Vitamin E and Macrophage Cyclooxygenase Regulation in the Aged1,2

Dayong Wu,*† Michael G. Hayek** and Simin Nikbin Meydani*3

*Nutritional Immunology Laboratory, Jean Mayer Human Research Center on Aging at Tufts University, Boston, MA 02111; †Department of Immunology, Norman Bethune University of Medical Sciences, Changchun, China; and **Iams Company, Lewisburg, OH 45338

ABSTRACT Aging is associated with increased evidence of cardiovascular disease (CVD). Atherosclerosis, a major cause of CVD, is an inflammatory process whose development is influenced by several proinflammatory mediators. Products of arachidonic acid metabolism, in particular, prostaglandin (PG) E2 and thromboxane (TX) A2, play an important role in the development of atherosclerosis. We showed previously that the aged have higher PGE2 production compared with their young counterparts. This age-associated increase in PGE2 production is mainly a consequence of increased cyclooxygenase (COX) activity. We demonstrated further that increased COX activity in old mice is due to the increased expression of mRNA and protein for the inducible form of COX, COX-2. Vitamin E has been shown to reduce PGE2 production and risk of CVD. In aged mice, we showed that a vitamin E–induced decrease in PGE2 production is due to decreased COX activity. However, vitamin E had no effect on COX mRNA and protein levels, indicating a post-translational regulation of COX by vitamin E. Further experiments indicated that vitamin E decreases COX activity through reducing formation of peroxynitrite, a hydroperoxide shown to be involved in the activation of COX-2. Other homologues of tocopherols were also effective in inhibiting COX activity, but their degree of inhibition varied. The varied potency to inhibit COX activity was not explained totally by differences in their antioxidant capacity. Vitamin E–induced inhibition of COX activity might contribute to its effect of reducing CVD risk.


KEY WORDS: • vitamin E • cardiovascular disease • atherosclerosis • prostaglandin
Phospholipase A2. AA is metabolized to prostaglandin (PG)H2 by cyclooxygenase (COX). COX has bifunctional catalytic properties, i.e., catalyzing both formation of PGG2 from AA via its cyclooxygenase activity and subsequent reduction of PGG2 to PGH2 via its peroxidase activity. PGH2 will be converted to different PG and thromboxane (TX) A2 by the action of different isomerases. COX is the rate-limiting enzyme in the biosynthesis of PG. AA can also be metabolized to various hydroxyeicosatetraenoic acids (HETE) and leukotrienes (LT) by the corresponding lipoxigenases (LO).

FIGURE 1 Arachidonic acid metabolism cascade. Arachidonic acid (AA) is released from membrane phospholipids under action of phospholipase A2. AA is metabolized to prostaglandin (PG)H2 by cyclooxygenase (COX). COX has bifunctional catalytic properties, i.e., catalyzing both formation of PGG2 from AA via its cyclooxygenase activity and subsequent reduction of PGG2 to PGH2 via its peroxidase activity. PGH2 will be converted to different PG and thromboxane (TX) A2 by the action of different isomerases. COX is the rate-limiting enzyme in the biosynthesis of PG. AA can also be metabolized to various hydroxyeicosatetraenoic acids (HETE) and leukotrienes (LT) by the corresponding lipoxigenases (LO).

and an inducible (COX-2) form. COX-1 is constitutively expressed and is involved mainly in control of normal physiologic functions, whereas COX-2 is regulated by growth factors, tumor promoters, cytokines, glucocorticoids and lipopolysaccharide (LPS). Overexpression of COX-2 has been indicated in the pathogenesis of inflammatory and neoplastic diseases. In recent years, a role for COX-2 in the pathogenesis of atherosclerosis has been defined. COX-2 is involved in inflammatory response via rapid and excessive production of prostanooids, which have proatherosclerotic effects. A COX-dependent, aspirin-reversible constricting factor was shown to contribute to the endothelial cell dysfunction in atherosclerosis (Husain et al. 1998). Cytomegalovirus (CMV) has been suggested to contribute causally to restenosis and atherosclerosis. CMV was shown to increase production of reactive oxygen species through a COX-2 dependent pathway (Speir et al. 1998). COX-2 in activated human monocytes generates the isoprostane 8-epi-PGF2α, which is mitogenic and vasoactive (Yan et al. 2000). COX-2 is expressed in atherosclerotic lesions, increases after vascular injury and has been detected in myocardia of patients with congestive heart failure. Monocyte-derived prostaglandins decreased the secretion of procollagen by human vascular smooth muscle cells by 60%. This can result in reduced plaque stability and plaque rupture (Fitzsimmons et al. 1999).

Immunocytochemical studies using anti-COX-2 showed that COX-2 and inducible nitric oxide synthase (iNOS) were localized to macrophages (Mφ) in atherosclerotic lesions of patients with native and transplant coronary disease. COX-2 expression was also found in medial smooth muscle cells and endothelial cells, including those of the vasa vasorum. Nitrotyrosine (as a measure of peroxynitrite) was found in the same distribution as iNOS and was colocalized with COX-2 in Mφ (Baker et al. 1999).

Benzo(a)pyrene, present in tobacco smoke and tar, has been implicated in the development of atherosclerosis as well as cancer. Increased expression of COX-2 has been detected in both atherosclerotic lesions and epithelial cancers. Yan et al. (2000) showed that benzo(a)pyrene increased expression (protein and mRNA) of COX-2 in vascular cells (human and rat arterial smooth muscle cells) and increased prostaglandin synthesis. Rimarachin et al. (1994) showed that mechanical injury induced COX-2 in vascular tissue and increased expression of COX-2 presented within vascular smooth muscle cells during development of proliferative lesions in the injured vessels. High levels of COX-2 in epithelial cells are associated with the inhibition of apoptosis. In the development of atherosclerotic plaques, it is possible that a similar antiapoptotic effect of high levels of COX-2 could augment plaque growth by decreasing cell death rates and depressing normal vascular remodeling. Collectively, these data suggest that COX-2 and its AA-generated products may participate in the initiation and pathogenesis of atherosclerosis.

Upr egulation of PGE2 production with age

Several lines of evidence have suggested that eicosanoid production is altered with age. These have included increased PGE2 production from cultured adherent cells or spleen homogenates from old mice (Meydani et al. 1990b, Rosenstein and Strausser 1980) cultured mononuclear cells from elderly humans (Meydani et al. 1990a), kidney homogenates from old rats (Kim et al. 2000), urinary prostaglandin metabolites from older adults (Vericel et al. 1988, Wilson et al. 1989) and lung homogenates from old mice (Meydani et al. 1992). These data collectively suggest an alteration of eicosanoid synthesis during aging.

Eicosanoids generated from the AA cascade can result in a variety of metabolites, which are generated by several enzymes (Fig. 1). To determine whether aging upregulates the production of all or specific eicosanoid metabolites, a series of studies was conducted to characterize alterations in eicosanoid production with age (Hayek et al. 1994). Splenocytes were isolated from young (4 mo) and old (24 mo) mice and challenged with Ca++ ionophore or concanavalin A (ConA). As shown in Table 1, there was no difference in 12- or 15-hydroxyeicosatetraenoic acid (HETE) production between the two ages; however, there was an increase in leukotriene (LT) B4, LTC4, and PGE2 production in old mice compared with young mice. The data presented in Table 1 are for stimulation with CA++ ionophore. Similar results were observed when the splenocytes were stimulated with ConA. This suggested that the age-associated increase in eicosanoid synthesis was specific to the

<table>
<thead>
<tr>
<th>Eicosanoid</th>
<th>Young</th>
<th>Old</th>
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<tbody>
<tr>
<td>12-HETE</td>
<td>3766 ± 479</td>
<td>6606 ± 2816</td>
</tr>
<tr>
<td>15-HETE</td>
<td>362 ± 37</td>
<td>370 ± 49</td>
</tr>
<tr>
<td>LTC4</td>
<td>160 ± 37</td>
<td>476 ± 97*</td>
</tr>
<tr>
<td>LTB4</td>
<td>16 ± 13</td>
<td>58 ± 16*</td>
</tr>
<tr>
<td>PGE2</td>
<td>555 ± 10</td>
<td>3752 ± 1600*</td>
</tr>
</tbody>
</table>

1 Data adapted from Hayek et al. (1994).  
2 HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; PG, prostaglandin.  
3 Splenocytes were challenged with 2 µmol/L Ca++ ionophore A23187 for 20 min.  
* Significantly different than young at P < 0.01.
5-lipoxygenase (LTB₄ and LTC₄) and cyclooxygenase (PGE₂) enzymes. We further showed that the COX metabolite (PGE₂) and not the 5-lipoxygenase products contributed to the decline in T-cell-mediated function observed in the elderly (Beharka et al. 1997, Hayek et al. 1994).

These data along with the observation that there was no difference in fatty acid composition between splenocytes isolated from young and old mice (Hayek et al. 1994) suggested that aging results in alterations in the activity/expression of the COX enzyme. To further characterize age-related COX changes, Mφ were isolated from young and old mice and were stimulated with LPS and cultured for 48 h (Hayek et al. 1997). It was observed that PGE₂ production was significantly higher in Mφ isolated from old mice, and this was accompanied by higher COX activity at 0, 6, and 12 h of culture (Fig. 2). This increase in activity was due to increased protein and mRNA expression of COX-2 in Mφ of old mice with no apparent difference in COX-1 expression (Fig. 3). We are currently determining the mechanism(s) of age-associated increase in Mφ COX-2 mRNA expression.

**Vitamin E decreases in the macrophage PGE₂ production of the aged**

As discussed above, Mφ produce more PGE₂ with the progress of aging, and this inflammatory mediator contributes to a number of age-associated diseases. The physiologic and pathophysiologic changes associated with aging are particularly manifested in the cardiovascular and immune systems. It is interesting to note that these disorders are characterized by an excessive production of inflammatory mediators. In addition, oxidative stress has been causally linked to the inflammatory process. Antioxidants, particularly vitamin E, are reported to alleviate the symptoms of and reduce the pathogenesis of inflammatory diseases.

Atherosclerosis has been referred to as an inflammatory process. The dysregulated interaction between blood monocytes/Mφ and endothelium of blood vessels is believed to be an initial step in the process leading to the pathogenesis of atherosclerosis. Evidence from epidemiologic and some clinical intervention trials indicates that vitamin E may reduce the risk of CVD and might potentially be used to prevent CVD. Many mechanistic studies have revealed that vitamin E can

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**FIGURE 2** Prostaglandin (PG)E₂ production (A) and cyclooxygenase (COX) activity (B) in peritoneal macrophages (Mφ) from young and old C57BL/6NIA mice. Mφ were incubated in the presence of 5 µg/mL lipopolysaccharide for different lengths of time as indicated. PGE₂ in the supernatants was determined by RIA. COX activity was determined by measuring PGE₂ production from 30 µmol/L exogenous AA within 10 min. Results are means ± SEM, n = 10. *Significantly different from young mice at P < 0.01. Data adapted from Hayek et al. (1997).

**FIGURE 3** Cyclooxygenase (COX) mRNA (A) and protein (B) expression in peritoneal macrophages (Mφ) from young and old C57BL/6NIA mice. Mφ were incubated in the presence of 5 µg/mL lipopolysaccharide for different lengths of time as indicated. COX mRNA was assessed by RNase protection assay. tRNA was the negative control. COX protein was determined by Western blot. Values in (A) are the means of three independent experiments. *Significantly different from young mice at P < 0.03. Data adapted from Hayek et al. (1997).
modulate various factors that contribute to the progression of atherosclerosis. Of interest to this paper is the ability of vitamin E to regulate Mϕ eicosanoid production. Thus, we conducted a study (Wu et al. 1998b) to determine whether Mϕ production of PGE$_2$, especially its increase with age, can be modified by dietary vitamin E supplementation, and if so, to identify its mechanism of action. In this study, we used specific pathogen-free male young (6 mo), and old (24 mo) C57BL/6NIA mice. These mice were fed purified diets containing 30 ppm (adequate level) or 500 ppm vitamin E (RRR-α-tocopherol acetate) for 30 d. At the end of the feeding period, mice were killed and peritoneal resident Mϕ were collected. Mϕ were incubated in the presence or absence of LPS for 0, 6, 12, or 24 h at 37°C. PGE$_2$ produced by Mϕ were measured in culture medium by RIA. COX activity of Mϕ was determined by measuring PGE$_2$ that was produced in the presence of exogenously added AA. Results showed that unstimulated Mϕ produced very low levels of PGE$_2$ and there was no significant difference between young and old mice at any of the time points tested. Vitamin E supplementation did not cause a significant change in unstimulated PGE$_2$ production in either age group. In LPS-stimulated Mϕ, as shown in Figure 4A, PGE$_2$ production by Mϕ significantly increased with time in both age groups. Mϕ from old mice fed the control diet had significantly higher production of PGE$_2$ at 12 and 24 h compared with those from young mice fed the control diet. Vitamin E supplementation completely eliminated this age-related increase in PGE$_2$ production so that there was no significant difference in PGE$_2$ production between old mice fed 500 ppm vitamin E and young mice fed control or vitamin E-supplemented diets. Vitamin E supplementation, however, did not have a significant effect on PGE$_2$ production in young mice.

Previously, we reported (Hayek et al. 1997) that the age-associated increase in PGE$_2$ production by mouse Mϕ is a consequence of increased COX activity. The increased COX activity is, in turn, due to the age-associated upregulation of COX-2 protein and mRNA expression. Thus, to determine the mechanism of the vitamin E-induced decrease in PGE$_2$ production, we determined its effect on COX activity and expression. As shown in Figure 4B, Mϕ from old mice fed the control diet showed significantly higher COX activity than those from young mice fed the control diet at all time points. Furthermore, vitamin E supplementation completely suppressed the age-related increase in COX activity, but had no effect on COX activity of Mϕ from young mice. It is interesting to note that, for up to 24 h of culture, vitamin E inhibited LPS-stimulated PGE$_2$ accumulation and COX activity to the same extent (60%), suggesting that vitamin E inhibition of PGE$_2$ production is mainly through its inhibition of COX activity rather than an effect on substrate release. To show that the effect of vitamin E is exerted on COX and not on the activity of the downstream enzyme PGE$_2$ isomerase, we investigated the effect of vitamin E on another COX product, TXA$_2$ (measured as its stable hydrolytic product, TXB$_2$). The results showed that vitamin E had no effect on the conversion of exogenous AA to TXA$_2$ in LPS-stimulated Mϕ from young mice [10.6 ± 1.8 and 8.1 ± 1.4 pg TXB$_2$/g protein • 10 min] in control and vitamin E groups, respectively. Vitamin E, however, significantly decreased the conversion of AA to TXA$_2$ in LPS-stimulated Mϕ from old mice [19.5 ± 4.7 and 7.8 ± 1.4 pg TXB$_2$/g protein • 10 min] in control and vitamin E groups, respectively. Similar to the results for PGE$_2$, Mϕ from old mice fed the control diet produced significantly more TXB$_2$ than those from the younger mice. The magnitude of the inhibition of COX activity by vitamin E in old mice is similar whether measured as a conversion to PGE$_2$ or to TXA$_2$.

Inhibition of COX by vitamin E is post-translational

In many cases, the altered COX activity reflects changes in the rate of enzyme synthesis, the rate of mRNA transcription or both. Thus, to clarify further how vitamin E modifies COX activity in old mice, we examined COX protein and mRNA expression. As shown in Figure 4C, LPS-induced COX-2 protein expression was not affected by vitamin E supplementation. Similarly, COX-2 mRNA expression was not influenced by vitamin E (data not shown). Thus, the vitamin E-induced
decrease in COX activity of Mφ from old mice is not due to its regulation of COX transcription or translation; rather, it appears that vitamin E exerts its effect post-translationally. COX activity requires the presence of oxidant hydroperoxides (Hemler and Lands 1980, Kulmacz and Wang 1995, Smith et al. 1992). The lag phase in attaining maximal COX activity was shortened or eliminated by endogenous or exogenous hydroperoxides, whereas it was delayed by antioxidants (Hemler and Lands 1980). Vitamin E is an effective biological antioxidant and chain-breaking free radical scavenger; therefore, it may attenuate COX activity by scavenging the oxidant hydroperoxide necessary for COX activation. The observation that vitamin E inhibits COX activity in old but not young mice further supports this notion because many studies have demonstrated increased formation of lipid peroxides in different tissues of aged animals. Free radical nitric oxide (NO) has

**TABLE 2**

<table>
<thead>
<tr>
<th>Tocopherol isomers</th>
<th>PGE$_2$ production (% inhibition)$^{1,2}$</th>
<th>COX activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>12.5 µmol/L</td>
<td>25 µmol/L</td>
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<tr>
<td>α</td>
<td>60</td>
<td>56</td>
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<tr>
<td>β</td>
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<td>γ</td>
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<tr>
<td>δ</td>
<td>75</td>
<td>NA</td>
</tr>
</tbody>
</table>

$^1$ Adapted from Wu et al. (2000).
$^2$ PG, prostaglandin; COX, cyclooxygenase; NE, no effect; NA, not analyzed.
$^3$ Tocopherol concentration.

**TABLE 3**

<table>
<thead>
<tr>
<th>Tocopherol isomers</th>
<th>PGE$_2$ production$^3$</th>
<th>COX activity$^3$</th>
<th>Phospholipase activity$^4$</th>
</tr>
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<tbody>
<tr>
<td>α</td>
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<tr>
<td>δ</td>
<td>↓</td>
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$^1$ Adapted from Wu et al. (2000).
$^2$ PG, prostaglandin; COX, cyclooxygenase.
$^3$ Experimentally measured.
$^4$ Speculated.
been shown to be involved in regulation of COX activity and eicosanoid metabolism. It has been suggested that NO stimulates COX activity via direct stimulation of the enzyme (Salvemini et al. 1995). We have reported (Beharka et al. 1997) that LPS-stimulated peritoneal Mφ from old mice produced more NO than those from young mice, and dietary supplementation with vitamin E reduced NO production in Mφ from old mice. NO can be further metabolized to peroxynitrite (ONOO) in the presence of superoxide (SO), and ONOO has been shown to increase the activity of COX without affecting its expression. Therefore we hypothesized that decreased NO and thus ONOO formation may mediate the inhibition of COX activity by vitamin E. To test this hypothesis, we conducted a study (Beharka et al. 2000) in which young (6 mo) and old (24 mo) mice were fed 30 or 500 ppm vitamin E for 30 d. The results confirmed the previous findings that Mφ from old mice produced more NO than those from young mice, and this age-associated increase of NO was reduced by vitamin E supplementation. However, SO levels were not affected by age or vitamin E supplementation. Addition of NO donor to the cell culture to increase NO levels did not change PGE2 production and COX activity in either young or old mice. However, when NO donor was added in the presence of SO to elevate ONOO levels in the culture, COX activity was increased in the Mφ from old mice fed 500 ppm but not 30 ppm vitamin E. On the other hand when NO and SO inhibitors were added to Mφ from old mice fed 30 ppm vitamin E to block the generation of ONOO, COX activity was reduced significantly. These results suggest that vitamin E reduces COX activity in old Mφ by decreasing NO production, which leads to lower production of ONOO in Mφ from old mice.

**In vitro supplementation with various tocopherol homologues differentially affects COX activity**

A majority of studies on vitamin E, including those mentioned above, have used α-tocopherol (α-T). However, in recent years, interest in determining the biological effects of non-α-tocopherols has increased. It is generally agreed that the relative antioxidant activity of tocopherols is in the order $\alpha > \beta > \gamma > \delta$ (Burton and Ingold 1981, Kamal-Eldin and Appelqvist 1996), but recent studies have provided evidence that in some in vitro systems, other tocopherols might be more effective antioxidants than α-tocopherol. For example, γ-T was shown to inhibit peroxynitrite-induced lipid peroxidation more effectively than α-T (Christen et al. 1997). It has been suggested that the varied biological activity of tocopherols is explained only in part by their antioxidant activity (Azzi et al. 1993, Kasparek 1980). α-T has been studied extensively and is well documented for its beneficial effect in several bodily systems, in particular, the cardiovascular and immune systems (Diaz et al. 1997, Meydani 1995, Meydani and Beharka 1998, Weber et al. 1997). In contrast, the other forms of tocopherols, especially β- and δ-T have received little attention for their biological effects other than their antioxidant property. In fact, non-α-tocopherols are abundant in certain plant oils and thus may contribute to the total tocopherol bioactivity in food. Although the concentrations of these non-α-tocopherols in tissues and body fluids are much lower and they are less effective as biological antioxidants compared with α-T, their ability to modulate various other aspects of cell function has not been studied. We have shown that dietary or in vitro supplementation with α-T inhibited PGE2 production and COX activity in Mφ from old mice. We, therefore, conducted another study (Wu et al. 2000) to compare the effect of in vitro supplementation with all four natural tocopherols on the function of immune cells in old mice, including PGE2 production and COX activity. In this study, peritoneal Mφ were obtained from 26-mo-old male C57BL mice. After 4 h of preincubation with graded levels of each of the four tocopherols, Mφ were stimulated with LPS for 24 h. As shown in Figure 5 and Table 2, accumulated PGE2 production using the endogenous substrate, AA, was inhibited by all concentrations of α-T tested; there was no difference among the doses tested. β-T did not have an effect on accumulated PGE2 production, whereas γ- and δ-T inhibited PGE2 production in a dose-dependent manner. However, COX activity, as determined by the synthesis of PGE2 from exogenous AA, was inhibited by all four tocopherols including β-T. These results indicate that non-α-T homologues can also inhibit COX activity, and this effect does not seem to depend solely on their antioxidant capacity. The inconsistent effect of β-T on accumulated PGE2 and COX activity suggests that some mechanism other than COX, such as modulation of phospholipase A2, might also be involved in the net effect of various tocopherol isomer aggregation on PGE2 production (Table 3).

**SUMMARY**

Aging is associated with increased incidence of CVD which is the leading cause of mortality in the elderly. We, as well as others, have shown that the production of cyclooxygenase products increases with age. The age-associated increase in production of Mφ proinflammatory mediators, such as cytokines, PG, NO and other reactive oxygen species (Beharka et al. 1997, Chen et al. 1996, Hayek et al. 1999, Meydani et al. 1986) contributes to many diseases of aging including CVD. α-Tocopherol significantly reduced PGE2, TXB2, and NO production by Mφ from aged mice. α-Tocopherol-induced reduction in PGE2 production is through post-translational inhibition of COX activity. Other tocopherol homologues also inhibit PGE2 production, but their mechanisms of action vary and might not be dependent solely on their antioxidant property. The vitamin E-induced decrease in COX products, in addition to its demonstrated beneficial effect on immune response, might be important in maintaining cardiovascular health during aging.

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**LITERATURE CITED**


Chen, L.-C., Pace, J. L., Russell, S. W. & Morrison, D. C. (1996) Altered...


