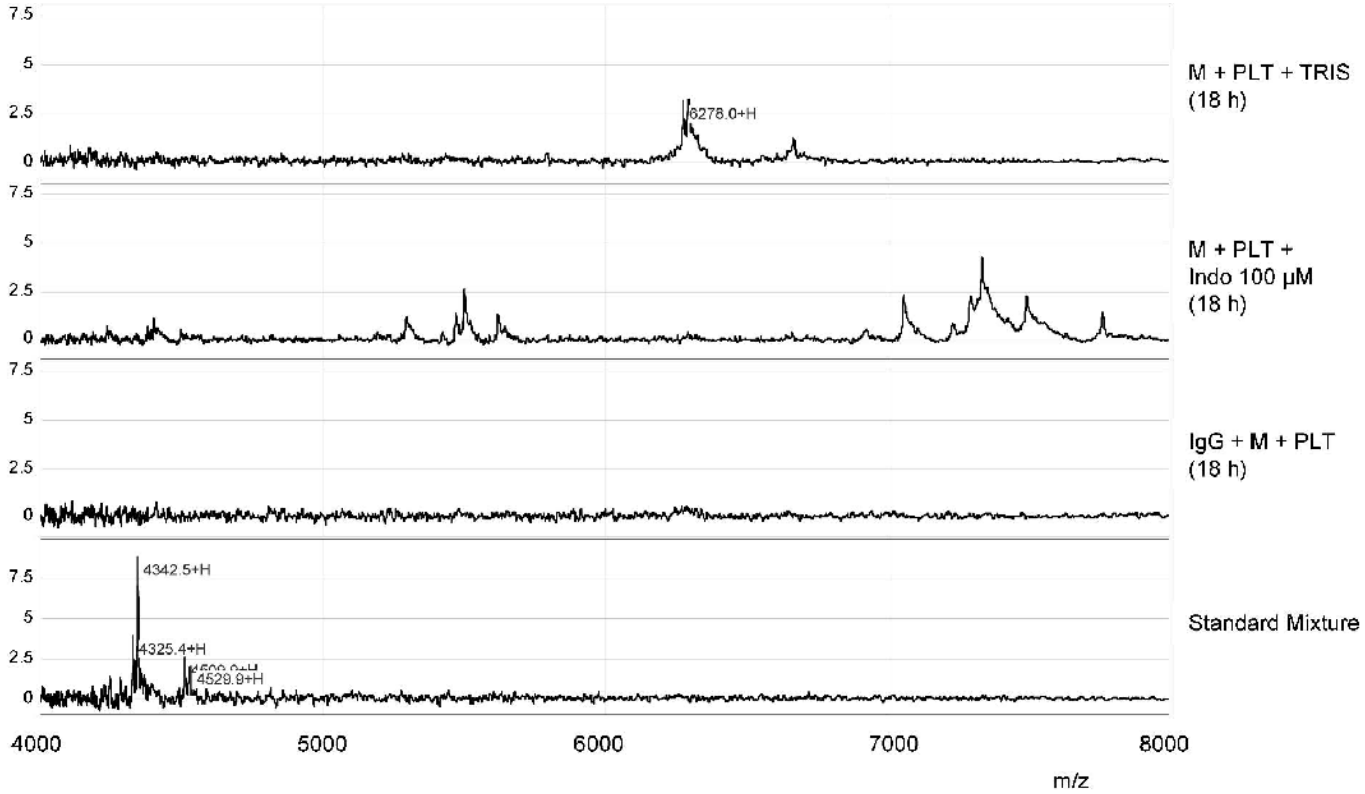


**FIGURE 4.** SELDI-TOF mass spectra of the lysate of co-incubations of J774 macrophages and blood platelets (M + PLT) for 18 hours in the presence (M + PLT + Indo 100 µM) or absence (M + PLT + TRIS) of 100 µM indomethacin. Incubation of macrophages and platelets for 18 hours in the presence of indomethacin inhibited the formation of the $[M + H]^+$ peak at m/z 6277.5. The standard, a mixture of $A\beta_{1\text{-}16}$, $A\beta_{1\text{-}40}$, and $A\beta_{1\text{-}42}$, gave the expected profile with $[M + H]^+$ peaks at m/z 1953.5 (not shown), 4343.5, and 4527.6, respectively. The IgG control was negative.

primed macrophages after stimulation with either peptide was too low to study the effect of inhibitors.[1] Since Ogawa et al[24] did not investigate whether the inhibition by the NSAIDs was specific for $A\beta_{1\text{-}40}$ by including an alternative stimulus, such as LPS, their experiments cannot be compared with the present study in which we used platelets as a stimulus. Furthermore, the active concentrations of indomethacin and ibuprofen were even higher than in the present study and could have interfered directly with the activity of iNOS, as reported by Aeberhard et al.[22] Since NSAIDs used in the present study did not interfere with the LPS-induced expression or activity of iNOS, it seems less likely that the concentrations used in the present study interfered with PPAR-γ nuclear receptors as proposed by Ogawa et al[24]. However, it cannot be excluded that some of their inhibitory effects were due to interference with iNOS- inducing pathways distal from Aβ-production.

Subsequently, SELDI-TOF mass spectrometry was used to substantiate the γ-secretase involvement. This technique demonstrated that incubation of macrophages with platelets resulted in a time-dependent formation of a peptide with a molecular mass of 6276.5 Da, containing an Aβ-like domain. The properties and the importance of this peptide are still unclear. Only its molecular mass is known and the fact that it contains the epitope recognized by the 6E10 antibody. Six theoretically possible Aβ-like peptides with a molecular mass of 6276.5 ± 5 Da remained after excluding peptides without the epitope of the 6E10 antibody on the SELDI chip. Two of these candidates deserve further consideration. The first one, which has a mass of 6275.1 Da, is 58 amino acids long and contains the complete $A\beta_{1\text{-}42}$ sequence (ie, amino acids 672–713 of full-length APP) at its C-terminal site, ending just at the γ-secretase cleaving site (Table 1). The difference between the theoretical mass and the measured mass was the lowest for this peptide as compared with the other five candidates. Another interesting candidate has a mass of 6273.3 Da, is 57 amino acids long and contains the complete $A\beta_{1\text{-}42}$ sequence at its N-terminal site, starting with the β-secretase cleaving site (Table 1). The exact identity of the Aβ-like peptide with a mass of 6276.5 ± 5 Da formed during platelet phagocytosis by macrophages remains to be established. Also the importance of the presence of the complete $A\beta_{1\text{-}42}$ sequence in this Aβ-like peptide remains to be determined, since $A\beta_{1\text{-}42}$ itself had only a limited effect on the ac-

tivation of both J774 macrophages, as discussed before, and microglia cells.[25] Nevertheless, the present study showed that addition of indomethacin to the co-incubations of platelets and macrophages inhibited the formation of this peptide, as well as nitrite production. This strongly suggests that the Aβ-like peptide with a mass 6276.5 ± 5 Da participates in macrophage activation following platelet phagocytosis.

In summary, our results demonstrate that NSAIDs known to alter the γ-secretase cleaving site of APP, as well as meloxicam, reduced macrophage activation following platelet phagocytosis, but not after LPS stimulation. Furthermore, NSAIDs like acetylsalicylic acid and naproxen, which do not affect γ-secretase cleavage were also without effect on macrophage activation after platelet ingestion. This indicates that the reduced macrophage activation is independent of COX-inhibition and points to an effect on γ-secretase. The inhibition of the formation of Aβ-related peptides by indomethacin, measured by SELDI-TOF, gives further support to the idea that NSAIDs act via interference with a γ-secretase-related pathway, although further research is necessary to identify the peptide(s). Finally, our findings indicate that the novel platelet-induced pathway of macrophage activation can be treated in a pharmacological way. This may open new perspectives for the development of specific drugs for intervention in the complex matter of plaque physiology.[5]

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