Release of tumor necrosis factor-α and prostanoids in whole blood cultures after in vivo exposure to low-dose aspirin

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Background: The preventive effect of low-dose aspirin in cardiovascular disease is generally attributed to its antiplatelet action caused by differential inhibition of platelet cyclooxygenase-1. However, there is evidence that aspirin also affects release of inflammatory cytokines, including tumor necrosis factor-α (TNF-α). It is not known whether this is caused by direct action on the cytokine pathway or indirectly through cyclooxygenase inhibition and altered prostanoid synthesis, or both.

Methods: We assessed the capacity of lipopolysaccharide-activated leukocytes in whole blood cultures of eight healthy subjects following a single oral dose of 80 mg aspirin to release TNF-α, prostanooid E2 (PGE2) and prostanooid I2 (PGI2), and thromboxane A2 (TXA2). TNF-α and prostanoids were determined by enzyme-linked immunoassays.

Results: In seven subjects, TNF-α release in blood cultures decreased 24 h after intake of aspirin. The effect of aspirin on prostanooid release was assessed in three individuals: PGE2 increased in all subjects, PGI2 increased in two and remained unchanged in one, and TXA2 was reduced in two and unchanged in one individual. The presence of DFU, a specific inhibitor of cyclooxygenase 2, did not affect the reduction of TNF-α release by aspirin, but abolished prostanooid production in all three individuals.

Conclusion: The capacity of activated leukocytes to release TNF-α is reduced by ingestion of low-dose aspirin, independent of changes in prostanooid biosynthesis.

Key words: Low-dose aspirin, Tumor necrosis factor-α, Cyclooxygenase inhibition, Prostanoids

Introduction

The demonstrated benefits of low-dose aspirin in the prevention of cardiovascular disease are generally explained by differential inhibition of the constitutive cyclooxygenase enzyme cyclooxygenase-1 (COX-1) in platelets, resulting in a reduced synthesis of vasoconstrictive and platelet aggregation stimulating thromboxane A2 (TXA2).1,2 The same mechanism is thought to be involved in the still disputed prevention of pre-eclampsia and fetal growth retardation (FGR) by low-dose aspirin.3-5 Recent studies have focused on the importance of release of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), in the pathophysiology of these vascular events.6-9 It is known that the biosynthesis and release of inflammatory cytokines by activated leukocytes are affected by prostanooid E2 (PGE2), prostanooid I2 (PGI2) and TXA2,10,11 produced simultaneously in activated mononuclear cells through induced cyclooxygenase COX-2. Results of two studies indicate that in vivo exposure to aspirin alters the release of TNF-α by peripheral blood mononuclear cells,12,13 but it is not clear whether this is a direct effect caused by inhibition of the cytokine pathway, an indirect effect due to inhibition of COX-1 and/or COX-2, or whether both mechanisms are involved.

Our study was designed to assess the effect of ‘in vivo’ exposure to a single low dose of aspirin on the release of TNFα, PGE2, PGI2 and TXA2 by lipopolysaccharide (LPS)-activated leukocytes in whole blood culture, and to investigate their interaction under the influence of aspirin. In some of our experiments, the effect of prostanooids TNFα synthesis was eliminated by blocking the synthesis of PGE2, PGI2 and TXA2 in LPS-activated monocytes with DFU (5,5'-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)furanone), a specific inhibitor of inducible COX-2.14
Materials and methods

Materials

Aspirin (acetylsalicylic acid, non-coated, 80 mg tablets) was obtained from Pharmachemie B.V. (Haarlem, The Netherlands). DFU was kindly provided by Merck Frosst (Quebec, Canada) through the courtesy of Dr. A. W. Ford-Hutchinson. TNF-α was obtained from Knoll (Ludwigshafen, Germany). Mouse anti-human TNF-α monoclonal antibody (mAb1) and biotinylated mouse anti-human TNF-α monoclonal antibody (mAb11) for enzyme-linked immunosorbent assay (ELISA) determinations were purchased from PharMingen (San Diego, CA, USA). Cell culture medium RPMI and supplements were obtained from Life Technologies B.V. (Breda, The Netherlands). Streptavidin-horseradish peroxide conjugate was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). LPS Escherichia coli O111 B4, bovine serum albumin (BSA), 3,3′,5,5′-tetramethylbenzidine (TMB) and Tween 20 (polyoxyethylene sorbitan monolaureate) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were from Merck (Darmstadt, Germany).

Study subjects

Nine healthy female, non-pregnant volunteers aged 24–35 years participated in the study. Informed consent was obtained from all subjects. Participants used oral contraceptives but no other drugs during at least 2 weeks before and during the experiments.

Sampling

Venous blood was obtained from all subjects immediately before (day 1, 8.00 a.m.) and 24 h after oral intake of 80 mg aspirin (day 2). One subject ingested 80 mg aspirin for 15 consecutive days and was sampled on days 1, 2, 9, 13 and 16. Blood samples were drawn into endotoxin-free vacutainers (Becton-Dickinson) with ethylenediamine tetraacetic acid as anticoagulant and processed immediately after sampling.

Whole blood cultures

Seven hundred and fifty microliters of blood were diluted with RPMI 1640 culture medium as already described and incubated with 4 μl DFU solution (5 μM in dimethylsulfoxide (DMSO)) for 15 min at 37°C and 5% CO2. LPS was added and blood cultures were processed as already described.

Inhibition of COX-2 in whole blood cultures

Seven hundred and fifty microliters of blood were diluted with RPMI 1640 culture medium as already described and incubated with 4 μl DFU solution (5 μM in dimethylsulfoxide (DMSO)) for 15 min at 37°C and 5% CO2. LPS was added and blood cultures were processed as already described.

Determination of TNF-α

TNF-α in blood culture supernatants was determined by an enzyme-linked immunosorbent assay (PharMingen ELISA) using one capturing monoclonal antibody. Briefly, 96-well microtiter plates were coated overnight at 4°C with mAb1 (25 ng/well) in 0.1 M NaHCO3 (pH 8.2). The plates were washed with washing buffer PBS (pH 7.4)/Tween 20 (PBS/Tween; 1000:1) and incubated for 2 h with a 3% (w/v) solution of BSA in PBS (200 μl/well). After washing with PBS/Tween, TNF-α standards and samples, diluted in complete RPMI 1640 culture medium, were applied and incubated overnight at 4°C. The microtiter plates were washed again with PBS/Tween and incubated with biotinylated monoclonal antibody mAb-11 (50 ng/well, incomplete RPMI 1640 culture medium) for 45 min at room temperature. After washing with PBS/Tween, the plates were incubated with horseradish peroxidase-labeled streptavidin (25 ng/well in Tris–HCl buffer (pH 8.0) with 0.1% gelatine, 0.1% caseine and 0.02% Tween). After incubation for 30 min at room temperature and washing with PBS/Tween, 100 μl TMB chromogene solution (100 μg TMB/ml sodium acetate solution (pH 5.5) with 1 μl of 3% H2O2) were added, incubated in the dark for 30 min at room temperature and the substrate reaction stopped with 1 M H2SO4 (100 μl/well). The absorbance per well was measured at 450 nm with a Medgenix EASIA reader, and TNF-α concentrations in samples calculated using the TNF-α standard calibration line. Intra-assay and interassay coefficients of variation were 10 and 15%, respectively, and the detection limit was 10 pg/ml. Results are expressed as nanograms of TNF-α per milliliter of blood or calculated as nanograms of TNF-α per 10^5 monocytes in the longitudinal experiment with subject 9. In subjects 5–7, TNF-α concentrations in culture supernatants were also determined with a commercially available enzyme amplified sensitivity immunoassay (EASIA; BioSource Europe S.A., Fleurus, Belgium) with three monoclonal antibodies detecting different epitopes on the TNF-α molecule. Intra-assay and interassay coefficients of variation were 6 and 10%, respectively, and the detection limit was 3 pg/ml.
Purification and determination of prostanoids

For purification of prostanoids in blood culture supernatants, a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) was prewashed with 10 ml absolute ethanol, 10 ml distilled water, and 2 ml air. One milliliter of culture supernatant was applied to the cartridge and the cartridge washed with 2 ml distilled water, and the prostanoids were eluted with 1 ml absolute ethanol, followed by 2 ml air. The eluate was dried at 40°C under vacuum in a Speed-Vacuum Concentrator, dissolved in assay buffer according to the instructions of the manufacturer, and analyzed.

TXA_2, with a half-life of approximately 30 sec under physiological conditions was measured as its stable metabolite TXB_2, and PGI_2 as its stable metabolite 6-keto-prostaglandin F_{1\alpha}. All determinations were performed using commercially available enzyme-linked immunoassays (EIA system; Amersham Pharma- cia Biotech UK, Little Chalfont, UK) with the following intra-assay and interassay coefficients of variation and detection limits, respectively: for PGE_2, 10%, 10%, and 40 pg/ml; for PGI_2, 7%, 15%, and 3 pg/ml; for TXB_2, 14%, 10%, and 3.6 pg/ml. Cross-reactivities with related substances were below 0.1%. The results were calculated as nanograms of prostanoid per milliliter of blood or per 10^5 monocytes.

Results

Change in TNF-α release in LPS-stimulated whole blood cultures after aspirin ingestion

In seven of the eight tested healthy women, oral ingestion of 80 mg aspirin induced a decrease of TNF-α release in LPS-stimulated blood cultures 24 h after aspirin intake when compared with values obtained before aspirin ingestion; in one subject, TNF-α secretion was unaffected by aspirin intake. Figure 1 shows a marked individual variation in time course and magnitude of the aspirin-induced change in TNF-α release, which was observed in five out of seven blood cultures already after 12 h of cultivation, and in seven cases after 24 and 48 h. The absolute values for TNF-α as determined for subjects 1–8 are summarized in Table 1.

In subjects 5, 6 and 7, in whom TNF-α determinations were repeated with the enzyme-amplified sensitivity immunoassay, absolute TNF-α values differed between the two immunoassays, but the trend in aspirin-induced changes of TNF-α release was identical in all three subjects.

Effect of COX-2 inhibition on changes in TNF-α release in LPS-stimulated blood cultures after aspirin ingestion

TNF-α release in LPS-stimulated whole blood cultures was investigated in the presence of the specific COX-

![Graph showing TNF-α changes](image)

### Table 1. Tumor necrosis factor-α (TNF-α) concentrations in blood culture supernatants after 12, 24 and 48 h of cultivation before and 24 h after intake of 80 mg aspirin in eight healthy women

| Subject number | TNF-α (ng/ml) after 12 h | TNF-α (ng/ml) after 24 h | TNF-α (ng/ml) after 48 h |
|----------------|-------------------------|-------------------------|-------------------------|
| 1              | 20.2, 16.7              | 16.7, 11.5              | 10.8, 6.7               |
| 2              | 61.1, 44.1              | 57.1, 29.7              | 30.1, 17.6              |
| 3              | 42.6, 39.3              | 38.3, 29.5              | 18.1, 5.8               |
| 4              | 65.3, 28.6              | 37.8, 12.7              | 11.1, 1.4               |
| 5              | 53.7, 51.2              | 43.6, 32.8              | 24.0, 12.4              |
| 6              | 99.7, 84.9              | 72.5, 53.9              | 23.1, 15.6              |
| 7              | 111.1, 73.4             | 72.7, 44.6              | 22.1, 13.6              |
| 8              | 30.6, 40.6              | 18.0, 18.5              | 5.1, 5.1                |

Data presented as before aspirin intake, after aspirin intake. TNF-α concentrations in lipopolysaccharide-stimulated blood cultures of subjects 1–8 were determined by an enzyme-linked immunoassay (Pharmingen) and are calculated as nanograms per milliliter of blood. In all subjects, TNF-α concentrations at 0 h were below the detection limit of the assay.
2 inhibitor, DFU, in subjects 5, 6 and 7 before and after intake of 80 mg aspirin. The maximal inhibitory dose of DFU was determined in preliminary tests (data not shown) and applied in a 10 times higher concentration in the experiments. Figure 2 shows that the presence of DFU during blood cultivation had no influence on the reduction of TNF-α release in blood cultures 24h after aspirin intake.

Effect of aspirin intake on release of PGE$_2$, PGI$_2$ and TXA$_2$ in LPS-stimulated whole blood cultures

The effect of a single dose of 80 mg aspirin on prostanoid release in LPS-stimulated whole blood cultures was investigated in the absence and presence of DFU in subjects 5, 6 and 7. All three subjects showed a marked increase in PGE$_2$ release already after 24h of cultivation time, whereas this increase rose with prolonged cultivation. The effect of aspirin on release of PGI$_2$ was less pronounced and differed individually; PGI$_2$ secretion showed a slight increase in subjects 5 and 6, and remained almost unchanged in subject 7. TXA$_2$ release was almost unchanged in subject 5, and fell slightly in subjects 6 and 7. In all three subjects, cultivation of blood in the presence of DFU reduced prostanoid production to a base level, and no effect of aspirin could be detected. In view of pronounced inter-individual differences, the results are presented for each case separately in Figure 3.

Effect of daily ingestion of 80 mg aspirin on release of TNF-α and prostanoids in LPS-stimulated whole blood cultures

In an additional experiment in one woman, designed to look into the consequences of continuous intake of 80 mg aspirin for the capacity of leukocytes to secrete TNF-α and prostanoids in LPS-stimulated blood cultures, subject 9 ingested aspirin for 15 consecutive days. Daily aspirin intake and preceding blood sampling were scheduled for 8.00 a.m. in order to avoid a circadian influence on cytokine secretion. Different white blood cell counts (Cymex NE 8000) performed for each blood sample showed a variation of leukocyte and leukocyte subtype numbers during the course of the experiment. Leukocytes varied between 4.9 × 10$^6$ and 6.1 × 10$^6$/ml, and monocytes between 3.7 × 10$^5$ and 6.8 × 10$^5$/ml. With the monocyte as the main source for release of TNF-α and prostanoids after LPS stimulation, analytical data were calculated per 10$^5$ monocytes and compared as values per 10$^5$ monocytes. TNF-α determinations were performed with the enzyme amplified sensitivity immunoassay (BioSource). Figure 4 shows the results of TNF-α determinations during the experiment. Daily ingestion of aspirin reduced the release of TNF-α in blood cultures after 12 days to a level of 48% of the value obtained before aspirin intake. In this subject, ingestion of aspirin for three more days achieved no further reduction of TNF-α secretion. The presence of DFU during blood cultivation did not change the results as presented in Figure 4. In subject 9, the release of all three prostanoids was reduced already after intake of one aspirin and reached a minimum after ingestion of 12 aspirin tablets, with no further reduction after intake of three more tablets. The presence of DFU in blood cultures abolished the release of prostanoids almost completely (Fig. 5).

Discussion

Preliminary experiments in unstimulated whole blood cultures of healthy individuals showed that TNF-α release was low, and aspirin ingestion reduced TNF-α levels to values below the detection level of the immunoassays. For these reasons, TNF-α release from leukocytes in blood cultures was stimulated with a maximal stimulating dose of *E. coli* LPS. The use of a whole blood culture model has advantages when compared with cultures of isolated monocytes or macrophages. It permits interaction between different leukocytes and between leukocytes and prostanoids released during cytokine biosynthesis, and it avoids depletion or enrichment of monocyte sub-populations during monocyte isolation or uncontrolled activation of monocytes by purification procedures.
Although the female volunteers in our study express the known inter-individual quantitative differences in LPS-induced TNF-α production, ingestion of one low dose of aspirin resulted in a reduction of TNF-α release by activated leukocytes in seven of eight subjects. This indicates a fundamental effect of aspirin intake on TNF-α biosynthesis. The observed inter-individual variability in aspirin-induced reduction of TNF-α release, varying from 20 to 66% at 24 h of blood culture, may be explained by differences in absorption, metabolism and clearance of the drug. It is questionable whether the observed reduction in TNF-α release in full blood cultures 24 h after ingestion of 80 mg aspirin is mediated by aspirin itself.
Aspirin has a half-life of 20–30 min in the circulation and is deacetylated in the liver to salicylic acid. Salicylate with a half-life of approximately 3 h at the usual therapeutic doses is cleared through the kidney as a glycine or glucuronic acid conjugate or metabolized to gentisic acid (2,5-dihydroxybenzoate). This suggests that salicylic acid and metabolites rather than acetylsalicylic acid may participate in the observed reduction of TNF-α release 24 h after aspirin ingestion. The inhibitory effect of aspirin on COX-2-induced prostaglandin biosynthesis in activated mononuclear cells is less pronounced than the blocking capacity of aspirin for constitutive COX-1, but aspirin and salicylate have been shown to inhibit PGE₂ release from LPS-stimulated monocytes in vitro, as well as in vivo after oral ingestion of aspirin. In contrast to these studies, our results show an increase of PGE₂ secretion in all three tested subjects after a single low dose of aspirin. The discrepancy in the effect on PGE₂ release could depend on the different dosage of aspirin and salicylate applied in other studies, where doses of 300 mg or more per day were used. The increase of PGİ₂ release after oral ingestion of 80 mg aspirin in two of the three subjects in our study was less pronounced than the effect on PGE₂ release, and of similar magnitude to the decrease in TXA₂ secretion. Inhibition of COX-2 with the specific COX-2 inhibitor DFU abolished prostanoïd release almost completely, which indicates that all three prostanoïds are products of LPS-induced COX-2 activity.

The three investigated prostanoïds are known to affect the biosynthesis of TNF-α. The effect of

FIG. 4. The effect of a daily intake of 80 mg aspirin on the release of tumor necrosis factor-α (TNF-α) from lipopolysaccharide (LPS)-stimulated blood cultures from a healthy woman. Subject 9 ingested 80 mg aspirin for 15 consecutive days. Blood was sampled immediately before aspirin intake (day 1) and on days 2, 9, 13 and 16. An automatic differential leukocyte count (Cysmex NE 8000) was performed in all blood samples. LPS-stimulated blood cultures were prepared, and TNF-α concentrations in blood culture supernatants measured by a Biosource enzyme immunoassay using three monoclonal antibodies for TNF-α detection and calculated as nanograms per 10⁵ monocytes.

FIG. 5. The effect of a daily intake of 80 mg aspirin on the release of prostanoïds from lipopolysaccharide (LPS)-stimulated blood cultures from a healthy woman. Subject 9 ingested 80 mg aspirin for 15 consecutive days. Blood was sampled immediately before (day 1) and after aspirin intake on days 2, 9, 13 and 16. Monocytes in blood samples were determined by differential leukocyte counting with a Cysmex NE 8000. LPS-stimulated blood cultures without and with the cyclooxygenase-2 inhibitor DFU (10⁻⁵ M in dimethylsulfoxide) were prepared, prostanoïds extracted from blood culture supernatants by absorption on C₁₈ cartridges and elution with absolute ethanol, and determined by immunoassays. Values for (A) prostanoïd E₂ (PGE₂), (B) prostanoïd I₂ (PGİ₂), and (C) thromboxane A₂ (TXA₂) were calculated as nanograms per 10⁵ monocytes. Concentrations of prostanoïds in blood cultures with DFU were all below the indicated reference lines.
PGE\textsubscript{2} on TNF-\(\alpha\) biosynthesis in activated monocytes is dose dependent: low concentrations of PGE\textsubscript{2} stimulate, and higher concentrations inhibit TNF-\(\alpha\) production.\textsuperscript{22} The stable PGI\textsubscript{2} analogs iloprost and cicaprost suppress TNF-\(\alpha\) synthesis in LPS-stimulated peripheral blood mononuclear cells.\textsuperscript{23} It has been shown that TXA\textsubscript{2} in activated monocytes functions as a paracrine or autocrine facilitator of TNF-\(\alpha\) synthesis, which is at least in part determined by the balance between PGE\textsubscript{2} and TXA\textsubscript{2} concentrations.\textsuperscript{11} Aspirin-induced changes in prostanthond concentrations could therefore affect TNF-\(\alpha\) release in stimulated blood cultures. Our results show that inhibition of prostanoïd biosynthesis by the specific COX-2 inhibitor DFU had no influence on the aspirin-induced reduction in TNF-\(\alpha\) release, indicating that it is induced through a pathway independent of prostanoid synthesis.

The production of TNF-\(\alpha\) in LPS-stimulated monocytes and macrophages is a multistep process that requires the liberation of the nuclear transcription factor KB (NF-KB)\textsuperscript{24} from an inhibitory protein, IKB. Release of NF-KB is achieved through phosphorylation and subsequent degradation of IKB by two cellular kinases, IKK-\(\alpha\) and IKK-\(\beta\).\textsuperscript{25} Aspirin and salicylate were shown to inhibit specifically the activity of IKK-\(\beta\), thus preventing the activation of NF-KB and the NF-KB-induced transcription of genes responsible for TNF-\(\alpha\) biosynthesis.\textsuperscript{21,26} It should be noted that inhibition of IKK-\(\beta\) activity by aspirin or salicylic acid in both \textit{in vitro} studies was achieved with concentrations equivalent to anti-inflammatory doses \textit{in vivo}. Our results, showing an aspirin-induced decrease in TNF-\(\alpha\) release from LPS-stimulated full blood, are in agreement with these fundamental observations, but it remains questionable whether plasma levels achieved after ingestion of 80 mg are sufficient to inhibit IKK-\(\beta\) activity. Our results are contrary to the results of two studies,\textsuperscript{1,13} in which oral ingestion of aspirin increased TNF-\(\alpha\) secretion from LPS-stimulated monocytes. It is possible that the higher doses of aspirin (300 and 325 mg/day) that were used in these studies induce responses in mononuclear cells that are different from those obtained with low-dose aspirin. The influence of LPS concentrations during stimulation of isolated monocytes or whole blood cultures on aspirin-induced changes of TNF-\(\alpha\) release was recently discussed by Osnes \textit{et al.}\textsuperscript{17} Stimulation with low concentrations of LPS as used in the aforementioned studies could activate signalling pathways different from those activated by high LPS concentrations that were used in our study and by Osnes \textit{et al.} in earlier experiments,\textsuperscript{21} where aspirin reduced TNF-\(\alpha\) release from LPS-stimulated monocytes. We cannot exclude the possibility that eicosanoids like 15-(\textit{R})-hydroxyeicosatetraenoic acid (15-(\textit{R})-HETE), produced by aspirin-acetylated COX-\(\textit{\textsubscript{2}}\)\textsuperscript{28} and known to reduce TNF-\(\alpha\) secretion by human monocytes,\textsuperscript{29} and lipoxigenase-mediated lipoxins participate in the observed effect of aspirin ingestion on TNF-\(\alpha\) release. However, the induction of COX-2 by LPS in our experiments was initiated 24 h after oral low-dose aspirin intake. By this time, aspirin should be completely metabolized.\textsuperscript{30,31} Acetylated COX-1 seems to be unable to synthesize HETE-like products.\textsuperscript{32}

In the single experiment with multiple intake of low-dose aspirin, the inhibition of TNF-\(\alpha\) release became stable after 12 days; ingestion of three more aspirin did not further reduce TNF-\(\alpha\) release. The permanent reduction of TNF-\(\alpha\) secretion in this experiment after continuous low-dose aspirin ingestion might be involved in the beneficial effects of the drug that were observed in pathological conditions like pre-eclampsia and FGR, where increased TNF-\(\alpha\) release was detected. This requires confirmation by further experiments.

To our knowledge this is the first report of the effect of low-dose aspirin after single and multiple intake on the release of TNF-\(\alpha\), PGE\textsubscript{2}, PGI\textsubscript{2} and TXA\textsubscript{2} in LPS-stimulated full blood cultures. Further studies with more subjects are needed to confirm and extend these first results. The question of whether salicylate and gentisic acid as metabolites of aspirin and/or lipid products of acetylated COX isoenzymes participate in the described effects on peripheral mononuclear cells remains to be answered.

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