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ALTERATION OF UPTAKE AND DISTRIBUTION OF EICOSANOID PRECURSOR FATTY ACIDS BY ASPIRIN*

JOHN S. BOMALASKI,†‡§ JUAN ALVAREZ,‖ JOSEPH TOUCHSTONE‖ and ROBERT B. ZURIER†

†Rheumatology Section, Department of Medicine, and ‖Department of Obstetrics and Gynecology, University of Pennsylvania School of Medicine, Philadelphia, PA; and ‡V.A. Medical Center and Medical College of Pennsylvania, Philadelphia, PA, U.S.A.

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Abstract—Aspirin is an important drug in the treatment of numerous disorders, especially rheumatic diseases. Its several mechanisms of action include inhibition of prostaglandin production by acetylation of prostaglandin synthetase. To explore further the modulatory effect of aspirin on eicosanoid production, we examined its effect on uptake and incorporation of fatty acids into phospholipids of human peripheral blood monocytes. Aspirin ingestion by normal volunteers inhibited uptake of arachidonic acid and linoleic acid in monocytes cultured for 3 days. Similar inhibition was observed when cultured normal human peritoneal macrophages were treated with aspirin for 3 days. In contrast, monocytes cultured for 12 days from both normal volunteers who had ingested aspirin and normal cells treated with aspirin *in vitro* for the first 3 days of a 12-day culture period expressed an increased uptake of both arachidonic and linoleic acids. Similarly, incorporation of fatty acid into phosphatidylcholine was depressed in 3-day cultured cells but was increased in 12-day cultured cells. Thus, aspirin, whether administered *in vivo* or added *in vitro*, modulates cellular uptake and incorporation of eicosanoid precursor fatty acids and their insertion into membrane phospholipids in cultured human monocytes and macrophages.

Inhibition of prostaglandin synthesis is a well described mechanism of action of aspirin (ASA) and other nonsteroidal anti-inflammatory drugs (NSAID) [1–3]. However, the precise mechanisms responsible for this inhibition are not clear in all cases. ASA acetylates platelet prostaglandin synthetase (cyclooxygenase), and abolishes prostaglandin production [4–7]. However, nonacetylated salicylates may also inhibit prostaglandin production [1, 2] as do NSAIDs that are not salicylates [3]. Also, we have shown that ingestion by normal human volunteers of ASA in anti-inflammatory doses inhibits peripheral blood monocyte phospholipase C (PLC) activity, but not phospholipase A_2 (PLA_2) activity [8, 9].

Thus, the mechanism of ASA action appears to involve enzymatic alteration of both phospholipase C and prostaglandin synthetase activities. We therefore examined the effect of ASA on another factor important to control of PG production—cellular uptake and incorporation of fatty acids (FA) into membrane phospholipids (PL). This process helps regulate the amount of FA eventually available for hydrolysis from PL by lipases. Once cleaved from PL, these FA can serve as substrate for oxygenation by prostaglandin synthetase and lipoxygenase enzymes [10]. We found that ASA, whether administered *in vivo* or added *in vitro*, also altered cellular uptake of eicosanoid precursor FA and the site of their insertion into membrane PL of cultured human monocytes (M-MØ) and macrophages.

MATERIALS AND METHODS

Materials. Radiolabeled fatty acids were obtained from New England Nuclear (Boston, MA). Unlabeled phospholipids were obtained from Avanti Lipids (Birmingham, AL) and unlabeled fatty acids were from NuChek Prep (Elysian, MN). The solvents used were HPLC grade and obtained from Fisher Scientific (Philadelphia, PA) as was the acetylsalicylic acid. Thin-layer chromatography plates were from the Analtech Corp. (Newark, DE) or Whatman Chemical (Clifton, NJ). Cell culture media were obtained from GIBCO (Grand Island, NY). HP/b scintillation fluid was obtained from Beckman Diagnostics (Fullerton, CA). The Bradford protein dye reagent concentrate was obtained from Bio-Rad Laboratories (Richmond, CA).

Monocyte culture. Peripheral blood mononuclear cells were obtained from normal controls, from normal controls who had taken at least 3.25 g of aspirin per day for at least 3 days preceding the study (ASA *in vivo*), and from normal controls who had ingested 650 mg of acetaminophen for 3 days preceding phlebotomy. Monocytes were isolated using a standard centrifugation technique with Lymphocyte Separation Medium (Bionetics, Inc., Kensington, MD). Monocytes were further isolated by elution from plastic flasks coated with gelatin and serum as previously described [11]. Cells were pooled, centrifuged and resuspended at a concentration of 2×10^5

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§ Address correspondence to: John S. Bomalaski, M.D., Arthritis-Immunology Center, V.A. Medical Center, 39th and Woodland Ave., Philadelphia, PA 19104.

live cells/ml in Dulbecco's Modified Eagle's Medium (DME) supplemented with 10% heat-inactivated fetal calf serum (FCS), 10% heat-inactivated horse serum (HS), minimal essential amino acids, glutamine (4 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml) (DME 10/10).

Macrophage isolation. During the procedure of tubal ligation, the patient's peritoneal cavity is routinely washed with normal saline. The saline wash was provided by the Obstetrics and Gynecology Department (courtesy of Dr. S. Sondheimer) at the Hospital of the University of Pennsylvania. The cells were fed with DME 10/10 and centrifuged (500 *g*, 10 min, 4°), and the remaining cells were washed with ice-cold sterile distilled water to lyse red blood cells. Peritoneal macrophages (M-MØ) were incubated at 37° in a 5% CO_2/95% air incubator. After 3 hr supernatant fractions and nonadherent cells were removed, and adherent cells refed with DME 10/10.

Aspirin treatment in vitro. Fresh monocytes or peritoneal macrophages were allowed to adhere for 1 hr, and then nonadherent cells were removed by three washes with PBS. Cells from individuals who had ingested ASA (ASA *in vivo*) were overlaid with DME 10/10 and refed every 3 days for cells cultured 12 days. Peritoneal macrophages and monocytes from untreated controls were overlaid with ASA at a 5 mg/dl (5.5×10^{-4} M), 10 mg/dl or 30 mg/dl concentration in DEM 10/10 (ASA *in vitro*). At appropriate time points the media were removed; the cells were washed twice with PBS and then overlaid with fresh DME 10/10. For cells that were to be cultured for 12 days, medium was removed after 3 days and the cells were cultured for 9 more days (12-day cultured monocytes) or immediately used for determination of FA uptake as noted below. Thus, 12-day cultured monocytes from normal volunteers ingesting ASA (ASA *in vivo*) were 12 days away from ASA exposure, whereas 12-day cultured monocytes treated with ASA *in vitro* were 9 days away from ASA exposure.

Uptake of fatty acids. Uptake of fatty acids was performed as we have described [11]. Adherent cells were overlaid with 1 ml of fresh RPMI (Roswell Park Memorial Institute) 1640 medium containing 0.5 μCi of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (AA) ([^{3}H]AA, 87.4 Ci/mmol sp. act.) or [1-^{14}C]linoleic acid (LA) ([^{14}C]LA, 52.6 mCi/mmol). Medium was removed after 2-hr incubation periods at 37° under 5% CO_2/95% air. The cells were washed twice with cold PBS and overlaid with 1 ml of fresh 0.05% Triton X-100 for 1 hr or until cell disruption had occurred as determined by light microscopy. Aliquots of medium and lysate were removed for radioactivity determinations. Uptake (cell association) of FA is expressed as percent of added label that becomes cell associated per 10 μg cell protein. The average of all the controls in our experiments was 49,780 ± 480 dpm per 10 μg cell protein. Individual values were calculated using the control for that particular day; variations were about 1%.

Extraction of cellular lipids. Lipids were extracted following a modification of the procedure of Bligh and Dyer [12]. Cell lysate (0.8 vol.) was combined with 3 vol. of chloroform–methanol (1:2), followed by 1 vol. of chloroform and then 1 vol. of distilled water. The chloroform phase was removed, taken to dryness under nitrogen and resuspended in chloroform for thin-layer chromatography (TLC).

Thin-layer chromatography of lipids. Phospholipids (PL) were separated using 250 μm Whatman LK5 Silica Gel TLC plates (Whatman Chemical Separation, Inc., Clifton, NJ) by the method of Korte and Casey [13] with a solvent system of chloroform–ethanol–water–triethylamine (30:34:8:35) or by the method of Touchstone *et al.* [14] with a solvent system of chloroform–ethanol–water–triethylamine (30:34:8:30). Unlabeled PL standards (Avanti Polar-Lipids, Inc., Birmingham, AL) were cochromatographed and were visualized with fluorescein under an ultraviolet lamp (Ultra-violet Products, San Gabriel, CA) or processed for spectrodensitometry. For cells that had incorporated radioactive fatty acids, bands corresponding to PL standards were scraped from the plates into scintillation vials to which 0.5 ml of methanol and 10 ml of scintillation fluid were added, and counted in a scintillation counter.

Spectrodensitometric determination of lipids. Determination of lipids by this method was performed as we have described [15, 16]. In brief, cells in PBS were added directly to the TLC plates, air dried, and chromatographed. After drying, the plates were sprayed with $CuSO_4$, dried at room temperature, and then heated in an oven at 100°, and then at 170° for 10 min. The developed chro-

Table 1. Uptake of eicosanoid precursor fatty acids by 3-day cultured monocytes*

Fatty acid	Source	% Uptake
Linoleic	Control	5.3 ± 0.7
	Acetaminophen *in vivo*	5.2 ± 1.0
	ASA *in vivo*	1.5 ± 0.5†
	ASA *in vitro*	
	5 mg/dl	6.7 ± 1.2
	10 mg/dl	6.3 ± 0.9
	30 mg/dl	7.2 ± 1.4
Arachidonic	Control	5.0 ± 0.6
	Acetaminophen *in vivo*	4.9 ± 0.8
	ASA *in vivo*	2.3 ± 0.6†
	ASA *in vitro*	
	5 mg/dl	5.6 ± 1.2
	10 mg/dl	5.0 ± 1.1
	30 mg/dl	4.8 ± 1.1

* Uptake is expressed as mean ± SD (dpm recovered per dpm added per 10 μg cell protein) × 100%, as described previously [11]. N = 10 for control, 4 for ASA *in vivo*, 6 for ASA *in vitro*, and 2 for acetaminophen *in vivo*. Cells were obtained from normal donors and and from normal donors who had ingested either ASA *in vivo* (a minimum of 3.25 g/day) or acetaminophen (650 mg/day) for a minimum of 3 days before phlebotomy. Cells from normal individuals who had not ingested any drugs were also overlaid with ASA (ASA *in vitro*). Cells were cultured for 3 days. The supernatant fractions were removed, and the cells were washed with phosphate-buffered saline. Radiolabeled fatty acid uptake was then determined. The average control was 49,780 ± 480 dpm per 10 μg cell protein.

† $P < 0.05$ vs control.

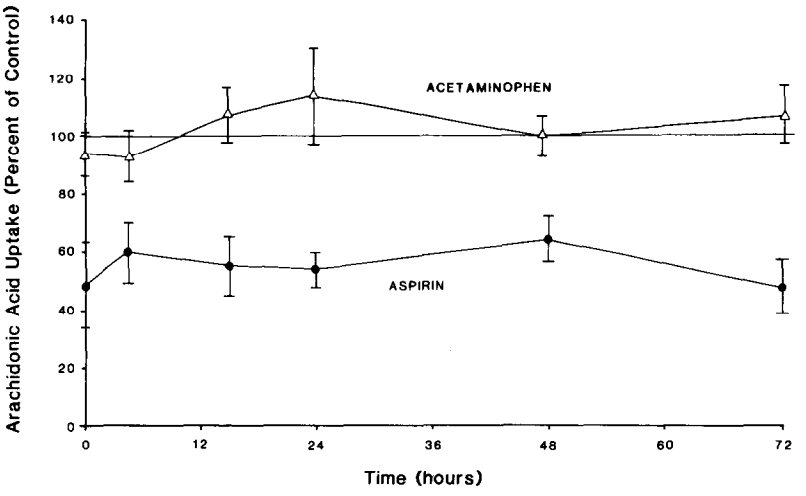

Fig. 1. Effect of aspirin and acetaminophen *in vivo* on [^{3}H]arachidonic acid uptake over time. Monocytes from normal controls, from normal donors ingesting a minimum of 3.25 g of ASA per day for at least 3 days preceding phlebotomy (●), and from normal donors ingesting 650 mg of acetaminophen for 3 days preceding phlebotomy (△) were separated, and [^{3}H]arachidonic acid uptake was determined as described in Materials and Methods. Time 0 was uptake in freshly processed cells exposed to [^{3}H]arachidonic acid for the 2-hr uptake period. Cells cultured for various time periods were also exposed to [^{3}H]arachidonic acid for the 2-hr uptake period. Results are expressed as percent of normal control cell uptake ± standard deviation. N = 3 for ASA *in vivo* and 2 for acetaminophen *in vivo*. Cells from normal donors ingesting aspirin (●) had significantly less uptake of [^{3}H]arachidonic acid ($P < 0.05$ by Student's *t*-test) compared to cells from normal control donors and cells from normal donors ingesting acetaminophen (△). The control value is stated in Table 1.

matograms were scanned in a Kontes Fiber Optic Scanner (model 800) using a 440 nm filter, and integration was accomplished with a Hewlett–Packard 3390A integrator.

Protein determination. Protein content of cell lysates was determined by the method of Lowry *et al.* [17] with bovine serum albumin as standard.

Statistical analysis. Statistical analysis was performed using Student's two-tailed *t*-test.

RESULTS

Uptake of fatty acids. The effects of ASA *in vivo* and *in vitro* on uptake of eicosanoid precursor FA linoleic acid (LA) and arachidonic acid (AA) in 3 day M-MØ are shown in Table 1. Uptake of FA—expressed as percent of added label that becomes cell-associated per 10 μg cell protein—by cultured monocytes from normal volunteers who had ingested ASA (ASA *in vivo*) was significantly lower for both LA (1.5 ± 0.5) and AA (2.3 ± 0.6) than for control cells (5.3 ± 0.7 LA; 5.0 ± 0.6 AA). This inhibitory effect of ASA *in vivo* was present immediately post-phlebotomy (time 0) and persisted to 3 days of culture (Fig. 1). ASA *in vitro* in various concentrations had no consistent effect on uptake of either LA or AA by cells cultured up to 3 days (data not shown). Acetaminophen, an analgesic compound that is not a cyclooxygenase inhibitor, did not affect radiolabeled FA uptake (Fig. 1). As cultured monocytes (M-MØ) express many biochemical, functional and morphological characteristics of normal macrophages (MØ) [18, 19], we examined the effect of ASA *in vitro* on peritoneal MØ from normal women undergoing elective tubal ligation (Table 2). ASA *in vitro* for 3 days at a concentration of 30 mg/dl significantly diminished the uptake of AA (6.5 ± 0.3 vs 15.4 ± 3.0).

Table 2. Uptake of fatty acid by normal peritoneal macrophages cultured for 3 days*

Fatty acid	Source	% Uptake
Linoleic	Control	12.7 ± 0.9
	ASA *in vitro* 30 mg/dl	11.1 ± 0.1
Arachidonic	Control	15.4 ± 3.0
	ASA *in vitro* 30 mg/dl	6.5 ± 0.3†

* Values are mean ± SD as described in Table 1. N = 10 for control cells and 3 for ASA-treated cells. Macrophages were cultured for 3 days, and then radiolabled fatty acid uptake was determined as described. See Table 1 for the control absolute value.

† $P < 0.01$ vs control cells.

To examine for the possibility of a rebound phenomenon following ASA exposure, uptake of LA and AA by 12-day M-MØ was also examined (Table 3). In contrast to uptake at 3 days, uptake of LA and AA by 12-day M-MØ was significantly greater for cells exposed to ASA *in vivo*, and to ASA *in vitro*.

Effects of aspirin on phospholipids. Following uptake, FA are incorporated into membrane PL. We therefore examined the effects of ASA on the distribution of radiolabeled AA in PL (Table 4). In 3-day ASA *in vivo* M-MØ, less AA was incorporated

Table 3. Uptake of eicosanoid precursor fatty acids by 12-day cultured monocytes*

Fatty acid	Source	% Uptake
Linoleic	Control	6.0 ± 0.5
	ASA *in vivo*	11.1 ± 0.4†
	ASA *in vitro*	
	5 mg/dl	14.2 ± 1.0†
	10 mg/dl	15.5 ± 2.4†
	30 mg/dl	13.7 ± 3.1†
Arachidonic	Control	6.1 ± 0.5
	ASA *in vivo*	10.8 ± 0.8†
	ASA *in vitro*	
	5 mg/dl	20.6 ± 1.3†
	10 mg/dl	22.8 ± 0.8†
	30 mg/dl	17.6 ± 4.0†

* Values are mean ± SD as described in Table 1. N = 10 for controls and 5 for ASA *in vitro* and 4 for ASA *in vivo*. Cells from normal individuals who had ingested ASA (ASA *in vivo*) were cultured for 12 days; thus, their last exposure to ASA was 12 days before, at the time of phlebotomy. Cells exposed to ASA *in vitro* were cultured in the presence of ASA for 3 days, and then the ASA-containing medium was washed away and replaced with fresh medium; thus, these cells were last exposed to ASA 9 days before radiolabeled uptake was determined. See Table 1 for the control absolute value.

† $P < 0.05$ vs controls.

into PC and PE, and more into PS compared to control cells. Similar results were seen in time course studies by 48 hr of culture (data not shown). ASA *in vitro* did not consistently affect incorporation of FA into PL in 3-day M-MØ. To examine further the effect of ASA *in vitro* on the composition of endogenous PL, we examined the effect of ASA *in vitro* on 3-day M-MØ PL by spectrodensitometry. We found that ASA *in vitro* significantly decreased the amount of endogenous PC (71.4 ± 16.2% of control; N = 4; $P < 0.05$), thus decreasing the PC/PE ratio.

Incorporation of radiolabeled AA into PL of 12-day M-MØ was also examined (Table 4). Cells treated with ASA both *in vivo* and *in vitro* incorporated significantly more AA into PC and less into PE compared to control cells.

DISCUSSION

Exposure of normal human monocytes to ASA *in vivo* or *in vitro* resulted in alterations in uptake of FA and their incorporation into membrane PL. Thus, ASA regulates the prostaglandin biosynthetic pathway at three sites: modulation of uptake and incorporation of FA into membrane PL, inhibition of phospholipase C activity and inhibition of prostaglandin synthetase.

Uptake of FA by cells cultured for up to 3 days from normal volunteers who had ingested ASA (ASA *in vivo*) was significantly less than control (Table 1 and Fig. 1). ASA *in vitro* had no consistent effect on FA uptake by 3-day cultured M-MØ (Table 1) nor 3-day cultured peritoneal macrophages from normal women undergoing tubal ligation (Table 2). However, uptake of LA and AA by 12-day M-MØ was significantly greater for ASA *in vivo* and ASA *in vitro* cells at all ASA concentrations tested (Table 3). Thus, 12-day cells, especially those from ASA *in vivo* donors, would appear to express a rebound phenomenon following the initial depression in uptake seen at 3 days (Table 1).

An ASA-induced rebound phenomenon in the prostaglandin biosynthetic pathway is not without precedent. It has been shown, for example, that prostacyclin production by human endothelial cells is enhanced approximately 36 hr after exposure to ASA *in vitro* [20, 21]. The ability of human platelets to regain prostacyclin and thromboxane synthetic

Table 4. Distribution of radiolabeled arachidonic acid into phospholipid*

	Radiolabeled AA (%)					
	Phospholipid					
Source	SP	PC	PS	PI	PE	PG
3 Day						
Control	9.5 ± 3.0	24.2 ± 4.5	15.6 ± 4.3	19.6 ± 4.1	17.3 ± 4.9	12.0 ± 3.9
ASA *in vivo*	10.9 ± 1.0	13.9 ± 1.2†	20.5 ± 0.5†	25.5 ± 2.6†	11.5 ± 0.4†	17.6 ± 1.6
ASA *in vitro*						
5 mg/dl	8.3 ± 2.1	25.2 ± 5.2	14.8 ± 3.9	20.2 ± 4.3	20.4 ± 3.4	11.2 ± 2.5
30 mg/dl	10.1 ± 2.1	18.1 ± 3.8	31.6 ± 6.0†	13.1 ± 1.7†	13.9 ± 3.5	12.4 ± 2.4
12 Day						
Control	7.1 ± 4.7	23.9 ± 6.9	10.2 ± 2.5	16.1 ± 3.1	25.2 ± 9.9	15.5 ± 8.0
ASA *in vivo*	10.3 ± 0.6	43.7 ± 3.2†	10.4 ± 1.4	16.8 ± 2.9	9.4 ± 1.3†	9.4 ± 0.1
ASA *in vitro*						
5 mg/dl	3.7 ± 0.9	53.6 ± 6.0†	11.3 ± 4.6	12.2 ± 8.6	12.7 ± 7.2†	5.5 ± 0.8
30 mg/dl	4.1 ± 0.5	56.7 ± 7.8†	8.6 ± 0.8	11.8 ± 5.1	15.8 ± 2.2†	4.2 ± 0.2

* Values are mean percent ± SD of total arachidonic acid incorporated into phospholipids for cells cultured for 3 and 12 days. N = 6 for control, 4 for ASA *in vitro* and 4 for ASA *in vivo*. See Table 1 for the control absolute value. Abbreviations: SP, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; and PG, phosphatidylglycerol.

† $P < 0.05$.

capacity varies with ASA dose, and a rebound phenomenon has been suspected to also occur in these cells [22, 23]. This may have clinical relevance as patients with rheumatoid arthritis who discontinue ASA compounds experience worsening of their disease following cessation of ingestion of cyclooxygenase inhibitors [24].

Studies presented here indicate that ASA also modulates the incorporation of FA into PL. In 3-day ASA *in vivo* M-MØ, incorporation of AA into PL was restricted (Table 4). Furthermore, this restriction of incorporation of FA into PC correlates with a reduction of total PC observed with spectrodensitometry. In contrast, in 12-day M-MØ ASA *in vivo* and *in vitro* significantly enhanced AA incorporation into PC (Table 4), again suggesting a rebound phenomenon. ASA at 1 mM has also been shown to increase radiolabeled AA incorporation into PL in female hamster lung [25].

PC is a major structural PL in normal human monocytes [26]. The eicosanoid precursors LA and AA account for 25–64% of PC acyl groups in rabbit alveolar MØ [27, 28]. Selective inhibitors of PC synthesis interfere with production of prostaglandins and leukotrienes by macrophages [29], demonstrating that eicosanoid production is linked to the availability of PC. PC is also the precursor of platelet-activating factor, another important modulatory lipid [30]. Interaction of the catalytic and regulatory proteins of adenylate cyclase is promoted by or requires PC and is, therefore, necessary for optimal production of cyclic AMP [31]. PC biosynthetic mechanisms also are linked to important biological events. Formation of PC from PE by the *S*-adenosyl-methionine pathway helps regulate phospholipase activation, prostaglandin production, and chemotaxis in monocytes, macrophages and other cells [32, 33]. Thus, changes in FA incorporation into PL and PC turnover in M-MØ may alter a variety of cell functions.

In summary, we have demonstrated that ASA *in vivo* and *in vitro* modulated the incorporation of eicosanoid precursor fatty acids into PL in cultured human peripheral blood monocytes and fresh human peritoneal macrophages. An initial inhibitory effect on FA uptake in cell cultured for up to 3 days was followed by enhanced uptake in 12-day cells. Furthermore, ASA modulated the composition of cell PL by decreasing total phosphatidylcholine content. Thus, ASA not only inhibits prostaglandin synthetase but regulates cellular lipid metabolism by altering both fatty acid uptake into cell phospholipids and the composition of membrane PL.

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