

*Original Contribution*SYNERGISTIC INHIBITION OF CYCLOOXYGENASE-2 EXPRESSION BY
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Abstract—The use of aspirin in rheumatoid arthritis is limited since inhibition of the pro-inflammatory enzyme cyclooxygenase-2 occurs only at higher aspirin doses that are often associated with side effects such as gastric toxicity. Using a macrophage cell line (J774.1A), the present study explores possible synergistic effects of aspirin and vitamin E on the expression and activity of cyclooxygenase-2. Lipopolysaccharide-induced prostaglandin E₂ formation was significantly reduced by aspirin (1–100 μ M) or vitamin E (100–300 μ M). When combined with vitamin E, aspirin-dependent inhibition of prostaglandin E₂ formation was increased from 59% to 95% of control. Likewise, lipopolysaccharide-induced cyclooxygenase-2 protein and mRNA expression were virtually abolished by the combined treatment of aspirin and vitamin E, whereas the two agents alone were only modestly effective. Vitamin C did not mimic the actions of vitamin E under these conditions, suggesting that redox-independent mechanisms underlie the action of vitamin E. In agreement with this, vitamin E and aspirin were without effect on lipopolysaccharide-induced translocation of the redox-sensitive transcription factor NF- κ B. Our results show that co-administration of vitamin E renders cyclooxygenase-2 more sensitive to inhibition by aspirin by as yet unknown mechanisms. Thus, anti-inflammatory therapy might be successful with lower aspirin doses when combined with vitamin E, thereby possibly avoiding the side effects of the usually required high dose aspirin treatment. © 2000 Elsevier Science Inc.

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INTRODUCTION

Aspirin is one of the most widely used drugs in the treatment of pain and fever. Although other mechanisms may play a role, inhibition of cyclooxygenase (COX) activity and ensuing prostaglandin synthesis is still considered essential to its analgesic and antipyretic action [1]. A reduced synthesis of pro-inflammatory prostaglandins also accounts for the beneficial effect of aspirin in patients with rheumatoid arthritis. Under this condition prostaglandin formation is predominantly catalyzed by the inducible COX isozyme COX-2. COX-2 is expressed in rheumatoid tissue and promotes joint pain, swelling, and damage [2,3]. Aspirin inhibits COX-2 activity in the inflamed synovium and, in addition, suppresses pro-in-

flammatory pathways such as leukocyte accumulation. The dose required to relieve joint symptoms is rather high compared to its use in pain and fever [4,5]. In rheumatoid patients who can tolerate high doses, aspirin is considered a first-line agent, since it is safe, effective, and cheap [6]. However, at the usual anti-rheumatic dosing regimen of 3–6 g aspirin per day, a substantial number of patients experience gastric irritation [6,7]. This side effect is believed to result from the blockade of COX-1. COX-1 is constitutively expressed and has clear physiological functions in many tissues including the gastric mucosa, where it generates cytoprotective mediators such as prostacyclin [5].

The gastric toxicity of aspirin can be diminished by using enteric-coated formulations [6] or by chemically altering the molecule as in NCX-4016, a novel experimental aspirin derivative that contains and releases gastroprotective nitric oxide [8]. But despite these efforts, gastrointestinal side effects remain the major

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limitation to a wider use of aspirin in rheumatoid arthritis.

Recent studies have shown that patients with low intake or serum levels of the antioxidant vitamin E have an increased risk of developing rheumatoid arthritis [9,10] and that treatment with vitamin E alleviates some rheumatoid symptoms, in particular, pain [11,12]. Moreover, clinical evidence points to a reduced need for non-steroidal anti-inflammatory drugs in patients who are concurrently treated with vitamin E [12–14]. Additive anti-inflammatory effects of aspirin and vitamin E have also been demonstrated in rat adjuvant arthritis, an animal model of rheumatoid arthritis with a proven track record of predictability in humans [15,16].

Although beneficial actions of vitamin E in the therapy of osteoarthritis were described as early as the 1960s [13], the underlying mechanisms have remained obscure. COX-2 induction in immune cells and the ensuing formation of arachidonic acid metabolites are of crucial importance in the pathophysiology of rheumatoid arthritis. Therefore, and in order to explore potential synergisms, the present study investigates the effect of vitamin E, alone and in combination with aspirin, on COX-2 expression and activity in the murine macrophage cell line J774.1A.

METHODS

Materials

Lipopolysaccharide (LPS) from *Escherichia coli* (Sero type 0111:B4), Dulbecco's modified Eagle Medium (DMEM), the polyclonal antibody against prostaglandin (PG) E₂, aspirin and vitamin E (*d*- α -tocopherol acetate) were purchased from Sigma, Deisenhofen, Germany. Fetal bovine serum (FBS) was from Gibco, Eggenstein, Germany; 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Merck, Darmstadt, Germany. Nitrocellulose membranes and H³-PGE₂ were purchased from Amersham Buchler GmbH, Braunschweig, Germany. Polyclonal COX-2 antibody (rabbit antibody raised against murine COX-2) was obtained from Cayman, MI, USA. The COX-2 cDNA probe was a generous gift of Prof. Margarete Goppelt-Strübe, Department of Medicine, Erlangen-Nürnberg University, Germany. The anti-rabbit peroxidase conjugated secondary antibody (anti-rabbit IgG developed in sheep and linked to horseradish peroxidase) and enhanced chemiluminescence Western blotting kit as well as the DIG DNA labeling and detection kit were from Boehringer Mannheim, Mannheim, Germany. All other standard reagents and chemicals were of analytical grade and were obtained from different sources.

Cell culture

The murine macrophage cell line J774.1A was obtained from the American Type Culture Collection. The cells were grown in DMEM containing 10% FBS [17]. Cells were grown in a humidified incubator at 37°C and 5% CO₂.

Measurement of PGE₂ accumulation

The formation and release of PGE₂ into the cell supernatant was measured by radioimmunoassay as previously described [18,19]. Cells were grown in 96 well plates and treated with LPS (1 μ g/ml) for 24 h. Aspirin and/or vitamin E were added to the cells 30 min prior to LPS. After the incubation, aliquots were drawn from the supernatant and analyzed for PGE₂ content. Basal PGE₂ formation under control conditions was 44.59 ng/ml.

Cell viability analysis

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT to formazan [20]. At the end of each incubation, cells were incubated at 37°C with MTT (0.4 mg/ml, dissolved in culture medium) for 20 min. After that, the medium was removed and cells were solubilized in DMSO (200 μ l). The extent of cellular MTT reduction to formazan was quantitated by measurement of optical density at 540 nm to 630 nm (OD₅₄₀–OD₆₃₀) using a microplate reader (Biotek, EL 311c). Under the experimental conditions and various incubation protocols used in this study, cell viability remained unaffected.

COX-2 protein analysis

Cells were cultured in 6 well plates as described above. Aspirin and other potential modulators of COX-2 induction were added 30 min before LPS (1 μ g/ml). After a 24 h incubation, cells were washed and extracted as previously described [19]. The cell extract was boiled (10 min) at a 1:1 ratio with gel loading buffer and loaded onto discontinuous gels (4–10% Tris-glycine) for electrophoresis. The separated proteins were transferred to nitrocellulose membranes. The blot was incubated overnight at 4°C in blocking solution and then treated with the polyclonal COX-2 antibody at room temperature for 1 h. Thereafter the blot was incubated for 1 h with the secondary antibody. Finally the blot was developed for approximately 1 min with the enhanced chemiluminescence detection kit.

COX-2 mRNA analysis

COX-2 mRNA content was analyzed in macrophages grown in 150 mm culture dishes after treatment with LPS

and other agents for 6 h. Then cells were washed with PBS and pelleted by centrifugation. Total RNA was isolated using an RNA extraction kit (QuickPrep) according to the instructions of the supplier (Pharmacia, Freiburg, Germany). Northern blot analysis was performed as previously described [21]. Briefly, samples containing equal amounts of RNA (10–20 μ g) were separated in a 1% denaturing formaldehyde gel. Separated RNA was transferred onto a positively charged nylon membrane by vacuum transfer (500 mbar). The transferred RNA was fixed by baking at 120°C for 30 min. After that, the membranes were hybridized with a random primed digoxigenin-labeled murine COX-2 cDNA probe overnight at 50°C. The detection was carried out using the DIG detection kit from Boehringer Mannheim according to the instructions of the supplier. Equal loading was assessed by comparing the 28s ribosomal RNA bands after ethidium bromide staining of gels [19,22].

Nuclear extraction

After a 1 h incubation of cells with LPS, cells were scraped in cold PBS and centrifuged at $13,000 \times g$ for 10 min. Vitamin E and/or aspirin were given to the cells 30 min prior to LPS. The cell pellet was lysed in lysis buffer containing 10 mM Hepes-NaOH buffered to pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM DTT, 0.5% NP-40 (nonylphenoxy polyethoxy ethanol), and 1 mM PMSF (phenylmethylsulfonyl fluoride). The lysate was chilled in ice for 5 min and then centrifuged at $1500 \times g$ for 5 min to obtain nuclei. The nuclei were washed in lysis buffer without NP-40 and centrifuged again at $1500 \times g$ for 5 min. The pellet was resuspended in nuclear resuspension buffer containing 25 mM Tris-NaOH buffered to pH 7.8, then frozen and thawed three times to obtain nuclear protein.

Electrophoretic mobility shift assay

Mobility shift assays were performed as previously described [23]. DNA binding activity was determined after incubation of 5 μ g of nuclear protein extract with 10 fmol of a 32 P-labeled 22-mer oligonucleotide encompassing the NF- κ B site (5'AGT TGA GGG GAC TTT CCC AGG C3', Santa Cruz, CA, USA) in reaction buffer containing 10 mM Hepes-NaOH (pH 7.9), 1 mM DTT, 1 mM EDTA, 80 mM KCl, 1 μ g poly [dIdC][dIdC], and 4% Ficoll. After a 20 min incubation, the reaction mixture was electrophoresed on a 6% polyacrylamide gel. The gel was transferred to DE81 ion exchange chromatography paper (Whatman, Maidstone, UK) and dried down before exposure to autoradiographic film. Self

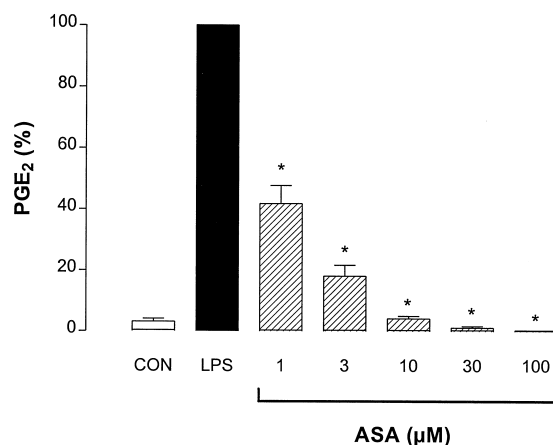


Fig. 1. Effect of aspirin (ASA) on LPS-induced PGE₂ formation in macrophages. Cells were incubated for 24 h with LPS. Aspirin was given to the cells 30 min prior to LPS. PGE₂ accumulation in the cell supernatant was carried out as described under Materials and Methods. * $p < .05$, treatment vs. control (CON), two-tailed t -test. All data shown are mean \pm SEM of $n = 12$ observations.

competitions were carried out under the same conditions using a 100-fold molar excess of the unlabeled NF- κ B oligonucleotide probe, which resulted in complete blockade of NF- κ B binding.

RESULTS

Effect of aspirin and vitamin E on the accumulation of PGE₂

A 24 h incubation with LPS (1 μ g/ml) induced PGE₂ accumulation in the supernatant of macrophages. Aspirin (1–100 μ M) or vitamin E (1–300 μ M) reduced PGE₂ accumulation by LPS in a concentration-dependent manner (Figs. 1 and 2). Simultaneous incubation with vitamin E (300 μ M) enhanced the inhibitory action of aspirin (1 μ M) and led to virtually complete blockade of LPS-induced PGE₂ formation (59% inhibition in the presence of aspirin alone, 95% inhibition in the presence of aspirin and vitamin E, Fig. 3).

Effect of vitamin E on inhibition of COX-2 protein expression by aspirin and salicylate

At concentrations between 10–100 μ M, aspirin and vitamin E did not affect the induction of COX-2 protein by LPS (data not shown). A marked inhibition of LPS-mediated COX-2 induction was observed when cells were incubated with 300 μ M of either aspirin or vitamin E. COX-2 protein induction was almost abolished when both substances were simultaneously added to the cells 30 min before LPS (Fig. 4A). Similar results were obtained with the aspirin metabolite salicylate under these conditions: Salicylate alone (300 μ M) elicited a substan-

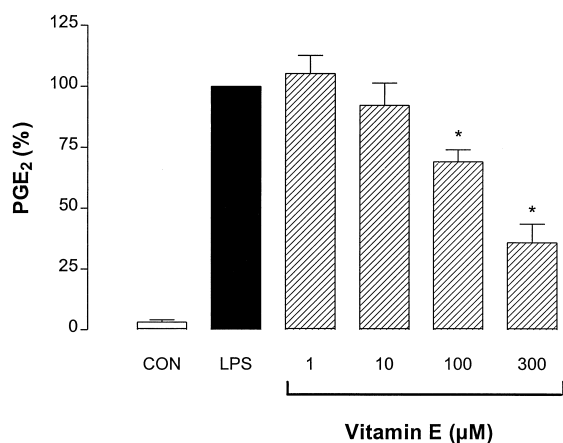


Fig. 2. Effect of vitamin E on LPS-induced PGE₂ formation in macrophages. Cells were incubated for 24 h with LPS. Vitamin E was given to the cells 30 min prior to LPS. PGE₂ accumulation in the cell supernatant was carried out as described under Materials and Methods. * $p < .05$, treatment vs. control (CON), two-tailed t -test. All data shown are mean \pm SEM of $n = 12$ observations.

tial inhibition of COX-2 protein induction, which was further enhanced to complete suppression upon simultaneous incubation with vitamin E (Fig. 4B).

Effect of vitamin C on inhibition of COX-2 protein expression by aspirin and vitamin E

In order to explore the effects of antioxidants other than vitamin E under these conditions, vitamin C (300 μ M) alone or in combination with aspirin was given to the cells 30 min prior to stimulation with LPS. In contrast to the inhibitory action of vitamin E, vitamin C alone

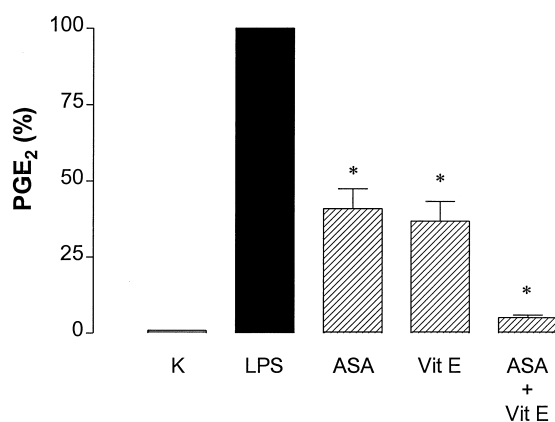


Fig. 3. Effect of aspirin (ASA) and/or vitamin (Vit) E on LPS-induced PGE₂ formation in macrophages. Cells were incubated for 24 h with LPS. Aspirin and vitamin E, alone or in combination, were administered 30 min prior to LPS. PGE₂ accumulation in the cell supernatant was carried out as described under Materials and Methods. * $p < .05$, treatment vs. control (CON), two-tailed t -test. All data shown are mean \pm SEM of $n = 12$ observations.

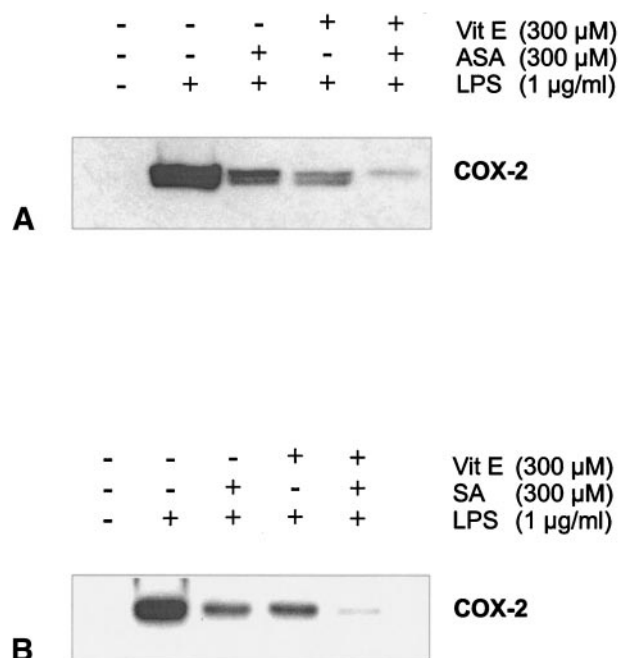


Fig. 4. Effect of vitamin (Vit) E on inhibition of COX-2 protein synthesis by aspirin (ASA) (A) and salicylate (SA) (B). Cells were incubated for 24 h with LPS. Aspirin, salicylate, and vitamin E, alone or combined as indicated, were given to the cells 30 min prior to LPS. Western blot analysis was performed as described under Materials and Methods. The data shown are representative of three experiments with similar results.

tended to augment LPS-induced COX-2 expression (1.4 ± 0.2 , fold increase LPS/vitamin C versus LPS alone, * $p < .05$, two-tailed t -test, densitometric data are mean \pm SEM of three independent experiments, see Fig. 5). Moreover, the combined treatment with vitamin C did not further enhance the inhibitory effect of aspirin on COX-2 expression (Fig. 5A). Since vitamin C is known to regenerate oxidized vitamin E and thus potentiate its radical scavenging action, COX-2 induction was measured after combined treatment with vitamins E and C. However, inhibition of COX-2 induction by vitamin E was not further enhanced in the presence of vitamin C (Fig. 5B).

Effect of aspirin and vitamin E on COX-2 mRNA expression

A pattern of action analogous to COX-2 protein synthesis was found when the effect of aspirin and vitamin E on COX-2 mRNA induction by LPS was assessed. Aspirin (300 μ M) or vitamin E (300 μ M) alone produced modest inhibitions of COX-2 mRNA expression. However, when administered in combination, vitamin E and aspirin nearly abolished COX-2 mRNA induction (Fig. 6).

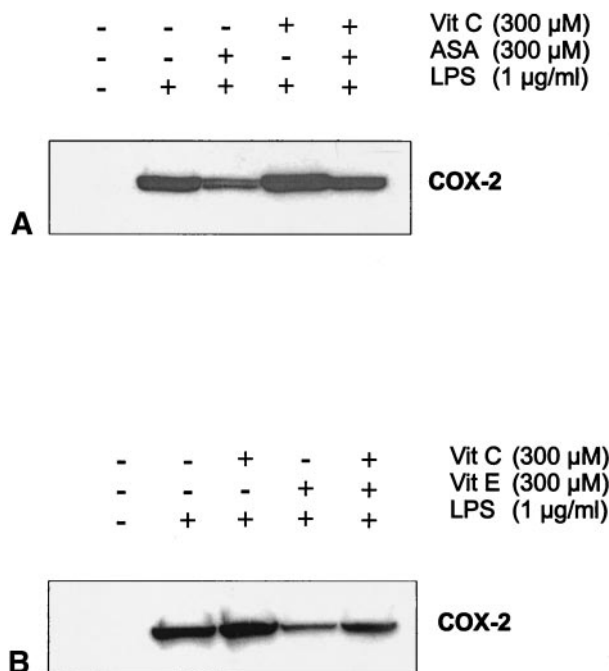


Fig. 5. Effect of vitamin (Vit) C on inhibition of COX-2 protein synthesis by aspirin (ASA) (A) and vitamin E (B). Cells were incubated for 24 h with LPS. Aspirin, vitamin E, and vitamin C, alone or combined as indicated, were given to the cells 30 min prior to LPS. Western blot analysis was performed as described under Materials and Methods. The data shown are representative of three experiments with similar results.

Effect of aspirin and vitamin E on NF- κ B translocation

Electrophoretic mobility shift assays demonstrated increased NF- κ B binding activity in nuclear extracts after

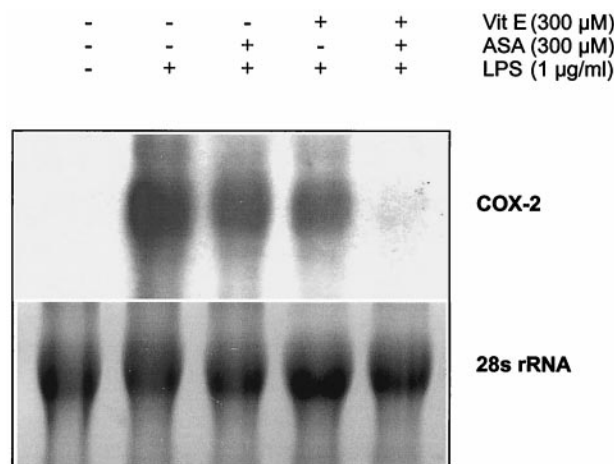


Fig. 6. Effect of aspirin (ASA) and vitamin (Vit) E on LPS-induced COX-2 mRNA expression in macrophages. Cells were incubated for 24 h with LPS. Aspirin and/or vitamin E were administered 30 min prior to LPS. Northern blot analysis was performed as described under Materials and Methods. The data shown are representative of three experiments with similar results.

a 1 h treatment of cells with LPS. Aspirin (300 μ M) or Vitamin E (300 μ M), administered alone or in combination 30 min before LPS, did not influence NF- κ B translocation. Results were verified by using a 100-fold molar excess of the unlabeled NF- κ B oligonucleotide probe, which resulted in complete suppression of the NF- κ B binding signal (Fig. 7).

DISCUSSION

In the present study we demonstrate the synergistic inhibition of LPS-mediated COX-2 induction by aspirin and vitamin E in macrophages. This finding may provide a mechanistic explanation for experimental and clinical observations showing that vitamin E alleviates rheumatic symptoms and that concurrent administration of vitamin E allows for dose reduction of NSAIDs without worsening of therapeutic efficacy [12–15].

Vitamin E was found to inhibit LPS-induced formation of PGE₂ at 100–300 μ M, which is within the range of plasma or tissue concentrations that can be expected during high-dose oral therapy [12,24–26]. Inhibition of prostaglandin formation was accompanied by a comparable decrease in COX-2 expression at the translational and transcriptional level. These observations are supported by a previous study reporting the prevention of age-associated increases in macrophage PGE₂ production and COX activity by vitamin E supplementation in rats [27]. Our findings, however, demonstrate for the first time that vitamin E is capable of inhibiting the induction of COX-2 in response to a pro-inflammatory stimulus. Since both enzyme activity and expression were reduced to a similar extent at the vitamin E concentrations tested, it can be assumed that inhibition of macrophage PGE₂ formation was largely a result of the diminished COX-2 expression. These findings, of course, do not entirely preclude an additional direct blockade of COX activity in the presence of vitamin E, as suggested by others [27]. In addition to the direct scavenging of tissue-destructive free radicals, a preventive effect of vitamin E on COX-2 induction, as shown here, may substantially contribute to the antirheumatoid action of vitamin E. Interestingly, inhibition of COX-2 expression by vitamin E appears unrelated to its antioxidant properties, because another potent antioxidant, vitamin C, did not reduce LPS-dependent COX-2 induction. In contrast to vitamin E, vitamin C tended to rather increase COX-2 expression in the presence of LPS, which points to a clear difference in the underlying mechanisms of action under these conditions.

Aspirin, at concentrations as low as 1 μ M, significantly reduced the accumulation of PGE₂ in macrophage cell supernatants. However, inhibitory effects on COX-2 mRNA and protein induction were seen at aspirin con-

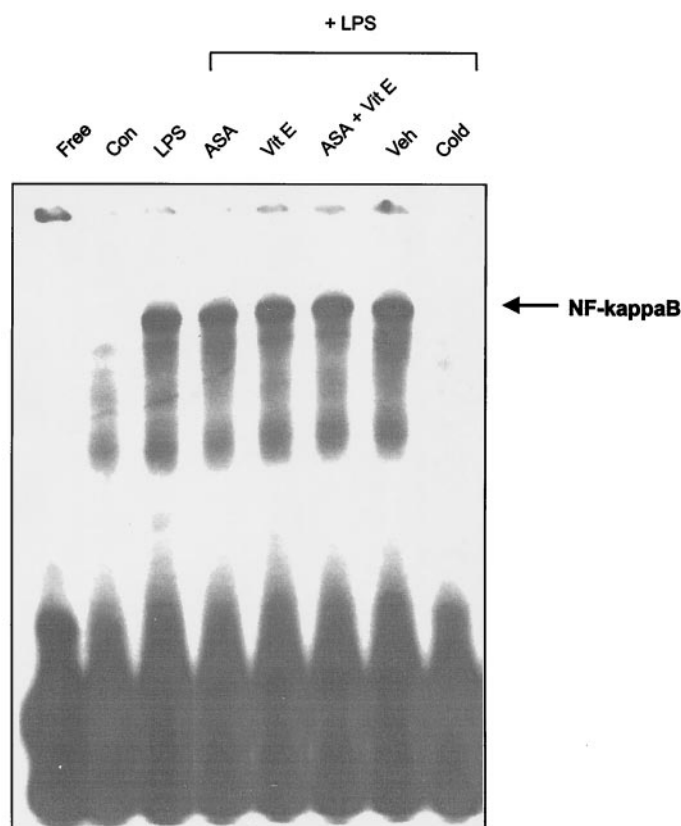


Fig. 7. Effect of aspirin (ASA) and vitamin (Vit) E on NF- κ B translocation. Cells were incubated for 1 h with LPS. Aspirin (ASA) and/or vitamin (Vit) E, or vehicle (Veh) were given to the cells 30 min prior to LPS. Nuclear extraction and electrophoretic mobility shift assays were performed as described under Materials and Methods. Results were verified by using a 100-fold molar excess of the unlabeled NF- κ B oligonucleotide probe (Cold), which resulted in complete suppression of the NF- κ B binding signal. The data shown are representative of three experiments with similar results (Free, labeled NF- κ B oligonucleotide probe in the absence of nuclear extract).

centrations two orders of magnitude above those required for a reduction of catalytic activity, indicating that in macrophages, COX-2 induction is far less sensitive to blockade by aspirin. Similar results were reported by Mitchell and co-workers [28], who observed diminished COX-2 transcription in lung epithelium at salicylate doses that were much higher than those leading to direct attenuation of enzyme activity. In contrast, expression of endothelial COX-2 appears to be more susceptible to blockade by aspirin or salicylate [29]. That aspirin does not readily suppress COX-2 induction in pro-inflammatory cells such as macrophages reflects the need for high doses in the treatment of inflammatory diseases such as rheumatoid arthritis.

The key finding of this investigation is our observation that vitamin E substantially enhances COX-2 inhibition by aspirin. At low aspirin doses (1 μ M) that only partially reduced PGE₂ formation, simultaneous addition of Vitamin E virtually abolished LPS-induced COX-2 activity. Moreover, combined application of vitamin E and higher aspirin doses (300 μ M) resulted in almost

complete suppression of COX-2 induction by LPS at both the transcriptional and translational levels. Again, vitamin C was without effect under these conditions and left aspirin-dependent inhibition of COX-2 protein synthesis unchanged, suggesting mechanisms other than those of antioxidant nature to underlie the action of vitamin E. Moreover, a combination with vitamin C did not further enhance the inhibitory effect of vitamin E on COX-2 protein expression. This finding provides additional evidence that suppression of COX-2 induction by vitamin E occurs through pathways unrelated to its antioxidant action, because the radical scavenging capacity of vitamin E is usually regenerated and potentiated in the presence of vitamin C. In agreement with this, vitamin E did not influence LPS-induced translocation of the redox sensitive transcription factor NF- κ B [30]. In most biological systems the nuclear transcription factor NF- κ B is involved in the regulation of COX-2 [1,31] and different groups including ours have shown that NF- κ B plays a decisive role in mediating LPS-induced COX-2 expression in macrophages such as the J774 cell line used in the

present study [19,32]. Although the role of aspirin as an inhibitor of NF- κ B activation and translocation is clearly established [33,34], mobility shift assays performed here showed that at COX-2 repressing concentrations, vitamin E and aspirin, alone or in combination, left NF- κ B translocation unaltered. It should be noted, however, that inhibitory effects on NF- κ B were previously detected only at concentrations between 2–10 mM aspirin [33–35]. This may explain why, at the far lower concentrations used in this study (100–300 μ M), aspirin did not change LPS-induced NF- κ B translocation. According to our results, the enhancement of aspirin action in the presence of vitamin E cannot be attributed to an increased affinity for, or inhibitory effect on, the NF- κ B system. Clearly, more research is needed in order to identify as yet unknown targets of aspirin and vitamin E in gene regulation and to further analyze the molecular mechanisms by which these agents exert their synergistic inhibition of COX-2 expression.

Additive and supra-additive actions of aspirin and vitamin E have been reported before, e.g., in endothelial protection and platelet inhibition [21,36,37], and are currently investigated in clinical trials aimed at the prevention of cancer and cardiovascular disease [38,39]. This study provides the first evidence for a synergism of the two compounds in immune cells at the level of COX-2 expression, which may have implications for the treatment of inflammatory disorders such as rheumatoid or osteoarthritis. Therapeutic relevance is also suggested by the fact that salicylate, the major anti-inflammatory metabolite of aspirin in vivo, interacted in a similar synergistic fashion with vitamin E. In particular, our findings reflect observations from clinical and experimental settings showing that vitamin E possesses anti-rheumatic activity and may reduce the need for concurrently used NSAIDs. According to the results presented here, co-administration of vitamin E constitutes a novel approach to render COX-2 more sensitive to inhibition by aspirin. Thus, anti-inflammatory therapy might be successful with lower aspirin doses when combined with vitamin E, thereby possibly avoiding the side effects of the usually required high dose aspirin treatment.

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ABBREVIATIONS

- COX-2—cyclooxygenase-2
- LPS—lipopolysaccharide
- DMEM—Dulbecco's modified Eagle Medium
- PG—prostaglandin
- FBS—fetal bovine serum
- MTT—3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromid