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A combination of aspirin and γ-tocopherol is superior to that of aspirin and α-tocopherol in anti-inflammatory action and attenuation of aspirin-induced adverse effects

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Abstract

Nonsteroidal anti-inflammatory drugs such as aspirin are used for pain relief and chemoprevention against cancer, but frequently cause gastric mucosal injury. We examined whether combinations of aspirin and α-tocopherol (αT) or aspirin and γ-tocopherol (γT), with αT and γT being the two major forms of vitamin E, are better anti-inflammatory agents than aspirin alone, and whether these combinations alleviate aspirin-associated side effects. In the carrageenan-induced air-pouch inflammation model in the rat, aspirin (150 mg/kg) or a combination of aspirin and γT (33 mg/kg) inhibited proinflammatory prostaglandin E_2 (PGE_2) by 70% ($P<.02$) at the inflammation site 6 h after inflammation was initiated. However, at 18 h, only the combination decreased exudate volume (15%; $P<.05$) and showed modest inhibition of PGE_2 (40%; $P<.07$) and lactate dehydrogenase activity (30%; $P=.07$) in the fluid collected at the inflammation site. γT, but not αT, spared aspirin-induced reduction in food intake, partially reversed aspirin-depressed gastric PGE_2 and attenuated stomach lesions. Surprisingly, the combination of aspirin and αT (33 mg/kg) did not show more benefits than aspirin alone, but worsened gastric injury and food intake reduction. Our study demonstrated that a combination of aspirin and γT, but not a combination of aspirin and αT, has some advantage over aspirin alone in terms of anti-inflammatory effects and attenuation of aspirin-induced adverse effects. This combination may be useful in complementing aspirin in the treatment of chronic inflammatory conditions and cancer.

Keywords: Vitamin E; NSAIDs; Cyclooxygenase; PGE_2; Inflammation

1. Introduction

Cyclooxygenase (COX)-catalyzed conversion of arachidonic acid into proinflammatory eicosanoids, including prostaglandin E_2 (PGE_2), plays a key role in the regulation of inflammatory response. During inflammation, inducible COX-2 is primarily responsible for PGE_2 generation at the inflammation site, in contrast to the constitutively expressed COX-1, which is found in many tissues, including the stomach, where prostaglandins are generated to help maintain physiological activities [1–3]. Nonsteroidal anti-inflammatory drugs (NSAIDs) targeting COX-1 and/or COX-2 have widely been used for the treatment of inflammatory diseases such as arthritis. However, NSAIDs, including aspirin, are known to have adverse effects, including upper gastrointestinal injuries such as ulcers [4,5]. Aspirin causes gastrointestinal problems, in part due to its irreversible inhibition of the COX-1-mediated formation of gastric PGE_2, which is important for cytoprotection [6,7]. In addition, the stomach damage caused by aspirin appears to involve oxidative stress [5,8]. Therefore, addition of antioxidants such as vitamin E has been proposed to alleviate the aspirin-associated side effect. The combination of aspirin and α-tocopherol (αT), the major form of vitamin E in tissues, has been investigated in animals and clinical trails, but these studies have yielded varied outcomes [9–12].

Recent studies indicate that γ-tocopherol (γT), the predominant form of vitamin E in US diets, has unique bioactivities compared with αT [13–15]. We have shown that γT, but not αT, has anti-inflammatory properties

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mediated through the inhibition of COX-2-catalyzed proinflammatory PGE_2 in cultured cells and in a rat inflammation model [16,17]. γT is better than αT in inhibiting lipid peroxidation and in scavenging electrophilic products of inflammation such as peroxynitrite and other reactive nitrogen species [18–20]. Because γT has both anti-inflammatory and antioxidant activities, we hypothesize that a combination of aspirin and γT may show a stronger anti-inflammatory effect and may alleviate aspirin-induced stomach injury. To test this hypothesis, we compared the anti-inflammatory action of a combination of aspirin and γT to the anti-inflammatory action of aspirin alone in the rat in a carrageenan-injected air-pouch model. The effect of γT on the attenuation of aspirin-related stomach injury was also examined. For comparison, the combination of aspirin and αT was tested.

2. Materials and methods

2.1. Chemicals

αT (~99%) and γT (95–97%) were purchased from Acros Organics (New Jersey, USA) or Sigma (St. Louis, MO, USA). Carrageenan, aspirin and all other chemicals were from Sigma.

2.2. Carrageenan-induced inflammation in the air-pouch model

The protocol on animal use was approved by the animal care committee at Purdue University and was strictly followed. Male Wistar rats (250–330 g; Charles River, California, USA) were caged singly and fed 2018 Teklad Global 18% Protein Rodent Maintenance Diet (Harlan Teklad, Indiana, USA) ad libitum, with free access to tap water. An air pouch was created by a subcutaneous injection of 12 ml of sterile air into the intrascapular area of the rat's back. Thirty hours later, 2 ml of 0.6% carrageenan or phosphate-buffered saline (PBS) (as noninflammed controls) was injected into the air pouch. αT and γT were first dissolved in tocopherol-stripped corn oil (Dyets, Inc., Bethlehem, PA, USA), and then aspirin was added. Prior to each feeding, the aspirin mixtures were vigorously mixed. Animals were fed aspirin (150 mg/kg body weight), aspirin+γT (33 mg/kg) or aspirin+αT (33 mg/kg) continuously for 3 days by gavage using 0.5 ml of tocopherol-stripped corn oil as vehicle. Control animals received the same volume of tocopherol-stripped corn oil. Immediately after the third gavage, carrageenan was injected into the pouch to initiate inflammation. Six or 18 h later, the rats were sacrificed, and pouch fluid was collected by lavage with Hanks-buffered saline solution containing 0.004% heparin, but no Ca^{2+}/Mg^{2+}. After a brief centrifugation, the supernatant was collected and frozen immediately for the measurement of PGE_2, leukotriene B4 (LTB_4), tumor necrosis factor-α (TNF-α) and so on. Total cells were counted with a hemocytometer.

2.3. Evaluation of stomach lesion

After the rats had been sacrificed, their stomach was removed, cut open along the greater curvature and rinsed in saline. Macroscopic lesions were assessed. Scores were given for different grades of injury: 0 (*no lesion*); 1 (*slight injury*; less than one gastric ulcer); 2 (*intermediate injury*; one to two gastric ulcers); 3 (*severe injury*; more than two gastric ulcers). Ulcer lesion is defined as a mucosal erosion equal to or greater than 0.3 cm.

2.4. Measurement of αT and γT

Plasma and exudate αT and γT were extracted using a mixture of methanol/hexane (2:5, vol/vol) in the presence of 0.8 mM butylated hydroxytoluene [21,22]. After a brief centrifugation at 4°C, the top hexane layer was dried under N_2, and the residue was resuspended in ethanol. Tocopherols were separated on a Supelcosil LC-18-DB column (150×4.6 mm, 5 μm; Supelco, Bellefonte, PA, USA) and eluted with 95:5 (vol/vol) methanol/0.1 M lithium acetate (final concentration of 25 mM, pH 4.75) at a flow rate of 1.3 ml/min. Tocopherols were monitored by coulometric detection (Model Coulochem II; ESA, Inc., Chelmsford, MA, USA) at 300 mV (upstream electrode) and at 500 mV (downstream electrode) using a Model 5011 analytical cell.

2.5. Measurement of PGE_2, LTB_4, 8-isoprostane, lactate dehydrogenase and TNF-α in exudate

The exudate was mixed vigorously with 2 ml of methanol to precipitate proteins, and with 5 ml of hexane to remove lipids. Following a brief centrifugation and aspiration of the hexane layer, the methanol layer was removed and evaporated under N_2. PGE_2, LTB_4 and 8-isoprostane were measured using the corresponding ELISA kits from Cayman Chemicals (Ann Arbor, MI, USA). TNF-α and lactate dehydrogenase (LDH) in the exudate were measured directly using an ELISA kit from R&D Systems (Minneapolis, MN, USA) and an analytical kit from Roche (Indianapolis, IN, USA), respectively.

2.6. Measurement of PGE_2 and 8-isoprostane in stomach mucosa

Gastric mucosal samples were weighed and homogenized in 400 μl of methanol at 4°C. After a brief centrifugation to remove unbroken tissues, the supernatant was collected and dried under N_2. The residues were reconstituted in 200 μl of PBS. PGE_2 and 8-isoprostane (pg/mg tissue) were measured with ELISA assays (Cayman Chemicals).

2.7. Statistics

One-way analysis of variance was performed in all data analyses, and unpaired Student's *t* tests were performed to compare the two treatment groups. Data are expressed as mean±S.E.M.

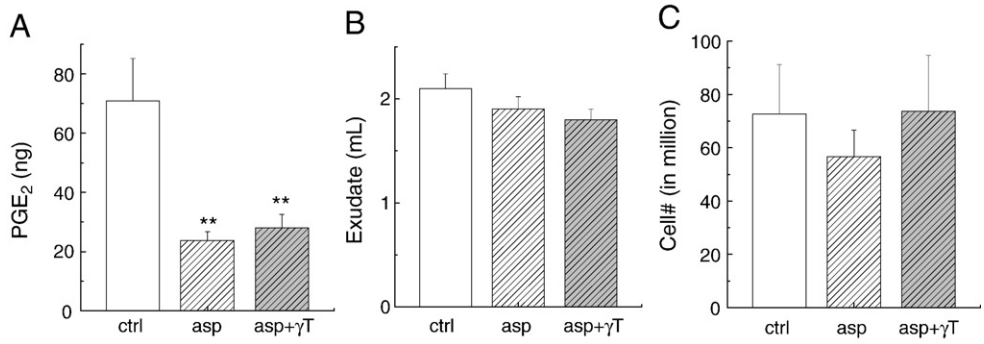

Fig. 1. The effects on PGE_2, exudate volume and number of infiltrating immune cells at the inflammation site at 6 h after carrageenan injection. Male Wistar rats were fed aspirin at 150 mg/kg (asp; n=6) or a combination of aspirin and γT at 33 mg/kg (asp+γT; n=6) by gavage using 0.5 ml of tocopherol-stripped corn oil as vehicle for 3 days before carrageenan injection. Control (ctrl; n=6) animals received the same volume of tocopherol-stripped corn oil. Six hours after the induction of inflammation, the effects on PGE_2 (A), exudate volume (B) and immune cell infiltration (in millions) (C) were determined. Results are expressed as mean±S.E.M. **A statistically significant difference between asp or asp+γT and ctrl (P<.02).

3. Results

3.1. Effect of aspirin or combinations of aspirin and γT or αT on inflammatory response

In a carrageenan-induced inflammation model in the rat, a single injection of carrageenan caused potent localized inflammation, as indicated by a marked increase in white cell infiltration, eicosanoid formation and tissue damage [23]. This model is known to mimic the pathological process occurring in joint diseases [23,24] and has been used to evaluate the anti-inflammatory efficacy of NSAIDs. In the current study, aspirin (150 mg/kg) or the combination of aspirin and γT or αT (33 mg/kg) was administered by gavage using corn oil as vehicle for 3 days. Aspirin at a similar dose is known to have anti-inflammatory effects and has been shown to cause stomach lesion. The dosage of γT represents a relatively high supplementation dose at which it exhibits anti-inflammatory effects in vivo [16].

Six hours after carrageenan injection, there was a marked increase in proinflammatory eicosanoids in the pouch compared with that in noninflammed controls, among which PGE_2 (70.9±14.3 vs. 0.1±0.06 ng/pouch) was quantitatively predominant to others such as LTB_4 (0.33±0.22 vs. 0.05±0.03 ng/pouch). Carrageenan treatment also caused a significant increase in TNF-α (4.7±2.1 vs. 0.3±0.2 ng/pouch). Administration of aspirin or aspirin+γT reduced PGE_2 by 70% (P<.02) at the site of inflammation (Fig. 1A), whereas neither showed a significant effect on exudate volume (Fig. 1B) or on the number of infiltrating immune cells (Fig. 1C). Aspirin alone or in combination with γT did not have a significant effect on LTB_4 or TNF-α (data not shown).

Eighteen hours after the initiation of inflammation, PGE_2 levels at the inflammation site remained elevated, whereas LTB_4 and TNF-α in the pouch dropped to the basal levels of noninflammed controls (data not shown). Compared with corn-oil-treated rats, animals fed the combination of aspirin and γT, but not aspirin alone or aspirin and αT, had 40% (P<.07) reduced PGE_2 (Fig. 2A) and 15% (P<.05) decreased exudate volume (Fig. 2B). None of the treatments affected immune cell infiltration (Fig. 2C). Inflammation-associated tissue damage was assayed by an increased

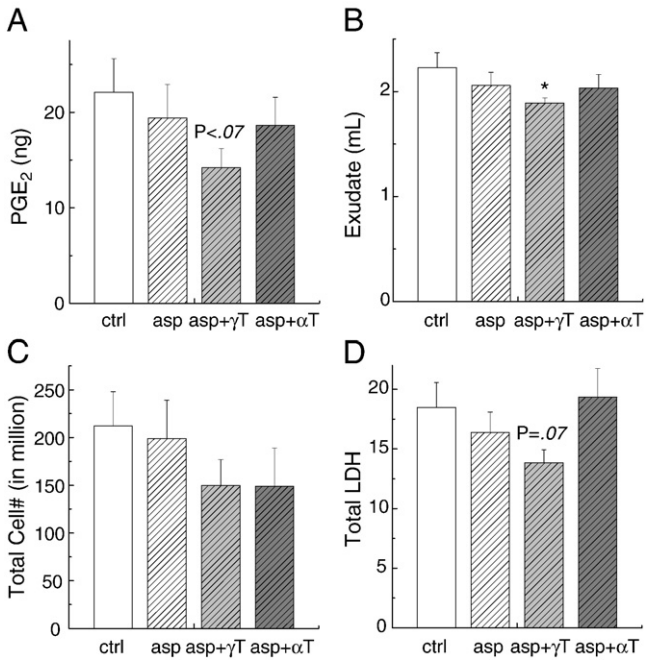

Fig. 2. The effects on PGE_2, exudate volume, immune cell infiltration and LDH at the inflammation site at 18 h after carrageenan injection. Male Wistar rats were fed aspirin at 150 mg/kg (asp; n=11), a combination of aspirin and γT at 33 mg/kg (asp+γT; n=11) or a combination of aspirin and αT at 33 mg/kg (asp+αT; n=11) by gavage using 0.5 ml of tocopherol-stripped corn oil as vehicle for 3 days before carrageenan injection. Control (ctrl; n=11) animals received the same volume of tocopherol-stripped corn oil. Eighteen hours after the induction of inflammation, the effects on PGE_2 (A), exudate volume (B), cell infiltration (C) and LDH (D) in the exudate were evaluated. Results are expressed as mean±S.E.M. *A statistically significant difference between treated rats and corn oil controls (P<.05).

release of cytosol LDH—a marker of cytotoxicity and tissue damage [23]. The combination of aspirin and γT showed a tendency towards decreasing (~30%; P=.07) LDH in the exudate (Fig. 2D).

3.2. Plasma and exudate concentrations of αT and γT

To evaluate the relative bioavailability of the administrated compounds, we measured the concentrations of γT and αT in the plasma and exudate. Feeding of αT or γT led to significant increases in the corresponding tocopherols in the plasma. Compared with aspirin-fed rats, αT-administrated animals had a 1.5-fold increase in this tocopherol, and γT-fed rats had a 3-fold elevation of γT in the plasma (Fig. 3A and B). Similarly, rats administered αT or γT had a twofold increase (P<.05) or a fivefold increase (P<.01) in the respective tocopherol in the exudate (Fig. 3C and D). Like previous studies [25,26], αT caused a significant decrease in γT in the plasma (73%; P<.01) and exudate (77%; P<.01). Aspirin alone nonsignificantly lowered αT, while aspirin+γT significantly decreased αT in the plasma (16%; P<.05) and exudate (24%; P<.05), respectively. This reduction in αT seems to be associated with aspirin because the concentrations of αT were similar in rats fed aspirin and in rats fed aspirin+γT (Fig. 3A and C), and γT has been shown to have no significant impact on αT or to sometimes increase αT [25,26].

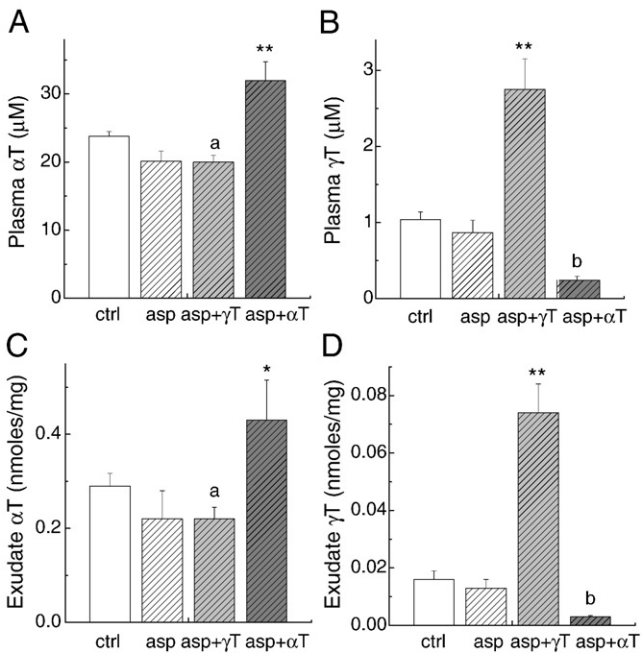

Fig. 3. Plasma and exudate concentrations of αT (A and C) and γT (B and D). The conditions of each treatment are the same as those indicated in Fig. 2. *P<.05 or **P<.01 (a significant difference between the combinations and aspirin alone). [a]A significant difference in αT concentrations between ctrl and asp+γT (P<.05). [b]A significant difference in γT concentrations between asp+αT and asp (P<.01).

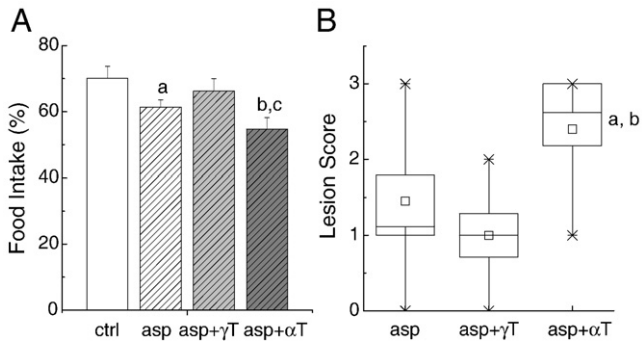

Fig. 4. Effects on food intake (A) and stomach injury (B). The conditions of each treatment are the same as those indicated in Fig. 2. The food consumption (%) of individual animals (A) is expressed as the ratio of food intake measured after carrageenan injection to food intake measured before carrageenan injection. Results are expressed as mean±S.E.M. [a]A statistically significant difference between the asp-treated rats and the corn-oil-administrated animals (P<.05). [b]A statistically significant difference between the asp+αT-treated rats and the corn-oil-administrated animals (P<.01). [c]A significant difference between the asp+γT-administered rats and the asp+αT-administered rats (P<.05). Stomach lesion (B) was expressed in a box plot showing the box as S.E. and whiskers of 5–95%. [a]A significant difference between treatment with asp and treatment with asp+αT (P<.05). [b]A significant difference between treatment with asp+γT and treatment with asp+αT (P<.01).

3.3. Effects on food intake, stomach lesion, and gastric PGE_2 and 8-isoprostane

We previously showed that carrageenan treatment led to a 30–40% reduction in food intake during the first 20 h after the initiation of inflammation [16]. Similarly, in the current study, carrageenan injection caused a 30% reduction in food intake (Fig. 4A). Aspirin treatment led to a further and significant decrease (12.4%; P<.05) in food intake compared with corn oil controls (Fig. 4A). γT supplementation spared aspirin-induced decrease in food intake (Fig. 4A). On the other hand, the combination of aspirin and αT appeared to aggravate aspirin-induced food reduction, as indicated by 22% (P<.01), 11% (P=.12) and 18% (P<.05) reductions in food consumption compared with corn oil controls, aspirin alone and aspirin+γT, respectively (Fig. 4A).

Consistent with reduced food intake, 3-day administration of aspirin induced lesions in the stomach, as indicated by macroscopic examination showing gastric ulcers. The combination of aspirin and γT nonsignificantly alleviated aspirin-induced lesions (Fig. 4B). In contrast, the combination of aspirin and αT showed more severe lesions compared with aspirin alone (P<.05) or the combination of aspirin and γT (P<.01) (Fig. 4B).

To explain the beneficial effect of γT on aspirin-induced food reduction and, seemingly, protection of stomach injury, we examined drug effects on gastric PGE_2, which is generated by COX-1-catalyzed reactions and is believed to provide mucosal cytoprotection [6,7]. Eighteen hours after the last dosing, rats fed aspirin and aspirin+αT had more than fourfold (P<.05) and threefold (P<.05) reduced

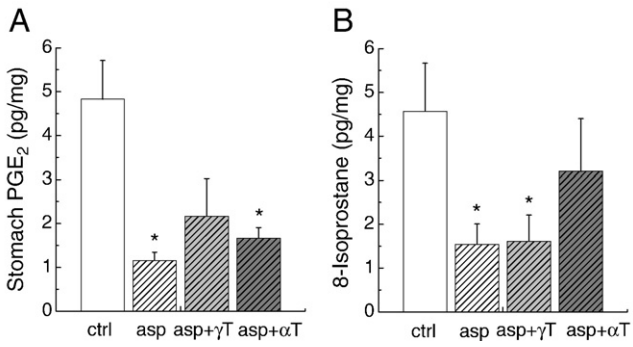

Fig. 5. Effects on gastric PGE_2 and 8-isoprostane. The conditions of each treatment are the same as those indicated in Fig. 2. Stomach PGE_2 (A) and 8-isoprostane (B) were measured with ELISA. Results (pg/mg tissue) are expressed as mean±S.E.M. *A significant difference between corn-oil-treated control rats and drug-treated animals ($P<.05$).

PGE_2, respectively, in the stomach mucosa (Fig. 5A). In contrast, the combination of aspirin and γT partially attenuated aspirin-induced reduction in gastric PGE_2. Because oxidative stress may play a role in aspirin-induced stomach injury [8,10], we measured 8-isoprostane, a lipid peroxidation marker. Compared with corn oil controls, treatment with aspirin or aspirin+γT led to an almost threefold reduction in 8-isoprostane ($P<.05$) in the stomach mucosa, whereas aspirin+αT did not significantly affect 8-isoprostane (Fig. 5B). We also measured gastric LTB_4, which is also believed to contribute to gastric injury [27]. The results indicated that LTB_4 was not affected by aspirin or its combinations compared with corn oil controls (data not shown).

4. Discussion

The present study shows that a combination of aspirin and γT appears to have some advantage over aspirin alone in terms of anti-inflammatory action and alleviation of aspirin-associated adverse effects. The combination of aspirin and γT, rather than aspirin alone or aspirin+αT, reduced exudate volume, which is an index of inflammation, and showed moderate inhibition of proinflammatory PGE_2 and tissue damage during the prolonged inflammation period. We previously showed that γT is more effective than αT in inhibiting proinflammatory PGE_2 in endotoxin-treated macrophages and interleukin-1β-stimulated epithelial cells [17]. We recently found that long-chain carboxychromanols, which are mainly generated from the metabolism of non-α-tocopherol forms of vitamin E such as γT [21,28], are potent COX inhibitors (Jiang et al., unpublished results). Consistent with this, in the similar air-pouch inflammation model in rats, γT (but not αT) at the same dose reduced proinflammatory PGE_2 and attenuated inflammation-associated damage [16]. In the present study, at 18 h, the concentrations of γT in the exudate are fivefold higher than those in corn-oil-treated controls. Therefore, the anti-inflammatory activity of γT and its metabolites may account for the prolonged anti-inflammatory effect in combination with aspirin. In contrast, aspirin alone only transiently reduced PGE_2 at the site of inflammation as a result of the rapid clearance of this drug and its metabolite, salicylate (half life in the plasma is reported to be between 20 min and 4.5 h). The prolonged anti-inflammatory effect was not observed with the combination of aspirin and αT.

Unlike the combination of aspirin and γT, aspirin alone or aspirin+αT caused prolonged depression of gastric PGE_2 and further reduction in food intake. It is well established that aspirin inhibits COX-1 in the stomach mucosa via acetylation of serine 530 at the substrate-binding site and therefore blunts gastric PGE_2, which is believed to be important for the maintenance of mucosal function and defense mechanisms [6,7]. The irreversible inhibition of COX-1 is responsible for the prolonged depletion of gastric PGE_2 even after aspirin had been washed out from the stomach. Aspirin-associated reduction in food intake is likely caused by the stomach injury induced by this drug. γT spared aspirin-induced reduction in food intake and partially attenuated stomach lesions. The observed beneficial effect of γT can be explained, in part, by its partial counteraction of aspirin-related reduction in gastric PGE_2, but the reason for this action is not clear. Besides depletion of gastric prostaglandins, other factors, such as oxidative-stress-associated increase in lipid peroxidation [10] and reduction in the hydrophobicity of mucosal surface [29], may contribute to stomach damage. Interestingly, both aspirin and its combination with γT significantly reduced 8-isoprostane, a lipid peroxidation marker. It is possible that the attenuation of PGE_2 depletion, together with inhibition of lipid peroxidation in the gastric mucosa, may account for the superior outcome from the combination of aspirin and γT, which is in sharp contrast to the effect of aspirin+αT.

It is unexpected that addition of αT would worsen aspirin-induced gastric injury, consistent with the accentuated decrease in food intake induced by this combination. Previous studies that investigated the potentially protective effect of αT supplementation on aspirin-induced damage revealed varied results. Fesharaki et al. [30] showed that supplementation of αT reversed aspirin-induced acute gastric erosions and restored aspirin-induced decrease in superoxide dismutase activity and glutathione levels in the stomach. However, other studies indicated that αT failed to attenuate aspirin-induced stomach injury [11] or provided protection limited to vitamin E (αT)-deficient animals, but not to those with adequate vitamin E intake [10,12]. For instance, Sugimoto et al. [12] reported that supplementation of αT (500 mg/kg) did not reduce aspirin+HCl-induced gastric mucosal injury compared with rats fed adequate αT (20 mg/kg), although rats with adequate or supplemented αT had reduced lesions compared with animals fed a vitamin-E-deficient diet. Stickel et al. [11] reported that supplementation of αT did not provide further protection against aspirin-induced gastric injury in 20-month-old rats.

Although αT is believed to be safe across a broad range of intakes [31], the surprisingly adverse effect of the addition of αT to aspirin in the current study is in line with the observation that individuals supplemented with αT and aspirin had increased gingival bleeding compared with individuals supplemented with aspirin alone, as observed in a controlled clinical trial, the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study [9]. This study also showed that αT supplementation was associated with an increased risk of death due to hemorrhagic stroke, compared with placebo controls [32]. The enhanced bleeding associated with αT supplementation may stem from its action on platelet aggregation via inhibition of protein kinase C activity [33] or modulation of vitamin K status [34]. Whether these mechanisms account for the adverse effect of αT observed in the current study awaits determination. We found that addition of αT did not have any impact on aspirin-induced reduction in gastric PGE_2, consistent with the study by Stickel et al. [11]. On the other hand, αT appeared to reverse aspirin-related reduction in 8-isoprostane, but the significance and mechanism of this effect are not clear.

NSAIDs, including aspirin, are commonly used for pain relief and have consistently been shown to reduce the risk of cancers [35,36]. Low-dose aspirin is effective in reducing cardiovascular disease risk because of its antithrombotic effect [37]. However, the long-term use of aspirin and other NSAIDs has been hindered by their associated adverse effects, including upper gastrointestinal bleeding and ulcers [37,38]. Our current study suggests that the combination of aspirin and γT may be superior to aspirin alone in the treatment of chronic inflammatory diseases and inflammation-associated disorders such as cancer due to prolonged anti-inflammatory action and protective effect against gastric injury, as well as the anticancer effect of γT itself [39]. The safety and efficacy of the chronic use of this combination should be further tested in animal models and human studies. Further investigation on the selection of the optimal dose of this combination is also needed. In addition, because of the varied outcomes of the combination of aspirin and αT, caution should be taken in the recommendation of this combination.

Acknowledgments

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