Age-associated increase in PGE$_2$ synthesis and COX activity in murine macrophages is reversed by vitamin E

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AGING IS ASSOCIATED WITH depressed immune function, and decline in immunologic vigor contributes to many age-associated infections and neoplastic diseases as well as to the prolonged postillness recovery often observed in the elderly. The age-associated changes are mostly observed in T cell-mediated functions, which are believed to be due to intrinsic changes in T cells and increased production of suppressive factors from macrophages (4, 15, 30). We, as well as others, have shown that macrophages and splenocytes from old mice and peripheral blood mononuclear cells from elderly human subjects synthesize significantly more PGE$_2$ (a compound with inhibitory effects on T cell-mediated function) than their young counterparts (2, 4, 15, 26, 28, 36). Furthermore, we showed that increased PGE$_2$ production contributes to the age-associated decline in T cell proliferation and interleukin (IL)-2 production (4). The increase in cyclooxygenase (COX) products has also been observed in other tissues (29, 43, 45). We recently showed that the age-associated increase in lipopolysaccharide (LPS)-stimulated PGE$_2$ production is due to an increase in COX activity, which is, in turn, mainly due to increased expression of COX-2 (16).

COX is the rate-limiting synthase in the biosynthesis of prostaglandins and has bifunctional catalytic properties. It catalyzes both formation of PGG$_2$ from arachidonic acid via its COX activity and subsequent reduction of PGG$_2$ to PGH$_2$ via its peroxidase activity (8). Many studies have shown that at least two forms of the enzyme COX exist, a constitutive form (COX-1) and an induced form (COX-2) (19, 31). The two enzymes appear to be functionally distinct, having different distributions and responsiveness patterns to different stimuli. COX-1 is constitutively expressed, whereas COX-2 is regulated by growth factors, tumor promoters, cytokines, and mitogens such as LPS and glucocorticoids (13, 42).

Prostaglandins are potent mediators of intercellular communication and as such modulate a variety of normal cellular responses as well as mediate some pathophysiological processes. At low concentrations PGE$_2$ is believed to be necessary for certain aspects of cellular immunity, whereas at higher concentrations PGE$_2$ has been reported to be immunosuppressive for the T cell-mediated response, as demonstrated by decreased T cell proliferation and IL-2 production (11, 12). PGE$_2$ has also been shown to inhibit immune response-related signals such as phosphatidylinositol turnover, phytomagglutinin- and anti-CD3-stimulated increases in Ca$^{2+}$, and tyrosine phosphorylation of a 100-kDa protein, which increases following mitogenic stimulation (1, 5, 33). PGE$_2$ has been implicated in maintaining the balance between T helper 1 and 2 cells as well (34).

We have previously reported that vitamin E supplementation significantly enhanced T cell proliferation, IL-2 production, and delayed-type hypersensitivity skin response in aged mice and humans (26, 28). In a recent study, we showed that vitamin E supplementation of healthy elderly subjects improved their in vivo indexes of cell-mediated immune function, including response to vaccine, while reducing the incidence of self-reported infections (27). We further showed (4) that vitamin E exerts its immunoenhancing effect in aged mice mainly...
through inhibition of macrophage PGE$_2$-mediated suppression.

The mechanism of the vitamin E-induced decrease in macrophage PGE$_2$ production is not known. The purposes of the present study were, therefore, 1) to determine the effect of vitamin E on macrophage PGE$_2$ production of young and old mice and 2) to determine whether the vitamin E-induced change in PGE$_2$ production is due to its effect on expression of COX-2. We report here that vitamin E supplementation decreases PGE$_2$ production and COX activity in macrophages from old but not young animals. This effect of vitamin E is not mediated through reduction in COX-1 or COX-2 expression; rather, vitamin E exerts its effect posttranslationally by affecting COX activity.

**MATERIALS AND METHODS**

Animals. Specific pathogen-free male young (6 mo) and old (24 mo) C57Bl/6NIA mice were obtained from National Institute on Aging colonies at Charles River Laboratories (Kingston, NY). The mean life span of these mice is 28 mo. Forty young and forty old mice were used to determine PGE$_2$ production and COX activity, and ninety-six old mice were used to determine the mRNA and protein expression in four experiments. Mice were housed singly in microisolator cages at constant temperature (23°C), with a 12:12-h light-dark cycle. Mice were daily fed semisynthetic diets (Table 1) containing 30 (adequate level) or 500 parts per million (ppm) vitamin E for 30 days. The leftover diet from the previous day was discarded. Mice were housed by age and euthanized via CO$_2$ asphyxiation. All conditions and handling of the animals were approved by the Animal Care and Use Committee of the Jean Mayer Human Nutrition Research Center on Aging at Tufts University and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice exhibiting visible tumors or splenomegaly were excluded from the study.

Peritoneal macrophage isolation. Peritoneal exudate cells were obtained by peritoneal lavage with cold Ca$^{2+}$- and Mg$^{2+}$-free Hanks’ balanced salt solution (GIBCO, Grand Island, NY). Cells from 2 mice were pooled for measurement of PGE$_2$ production and COX activity (final $n = 10$), and cells from 12 old mice per treatment group were pooled for analysis of COX mRNA and protein levels (final $n = 4$). Peritoneal exudate cells were enriched for macrophages using the method of Kumagai et al. (22). Briefly, peritoneal macrophages were suspended in endotoxin-free RPMI 1640 (ETRPMI; Bio Whitaker, Walkersville, MD) medium supplemented with 25 mM HEPES, 2 mM glutamine (GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO), and 5% FCS. The cells were plated on six-well plates (Falcon Labware, Lincoln Park, NJ) that had been previously blocked with FCS and allowed to adhere for 2 h at 37°C in 5% CO$_2$, at which time nonadherent cells were removed by vigorous washing. Peritoneal macrophages prepared in this manner were at least 90% macrophages, as assessed by Mac-1 and F4/80 cell surface antibody.

Table 1. Composition of basal diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Weight, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin-free casein</td>
<td>18.00</td>
</tr>
<tr>
<td>Corn starch</td>
<td>33.55</td>
</tr>
<tr>
<td>Sucrose</td>
<td>33.40</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.00</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>0.30</td>
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<tr>
<td>Choline bitartrate</td>
<td>0.25</td>
</tr>
<tr>
<td>AIN 76A salt mix</td>
<td>3.50</td>
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<tr>
<td>AIN 76A vitamin mix</td>
<td>1.00</td>
</tr>
<tr>
<td>Lard (tocopherol stripped)</td>
<td>3.80</td>
</tr>
<tr>
<td>Corn oil (tocopherol stripped)</td>
<td>1.20</td>
</tr>
</tbody>
</table>

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VITAMIN E AND COX IN MACROPHAGES FROM YOUNG AND OLD MICE

RESULTS

Production of PGE₂. The production of PGE₂ from peritoneal macrophages without and with LPS stimulation is presented in Fig. 1, A and B, respectively. PGE₂ production in unstimulated cultures was low and did not show a significant change with time in macrophages from either young or old mice. There was no significant difference between young and old mice in PGE₂ levels at any of the time points tested. Vitamin E supplementation did not cause a significant change in unstimulated PGE₂ production in either age group (Fig. 1A). In LPS-stimulated macrophages, PGE₂ production significantly increased with time in macrophages from both age groups. Macrophages from old mice fed the control diet had significantly higher production of PGE₂ at 12 and 24 h compared with macrophages from young mice fed the control diet. Vitamin E supplementation completely eliminated this age-related increase in PGE₂ production, so that there was no significant difference in PGE₂ 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 PGE₂ production in young mice (Fig. 1B).

COX activity. COX activity in unstimulated and LPS-stimulated macrophages is presented in Fig. 2, A and B, respectively. In unstimulated cultures, COX activity did not change with time in either age group. Macrophages 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. Vitamin E supplementation did not have a significant effect on COX activity in young mice; however, it significantly decreased COX activity in old mice, so that there was no significant difference in COX activity between old mice fed 500 ppm vitamin E and young mice fed control or vitamin E-supplemented diets (Fig. 2A). After macrophages were stimulated with LPS, COX activity increased with time in both age groups. Macrophages from old mice fed 30 ppm vitamin E demonstrated significantly higher COX activity than those from young mice at all time points, and again supplementation with 500 ppm vitamin E completely suppressed this age-related increase in COX activity but had no effect.
Cox activity of macrophages from young mice (Fig. 2). Because LPS does not induce COX-1, the activity in LPS-stimulated cultures is mainly due to COX-2 (inducible form). The activity in unstimulated cultures may be due to either COX-1 or COX-2. The latter is more likely, since, under these conditions, COX-1 mRNA and protein are barely detectable (see Fig. 4A), whereas COX-2 mRNA and protein can be detected at low levels (see Fig. 4A). Serum (present in our cultures) has been shown to induce COX-2 (14). Furthermore, we have previously shown that the age-related increase in COX-2 activity is mainly due to increased expression of COX-2 and not COX-1 (16).

COX-2 mRNA and protein expression. Because we previously showed that the age-associated increase in PGE$_2$ production is due to increased COX-2 expression (16), we investigated the possibility that vitamin E decreases PGE$_2$ production by reducing transcription and/or translation of COX-2 gene. To test this hypothesis, we compared COX-2 mRNA and protein levels between macrophages from mice fed 30 or 500 ppm vitamin E. As previously reported, COX-2 gene was induced by LPS, and the mRNA reached maximum levels 2–4 h after stimulation (Fig. 3, A and B). Dietary treatment did not significantly affect the level of mRNA detected before (0 h) or after (2, 4, or 6 h) LPS stimulation. Furthermore, neither the level of COX-2 protein (Western blot; Fig. 4A) nor de novo synthesis of COX-2 (immunoprecipitation; data not shown) following LPS stimulation was affected by the level of vitamin E in the diet. In contrast to COX-2, COX-1 mRNA (data not shown) and protein were barely detectable in macrophages from either treatment group. Consistent with reports that COX-1 acts as a “housekeeping” gene, LPS stimulation and dietary treatment had no effect (P = 0.62) on its expression (Fig. 4B). These results demonstrate that vitamin E does not affect expression of either COX-1 or COX-2 genes but decreases COX activity by influencing posttranslational regulation.

DISCUSSION

In this study, we confirmed our previously reported findings that PGE$_2$ production by mouse macrophages is increased with age and that this change is a consequence of age-associated upregulation of COX activity (16). Furthermore, we demonstrated that dietary supplementation with vitamin E reduces PGE$_2$ production by macrophages from old mice mainly through the suppression of age-associated increase in COX activity and not substrate availability. The vitamin E-induced suppression of COX activity was not due to a decrease in expression of protein or mRNA for COX-1 or COX-2, thus indicating regulation by vitamin E at the posttranslational level.

In the present study, we investigated the effect of vitamin E on PGE$_2$ production by macrophages from old C57BL/6NIA mice fed a semipurified diet containing 30 or 500 ppm vitamin E (means ± SE of 4 independent experiments). Macrophages were cultured in presence of 5 µg/ml LPS for specified times. RNase protection assay was used to assess mRNA levels. Autoradiographs were scanned, and each COX-2 signal was normalized to signal of its corresponding β-actin band.
young and old animals and examined the mechanism of vitamin E-induced inhibition of PGE$_2$ production. PGE$_2$ level is regulated by substrate availability, i.e., arachidonic acid level, and the activity of enzyme COX. The activity of the enzyme is determined by the level of the enzyme and requires hydroperoxide as an activator (17, 41). Because our previous study showed that the age-associated difference in PGE$_2$ production is not due to substrate availability but rather is a reflection of changes in COX activity (15, 16), we focused on COX activity in determining the mechanism of vitamin E-induced decrease of PGE$_2$ production in old mice. To show that the effect of vitamin E was due to decreased COX activity and not substrate availability, PGE$_2$ production was measured in the presence of excess exogenously added arachidonic acid. As can be seen in Fig. 2, vitamin E inhibited PGE$_2$ production when substrate was not limited. This is further supported by the fact that the inhibitory effect of vitamin E was age specific; no significant effect on LPS-stimulated PGE$_2$ production or COX activity was observed in young macrophages. However, vitamin E supplementation significantly decreased both LPS-stimulated PGE$_2$ production and COX activity in macrophages from old 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, i.e., 60%, further 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, thromboxane (TX) A$_2$ (measured as its stable hydrolytic product TXB$_2$), in the supernatant from COX activity cultures. The results show that vitamin E had no effect on the conversion of exogenous arachidonic acid to TXA$_2$ in LPS-stimulated macrophages of young mice ($10.6 \pm 1.8$ and $8.1 \pm 1.4$ pg·µg protein$^{-1}$·10 min$^{-1}$ in mice fed 30 and 500 ppm vitamin E, respectively). Vitamin E, however, significantly decreased conversion of exogenous arachidonic acid to TXA$_2$ in LPS-stimulated macrophages of old mice ($19.5 \pm 4.7$ and $7.8 \pm 1.4$ pg·µg protein$^{-1}$·10 min$^{-1}$ in mice fed 30 and 500 ppm vitamin E, respectively). Similar to the results for PGE$_2$ production, macrophages from old mice fed 30 ppm vitamin E had significantly higher TXA$_2$ production than young mice fed 30 ppm vitamin E. 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$_2$, i.e., ~60%. This further supports our conclusion that the effect of vitamin E is mediated through the reduction of COX activity rather than upstream or downstream enzymes.

In many cases, the altered COX activity reflects changes in the rate of enzyme synthesis, the rate of mRNA transcription, or both. Thus, to further clarify how vitamin E modifies COX activity in old mice, we examined COX protein and mRNA expression. In agreement with previous reports (9, 16, 24, 32) and as shown in Fig. 3, the expression of COX-2 mRNA reached a maximum after 2–4 h of stimulation with LPS. In contrast, COX-1 mRNA was barely detectable, and LPS stimulation did not potentiate its expression (data not shown). Accordingly, Western blot showed a low but detectable level of COX-1 protein, whereas COX-2 protein was detected in a substantial amount after 6 h of LPS stimulation. Our results are in concordance with previous studies showing that COX-1 is not activated...
by the different stimuli in such monocyctic cells as mouse peritoneal macrophages (35), rat alveolar macrophages (23), mouse RAW 264.7 macrophage cell line (35), and human monocytes and alveolar macrophages (18). In contrast, COX-2 mRNA and protein were barely detected in unstimulated cells but were induced by LPS (10, 23, 44). There was no significant effect of vitamin E on protein (as measured by Western blot analysis and immunoprecipitation) or mRNA level for either COX-1 or COX-2. Thus the vitamin E-induced decrease in COX activity of macrophages from old mice is not due to its regulation of COX transcription or translation; rather, it appears that vitamin E exerts its effect posttranslationally.

COX activity requires the presence of oxidant hydroperoxides (17, 21, 41). Hemler and Lands (17) showed that the lag phase in attaining maximal COX activity was shortened or eliminated by endogenous or exogenous hydroperoxide, whereas antioxidant phenol and α-naphthol, as well as glutathione peroxidase, inhibited COX catalysis and caused an extended lag period. These studies indicated that sufficient hydroperoxide is required to fully activate COX. It is interesting to note that Kulmacz and Wang (21) showed that COX initiation in COX-2 requires a lower level of hydroperoxide than that required by COX-1, allowing prostaglandin synthesis by COX-2 to proceed at hydroperoxide levels that will not support COX catalysis by COX-1. Vitamin E is an effective biological antioxidant and chain-breaking free radical scavenger, and therefore vitamin E might attenuate COX activity by scavenging the oxidant hydroperoxide necessary for COX activation. The fact that vitamin E inhibits COX activity in old but not young mice supports this notion because many studies have indicated increased formation of lipid peroxides in different tissues of the aged animals. The free radical NO has drawn increasing attention for its role in a variety of cell functions. Several studies have indicated its involvement in regulation of COX activity and eicosanoid metabolism. Some investigators have suggested that NO stimulates COX activity via direct stimulation of the enzyme (40). We recently (3) showed that LPS-stimulated peritoneal macrophages from old mice produced more NO than those from young mice, and dietary supplementation with vitamin E reduced NO production in macrophages from old mice. Thus inhibition of COX activity by vitamin E might be mediated through reduction of NO production. Further studies are needed to determine the role of NO and other free radical species in vitamin E-induced suppression of COX activity in aged mice.

Sakamoto et al. (39) reported that phorbol 12-myristate 13-acetate or A-23187-stimulated PGE2 production and arachidonic acid release from rat macrophages were inhibited following 6 days of intraperitoneal injection of vitamin E. They suggested and later confirmed (37) that the reduced PGE2 production resulted from inhibition of phospholipase A2 activity and the subsequent decrease in arachidonic acid release by vitamin E. This may represent one of the mechanisms underlying the modulation of prostaglandin synthesis by vitamin E and might have contributed to the vitamin E-decreased accumulation of PGE2 production in old mice reported here. This is not likely, however, since as mentioned above the effect of vitamin E on PGE2 production is observed when substrate (arachidonic acid) is not limited (see PGE2 production and COX enzyme activity). Furthermore, the decrease in PGE2 accumulation (60%) corresponds to the 60% decrease in COX activity. In the above-mentioned studies, Sakamoto’s group did not examine COX activity or expression of COX protein and mRNA. In contrast to their previous report (37), in a recent paper Sakamoto et al. (38) reported no effect of vitamin E on LPS-stimulated PGE2 production, COX activity, COX-2 protein, or mRNA levels of rat macrophages. The reason for this discrepancy is not clear. The age of the animals in the two studies by Sakamoto et al. (37, 38) was not defined. Because we observe the effect of vitamin E in old mice and not in young mice, one may speculate that there might have been age differences between the two studies of Sakamoto et al. (37, 38).

The age-associated increase in COX activity and PGE2 production and its reduction by vitamin E has significant biological and clinical implications for the aged. We, along with other groups, have shown that increased PGE2 production contributes to decreased T cell-mediated function with aging (2, 4, 11, 15, 26, 28, 36). Furthermore, we demonstrated that inhibition of PGE2 production by vitamin E can result in significant improvement in T cell proliferation, IL-2 production, delayed-type hypersensitivity skin response, and antibody production in aged mice and humans (4, 26–28). In addition to changes in T cell-mediated function with aging, there is dysregulation of other components of the immune system, resulting in increased inflammation and greater incidence of autoimmune diseases. Elderly humans and aged mice have higher IL-6 production than their young counterparts (7). Hinson et al. (20) showed that PGE2 induces IL-6 production in vivo. It is interesting to note that we previously showed that vitamin E supplementation significantly decreases stimulated IL-6 production by human peripheral blood mononuclear cells (6) and spontaneous IL-6 production by peritoneal macrophages of old mice (46). Furthermore, increased PGE2 production and COX activity have been implicated in the pathogenesis of several age-associated diseases such as arthritis, atherosclerosis, cancer, and autoimmune diseases. A beneficial effect of vitamin E in reducing the risk of cancer and heart diseases has been reported (25).

In summary, our results show that the age-associated increase in PGE2 production is reversed by vitamin E. Vitamin E exerts its effect posttranslationally through inhibition of COX activity. These findings can have significant implications for the development of preventive and therapeutic strategies to reduce age-associated pathogenesis, particularly that of immune and inflammatory diseases.

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