The effect of tocopheryl phosphates (TPM) on the development of atherosclerosis in apolipoprotein-E deficient mice

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Summary
α-Tocopheryl phosphate (TP) is a naturally occurring form of vitamin E found in the body. In the present study we compared the ability of an α-TP mixture (TPM) against a standard vitamin E supplement, α-tocopherol acetate (TA) on the development of atherosclerotic lesions in ApoE-deficient mice. Mice were maintained on either a normal chow diet for 24 weeks (Normal Diet), vs a group in which the final 8 weeks of the 24-week period mice were placed on a high fat (21%), high cholesterol (0.15%) challenge diet (HFHC), to exacerbate atherosclerotic lesion development. The difference in these two control groups established the extent of the diet-induced atherosclerotic lesion development. Mice in the various treatment groups received either TA (300 mg/kg chow) or TPM (6.7–200 mg/kg chow) for 24 weeks, with TPM treatment resulting in dose-dependent significant reductions in atherosclerotic lesion formation and plasma levels of pro-inflammatory cytokines. TA-treated mice, with the tocopherol equivalent TPM dose (200 mg/kg chow), showed no significant reduction in plasma lipid levels or evidence for aortic lesion regression. At this TPM equivalent TA dose, a 44% reduction in aortic lesion formation was observed. In addition, these TPM treated mice, also showed a marked reduction in aortic superoxide formation and decreased circulating plasma levels of known pro-inflammatory markers IL-6, MCP-1, IL-1β, IFN-γ and TNF-α. These findings indicate that TPM treatment slows progression of atherosclerotic lesions in ApoE-deficient mice with this effect potentially involving reduced oxidative stress and decreased inflammation.

KEYWORDS
ApoE-deficient mice, atherosclerosis, superoxide, tocopherol, tocopheryl phosphate

1 | INTRODUCTION

Vitamin E or tocopherol as a cardio-protective agent has been examined in a variety of studies in animals and humans. Elevated oxidative stress, lipids, inflammation and endothelial dysfunction are believed to contribute to the development of cardiovascular disease.1–4 Strategies targeting the reduction of these via antioxidant supplementation have been extensively studied in human and animal models.5–9 Increased intake of α-tocopherol (vitamin E supplementation), has been shown to have protective effects against heart disease, while low levels of α-tocopherol (vitamin E) has long been associated with increased incidence of coronary heart disease.5,7 Vitamin E supplementation in ApoE-deficient mice has previously been shown to suppress isoprostane generation and atherosclerosis.9
Inhibition of smooth muscle cell proliferation, preservation of endothelial function, inhibition of monocyte-endothelial adhesion, inhibition of monocyte reactive oxygen species and cytokine release, and inhibition of platelet adhesion and aggregation are all factors that have been demonstrated in vitro by the use of vitamin E and contribute to its potential anti-atherosclerotic properties. However, human clinical trials using vitamin E to examine potential anti-atherosclerotic properties in vivo have been equivocal and in most cases show no effect. Current vitamin E supplements alone are therefore not a useful clinical option to combat atherosclerosis and inflammation.

Novel tocopherol derivatives have been previously described by our laboratory and collaborators including the α-tocopheryl phosphate ester (TP) and the di-α-tocopheryl phosphate ester (T₈P). TP is the ester derivative of phosphate with the hydroxyl group of tocopherol, while T₈P is obtained by esterification of two tocopherol moieties with one phosphate molecule. Tocopheryl phosphate mixture (TPM), also referred to in the literature as mixed tocopheryl phosphates (MTP), is made up of a mixture of these two phosphorylated forms of α-tocopherol, (ie contains α-tocopheryl phosphate (TP) and α-di-tocopheryl phosphate T₈P in a 2:1 ratio by weight).

In this context, we have shown that TP, like α-tocopherol, is a naturally occurring compound. Many in vitro and in vivo effects of TP have been described, including its ability to inhibit the proliferation of a number of cell types including aortic smooth muscle cells and monocytes, apoptosis, signal transduction and gene expression, all of which are key players in the formation of atherosclerotic lesions. This activity appears to be more potent than that of its unphosphorylated counterpart, and provides a possible avenue for potential benefits for cardiovascular disease (CVD) intervention. Indeed, animal studies using TPM supplementation, but not α-tocopheryl acetate (TA), have been shown to prevent the progression of atherosclerosis and ischemia/reperfusion injury.

Therefore, the aim of the present study was to assess the effects of TPM and TA (at an equivalent upper TPM dose) in a commonly used murine model of atherosclerosis, the apolipoprotein E-deficient (ApoE⁻/⁻) mouse, treated for a total of 24 weeks, with mice placed on a high fat (21%), high cholesterol (0.15%) challenge (HFHC) diet in the final 8 weeks to exacerbate the atherosclerotic lesion development. In particular, we have examined the effects of the tocopherols on plasma lipid profiles and the progression of aortic lesions in ApoE⁻/⁻ mice.

2 | RESULTS

2.1 | Analysis of food consumption and dosing with the tocopherol compounds

Animals gained weight throughout the experimental period at a similar rate (Table 1), and food (Table 1) and water (data not shown) consumption were also similar and did not differ between treatments. As described earlier, the tocopherol equivalent dose delivered to each of the TA and TPM treatment groups were not considered significantly different (Table 1).

The average daily doses of the various tocopherol compounds were calculated for each of the five doses in terms of tocopherol equivalent (TE) doses based on molecular weight comparisons with α-tocopherol. Table 1 outlines how the 6.7, 33, 67, 133 and 200 mg/kg TPM doses in chow correspond to TE average doses of 0.61, 2.96, 6.11, 12.01 and 17.88 mg/kg doses respectively for the first 16 weeks of treatment (on the ‘normal’ chow 7% fat diet), and 0.47, 2.26, 4.71, 9.14 and 19.08 mg/kg doses respectively for the final 8 weeks (on the HFHC diet). The TA 300 mg/kg diet dose corresponded to a TE average daily dose of 19.48 mg/kg for the first 16 weeks and 20.22 mg/kg for the final 8 weeks HFHC diet phase, which was approximately equivalent to the highest TPM dose tested (ie 17.88 and 19.08 mg/kg).

2.2 | Plasma lipid concentrations

Following the 16 weeks of treatment (induction phase), TA had no effect on plasma lipid levels compared to control HFHC mice. In contrast, there appeared to be a dose dependent reduction in TC, TG and LDL-C levels in TPM treated mice, however, only the higher doses (TPM 67 mg/kg, TPM 133 mg/kg and TPM 200 mg/kg) caused significant reductions in plasma TC and LDL-C, with the highest reduction of 25% and 35% respectively after TPM 200 mg/kg treatment, compared to control HFHC treated ApoE⁻/⁻ mice (Table 2). Plasma TC, TG, LDL-C and HDL-C all increased at the end of the challenge period in all HFHC fed mice and TPM treatment at all doses had no long-lasting effects on final lipid profile of the mice (Table 2).

2.3 | Atherosclerotic lesion development

En face staining with oil red O, showed substantial deposits of lipids in vascular atherosclerotic lesions in aorta taken from all treatment groups (Figure 1A). Animals maintained on a normal diet exhibited lesions that represented 6.70 ± 1.05% of the aorta at the end of the 24 weeks period (Figure 1B). Animals that were placed on the HFHC diet for the final 8 weeks showed significantly elevated lesion development (12.83 ± 1.06%) compared to control ND-fed ApoE⁻/⁻ mice. Interestingly, although we observed a trend towards a reduction in lesion development following treatment with TA, this effect did not reach statistical significance. In contrast, TPM at 67, 133 and 200 mg/kg chow doses, significantly reduced atherosclerotic lesion development compared to control HFHC fed mice following the challenge phase, representing an approximate 40%-44% reduction in lesion formation (Figure 1B).

2.4 | Superoxide expression

Dihydroethidium staining was used to localise superoxide levels within aortic sections taken from control ND and control HFHC fed mice, and also mice fed a HFHC diet that were treated with tocopherol equivalent doses of TA (300 mg/kg chow) and TPM (200 mg/kg chow). Fluorescence was observed throughout the three layers of the vessel wall. The intensity of fluorescence was measured and expressed as total intensity (arbitrary units). Treatment with TPM...
The superoxide anion is well recognized as a major mediator of oxidative stress and subsequent pro-atherogenic effects such as LDL oxidation, regulation of adhesion molecules and cellular proliferation and inflammation.\(^\text{30–33}\) In addition to the known antioxidant effects of \(\alpha\)-tocopherol,\(^\text{34}\) the beneficial effects of TPM observed in the current study may reflect TP-mediated inhibition of the production of superoxide by predominant sources such as NADPH oxidase.\(^\text{35}\) This is well supported as \(\alpha\)-tocopherol has previously been shown to inhibit the assembly of NADPH oxidase subunits and subsequently superoxide production.\(^\text{36}\) Interestingly, beneficial effects of vitamin E have been reported in LDL receptor-deficient mice independent of a reduction in cholesterol levels, but was associated with significant suppression of oxidative and inflammatory reactions following vitamin E supplementation.\(^\text{5}\) Therefore, although lipids were not reduced following the challenge phase, reductions in superoxide following TPM treatment may account for the decreased lipid deposition as well as subsequent reductions in circulating plasma biomarker levels.

Our findings are consistent with a previous study in which a 2% cholesterol diet was fed to New Zealand albino rabbits for 4 weeks in the absence or presence of TPM or TA.\(^\text{20}\) TPM-treated rabbits showed no significant change in the plasma lipid levels but exhibited a reduction in plaque formation compared to controls. Thus, in both studies (current;\(^\text{25}\)), in the face of deliberately-elevated plasma lipids, the same dose of TPM in the 2 animal models exerted similar macroscopic protection against plaque formation in the aortae of these animals. However, the current study also identified changes in oxidative stress which may account for reductions in inflammation, as a possible mechanism in TP mediated atheroprotection.

Although oxidative stress has been linked with a pro-inflammatory response, non-antioxidant mediated effects of TP, including anti-inflammatory properties independent of reductions in superoxide cannot be excluded. In rabbits, TP caused a reduction in CD36 scavenger receptor expression.\(^\text{29}\) The CD36 scavenger receptor has a pivotal role in the uptake of oxidized LDLs (oxLDLs) from the bloodstream.
### TABLE 1  Body weight and food consumption (g; mean ± SEM) during the ‘normal diet’ and high fat, high cholesterol challenge phases of the study

<table>
<thead>
<tr>
<th></th>
<th>Control (normal diet) (n = 20)</th>
<th>Control (HFHC diet) (n = 16)</th>
<th>TA(^a) (HFHC diet) (n = 16)</th>
<th>TPM 6.7 mg/kg (HFHC diet) (n = 16)</th>
<th>TPM 33 mg/kg (HFHC diet) (n = 16)</th>
<th>TPM 67 mg/kg (HFHC diet) (n = 16)</th>
<th>TPM 133 mg/kg (HFHC diet) (n = 15)</th>
<th>TPM(^a) 200 mg/kg (HFHC diet) (n = 16)</th>
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<tr>
<td><strong>Body weight measurements</strong></td>
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<td>Baseline (week 0)</td>
<td>19.04 ± 0.57</td>
<td>21.81 ± 0.67</td>
<td>19.52 ± 0.81</td>
<td>19.28 ± 0.54</td>
<td>20.45 ± 0.37</td>
<td>19.57 ± 0.59</td>
<td>19.57 ± 0.46</td>
<td>18.07 ± 0.43</td>
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<tr>
<td>Induction phase (week 16)</td>
<td>27.69 ± 0.37</td>
<td>28.17 ± 0.51</td>
<td>27.30 ± 0.59</td>
<td>27.20 ± 0.31</td>
<td>26.91 ± 0.79</td>
<td>26.31 ± 0.50</td>
<td>26.89 ± 0.43</td>
<td>26.50 ± 0.37</td>
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<tr>
<td>Challenge phase (week 24)</td>
<td>29.66 ± 0.49</td>
<td>32.69 ± 0.62</td>
<td>31.76 ± 1.05</td>
<td>35.52 ± 0.93</td>
<td>33.52 ± 1.30</td>
<td>33.93 ± 1.10</td>
<td>34.20 ± 1.10</td>
<td>30.48 ± 0.60</td>
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<tr>
<td><strong>Food consumption measurements</strong></td>
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<tr>
<td>TA/TPM dose in the chow (mg/kg)</td>
<td>– –</td>
<td>300</td>
<td>6.7</td>
<td>33</td>
<td>67</td>
<td>133</td>
<td>200</td>
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<tr>
<td><strong>Induction phase (week 0-16)</strong></td>
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<tr>
<td>Average daily food (g/mouse)</td>
<td>4.18 ± 0.21</td>
<td>4.14 ± 0.30</td>
<td>3.99 ± 0.27</td>
<td>3.07 ± 0.06</td>
<td>3.07 ± 0.60</td>
<td>3.00 ± 0.05</td>
<td>3.04 ± 0.07</td>
<td>3.43 ± 0.23</td>
</tr>
<tr>
<td>Average daily TA/TPM dose (mg/kg)</td>
<td>– –</td>
<td>21.41 ± 1.16</td>
<td>0.86 ± 0.03</td>
<td>4.17 ± 0.13</td>
<td>8.61 ± 0.29</td>
<td>16.91 ± 0.71</td>
<td>25.18 ± 0.90</td>
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<tr>
<td>Average daily TE dose (mg/kg)</td>
<td>N/A</td>
<td>N/A</td>
<td>19.48</td>
<td>0.61</td>
<td>2.96</td>
<td>6.11</td>
<td>12.01</td>
<td>17.88</td>
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<td><strong>Challenge phase (week 16-24)</strong></td>
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<tr>
<td>Average daily food (g/mouse)</td>
<td>4.078 ± 0.44</td>
<td>2.93 ± 0.15</td>
<td>3.30 ± 0.20</td>
<td>3.10 ± 0.07</td>
<td>2.92 ± 0.05</td>
<td>2.94 ± 0.05</td>
<td>2.98 ± 0.06</td>
<td>2.89 ± 0.11</td>
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<tr>
<td>Average daily TA/TPM dose (mg/kg)</td>
<td>– –</td>
<td>22.22 ± 1.68</td>
<td>0.66 ± 0.03</td>
<td>3.19 ± 0.10</td>
<td>6.64 ± 0.22</td>
<td>12.88 ± 0.46</td>
<td>26.87 ± 2.46</td>
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<tr>
<td>Average daily TE dose (mg/kg)</td>
<td>N/A</td>
<td>N/A</td>
<td>20.22</td>
<td>0.47</td>
<td>2.26</td>
<td>4.71</td>
<td>9.14</td>
<td>19.08</td>
</tr>
</tbody>
</table>

Where appropriate, data are given as the mean ± SEM; N/A, not applicable; TA, tocopheryl acetate; TPM, tocopheryl phosphate mixture; TE, tocopherol equivalent, calculated based on molecular weight comparisons with α-tocopherol.

\(^a\)The TE equivalent TA and TPM doses, which are used in subsequent analysis (ie the TA 300 mg/kg chow dose and TPM 200 mg/kg chow dose).
### Table 2  Mouse plasma lipid level comparisons (mmol L⁻¹; mean ± SEM) during the induction and challenge phases of the study

<table>
<thead>
<tr>
<th></th>
<th>Control (normal diet) (n = 20)</th>
<th>Control (HFHC diet) (n = 16)</th>
<th>TA³ (HFHC diet) (n = 16)</th>
<th>TPM 6.7 mg/kg (HFHC diet) (n = 16)</th>
<th>TPM 33 mg/kg (HFHC diet) (n = 16)</th>
<th>TPM 67 mg/kg (HFHC diet) (n = 16)</th>
<th>TPM 133 mg/kg (HFHC diet) (n = 15)</th>
<th>TPM⁴ 200 mg/kg (HFHC diet) (n = 16)</th>
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<tr>
<td><strong>Total cholesterol (mmol L⁻¹)</strong></td>
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<tr>
<td>Baseline (week 0)</td>
<td>10.98 ± 0.77</td>
<td>11.60 ± 0.71</td>
<td>12.04 ± 0.90</td>
<td>10.72 ± 0.65</td>
<td>10.94 ± 0.59</td>
<td>11.06 ± 0.40</td>
<td>10.25 ± 0.34</td>
<td>10.70 ± 0.57</td>
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<tr>
<td>Induction phase (week 16)</td>
<td>12.90 ± 1.58</td>
<td>14.88 ± 0.70</td>
<td>15.45 ± 0.77</td>
<td>14.14 ± 0.63</td>
<td>13.39 ± 0.98</td>
<td>13.16 ± 0.62</td>
<td>12.74 ± 0.41</td>
<td>11.93 ± 0.85</td>
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<tr>
<td>Challenge phase (week 24)</td>
<td>14.33 ± 1.09**</td>
<td>48.90 ± 1.63</td>
<td>50.36 ± 2.37</td>
<td>55.37 ± 2.14</td>
<td>51.54 ± 1.94</td>
<td>52.86 ± 2.19</td>
<td>47.80 ± 3.17</td>
<td>52.30 ± 2.52</td>
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<td><strong>Triglycerides (mmol L⁻¹)</strong></td>
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<tr>
<td>Baseline (week 0)</td>
<td>1.65 ± 0.12</td>
<td>1.75 ± 0.20</td>
<td>1.68 ± 0.21</td>
<td>2.31 ± 0.20</td>
<td>1.88 ± 0.18</td>
<td>1.75 ± 0.14</td>
<td>1.66 ± 0.14</td>
<td>1.55 ± 0.17</td>
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<tr>
<td>Induction phase (week 16)</td>
<td>1.13 ± 0.20</td>
<td>2.00 ± 0.24</td>
<td>1.31 ± 0.13</td>
<td>1.61 ± 0.15</td>
<td>1.53 ± 0.20</td>
<td>1.45 ± 0.10</td>
<td>1.64 ± 0.13</td>
<td>1.50 ± 0.19</td>
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<tr>
<td>Challenge phase (week 24)</td>
<td>1.58 ± 0.26</td>
<td>2.48 ± 0.21</td>
<td>2.11 ± 0.16</td>
<td>2.68 ± 0.19</td>
<td>2.21 ± 0.18</td>
<td>2.31 ± 0.17</td>
<td>2.42 ± 0.24</td>
<td>2.18 ± 0.17</td>
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<td><strong>LDL-C (mmol L⁻¹)</strong></td>
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<tr>
<td>Baseline (week 0)</td>
<td>5.23 ± 0.60</td>
<td>5.92 ± 0.87</td>
<td>5.30 ± 0.99</td>
<td>3.63 ± 0.35</td>
<td>3.83 ± 0.31</td>
<td>3.84 ± 0.19</td>
<td>3.47 ± 0.18</td>
<td>4.90 ± 0.68</td>
</tr>
<tr>
<td>Induction phase (week 16)</td>
<td>5.57 ± 0.83</td>
<td>5.39 ± 0.25</td>
<td>5.31 ± 0.48</td>
<td>5.24 ± 0.43</td>
<td>4.78 ± 0.46</td>
<td>4.30 ± 0.3</td>
<td>4.20 ± 0.18</td>
<td>3.71 ± 0.34*</td>
</tr>
<tr>
<td>Challenge phase (week 24)</td>
<td>5.14 ± 0.73**</td>
<td>24.08 ± 1.22</td>
<td>20.81 ± 1.32</td>
<td>27.20 ± 1.58</td>
<td>25.45 ± 1.56</td>
<td>25.40 ± 1.70</td>
<td>23.93 ± 2.01</td>
<td>24.75 ± 1.61</td>
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<td><strong>HDL-C (mmol L⁻¹)</strong></td>
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<tr>
<td>Baseline (week 0)</td>
<td>5.23 ± 0.50</td>
<td>4.89 ± 0.48</td>
<td>1.96 ± 0.53</td>
<td>6.03 ± 0.24</td>
<td>6.25 ± 0.25</td>
<td>6.42 ± 0.19</td>
<td>6.03 ± 0.18</td>
<td>5.10 ± 0.37</td>
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<tr>
<td>Induction phase (week 16)</td>
<td>7.16 ± 0.69</td>
<td>8.80 ± 0.35</td>
<td>9.54 ± 0.66</td>
<td>8.17 ± 0.24</td>
<td>7.91 ± 0.50</td>
<td>8.20 ± 0.32</td>
<td>7.79 ± 0.25</td>
<td>7.53 ± 0.45</td>
</tr>
<tr>
<td>Challenge phase (week 24)</td>
<td>7.76 ± 0.89**</td>
<td>23.69 ± 0.78</td>
<td>28.58 ± 1.29</td>
<td>26.94 ± 0.87</td>
<td>25.08 ± 0.77</td>
<td>26.39 ± 0.92</td>
<td>22.77 ± 1.48</td>
<td>26.55 ± 1.14</td>
</tr>
</tbody>
</table>

*P < .05 vs Control (HFHC Diet) after induction phase; **P < .001 vs all other diets after challenge phase.

¹The TE equivalent TA and TPM doses, which are used in subsequent analysis (ie the TA 300 mg/kg chow dose and TPM 200 mg/kg chow dose).
Thus a reduction in CD36 expression, suggests an involvement of reduced uptake of oxLDLs and thereby a reduction in foam cell formation. These results are consistent with phosphorylated tocopherols causing reduction of CD36 scavenger receptor expression and key pro-inflammatory markers in mammalian cell lines. Since there is already precedence for modulation of CD36 expression by TP in the hypercholesterolemic rabbit model, we measured other pro-inflammatory biomarkers that are known to contribute to the progression of atherosclerosis and have been extensively reviewed elsewhere. Our current study demonstrated significant reductions in plasma levels of IL-6, MCP-1, IL-1β, IFN-γ and TNF-α following TPM treatment (200 mg/kg chow dose) which are all known contributors to lesion development. Importantly chronic TPM treatment, but not TA or TP, resulted in significant reductions in lesion area further highlighting the positive effect of this vitamin E mixture.

α-Tocopherol has the ability to act as an antioxidant or free radical scavenger, while at the same time may also be a potential source of free radicals. Interestingly at a cellular level α-tocopherol also has other functions not related to its redox chemistry which are commonly referred to as the non-antioxidant roles of vitamin E, however the exact molecular mechanism of α-tocopherol or vitamin E is still not completely understood. The discovery that TP is another natural form of vitamin E, and can be synthesized by human coronary artery cells and mast cells, along with increased levels of α-tocopherol in cells indicates that endogenously cells may convert α-tocopherol to TP (ie α-tocopherol can be phosphorylated to TP, and/or TP can be de-phosphorylated). TP’s potent non-antioxidant effects, and the ability for it to interconvert to α-tocopherol and vice versa in the body, has offered a potential hypothesis to explain the observed non-antioxidant roles of vitamin E. For example, in cultured human aortic smooth muscle cells (HASMC), TP but not α-tocopherol, inhibited CD36 mRNA expression, indicating possible disparate roles for the two compounds. This is in line with the fact that in the current study, TP was more effective than TA in reducing oxidative stress and levels of inflammatory markers (Figure 4).

Interestingly, an important observation from the current study was that TPM, but not TA at equivalent tocopherol doses, caused a marked...
inhibition of the progression of atherosclerotic lesions in HFHC-fed ApoE−/− mice. Oil red O staining is a well-recognized method of plaque quantification and we have previously shown that this method correlates well with changes in the luminal encroachment by atherosclerotic plaque. We have demonstrated an atheroprotective effect and future studies will be targeting the lesion complexity following TPM treatment. Doses of TA and TPM were monitored and adjusted to approximate equivalent tocopherol levels (at the highest TPM dose; 200 mg/kg chow), and particular care was taken to provide a modified diet that was low in vitamin E in order to avoid confounding effects on treatments. However, in contrast to previous reports by Pratico et al. and Cyrus et al., we did not observe protective effects of vitamin E (TA) in our model of atherosclerosis. An important distinction between our study and those previously documented, is that our dose of TA was substantially lower (5-10 fold) with mice receiving 200 mg/kg chow compared to 1000-2000 mg/kg chow. The dose of TA was chosen to approximate an equivalent tocopherol load as that of TPM. Since our previous reports have documented that TP at 200 mg/kg chow was protective in hypercholesterolemic rabbits, we used the same dose and thus our TA dose was also reduced accordingly. Importantly, we have demonstrated results consistent with previous rabbit studies which further highlights the potency of TP, but also may explain disparate effects of TA in our study compared with previous reports.

In conclusion our results indicate that TPM treatment reduced the development of lesion formation in ApoE-KO mice, and thus may represent a novel treatment strategy in the setting of atherosclerosis. Mechanistically, the present study also indicated anti-oxidative and/
or anti-inflammatory properties of TP which may have contributed to the atheroprotective effects. Taken together, the present study highlights the ability of TPM to inhibit the progression of the major aggravating elements involved in the development of atherosclerosis. The higher potency of TPM compared to TA was also highlighted and may be due to better uptake of the phosphate ester and to its intracellular direct activity and/or hydrolysis providing more α-tocopherol to sensitive sites.

**FIGURE 3** Plasma cytokine concentrations for (A) IL-1β, (B) MCP-1, (C) IL-6, (D) TNF-α and (E) INF-γ in ApoE−/− mice fed either control normal diet (ND), control high fat high cholesterol diet (HFHC) alone or in combination with α-tocopherol acetate (TA) or α-tocopheryl phosphate mixture (TPM) at the tocopherol equivalent TA dose (ie 200 mg/kg chow) for the final 8 weeks. Data are mean ± SEM. *P < .05 vs control HFHC-fed ApoE−/− mice (n = 8)

**FIGURE 4** Schematic representation showing the anti-inflammatory effects of α-tocopherol (α-Toc) and α-tocopheryl phosphate (α-TP)
4 | METHODS

4.1 | Animals and diets

One hundred and thirty-one male ApoE<sup>−/−</sup> mice were obtained at 6 weeks of age from the Animal Resource Centre (ARC) in Perth, WA, Australia. The animals were divided into eight treatment groups, for a total of 24 weeks. The groups were as follows: Group 1 (n = 20), control mice fed a ‘normal’ vitamin E stripped (ie less than 20 ppm vitamin E), mouse chow pellet, for the full 24 weeks treatment period (this chow diet was a modified version of the standard AIN93G rodent diet (SF05-040, produced by Specialty Feeds, Glen Forrest, WA Australia, with 7% total fat, containing less than 20 ppm of vitamin E). Group 2 (n = 16), control mice fed a ‘normal’ vitamin E stripped (ie less than 20 ppm vitamin E), mouse chow pellet, for the first 16 weeks followed by a high fat, high cholesterol specialised rodent pellet diet (this chow diet was a modified version of the standard SF00-219 diet (SF04-055), produced by Specialty Feeds, Glen Forrest, WA, Australia, containing 21% fat and 0.15% cholesterol with less than 20 ppm of vitamin E), for the final 8 weeks of the experimental period to exacerbate the atherosclerotic lesion development, in this animal model. This control group, referred to as the HFHC group, was the base diet into which subsequent TPM or TA treatment were formulated. Group 3 (n = 16), mice fed the HFHC control diet plus tocopherol acetate (TA; 300 mg/kg chow), Group 4 (n = 16), mice fed the HFHC control diet containing TPM at 6.7 mg/kg chow; Group 5 (n = 16), mice fed the HFHC control diet plus TPM at 33 mg/kg chow; Group 6 (n = 16), mice fed the HFHC control diet plus TPM at 67 mg/kg chow; Group 7 (n = 15), mice fed the HFHC control diet plus TPM at 133 mg/kg chow and Group 8 (n = 16), mice fed the HFHC control diet plus TPM at 200 mg/kg chow. The mice were housed individually with free access to food and water over the experimental period. The animal body weight, food and water consumption were monitored weekly, with calculated average daily consumption and subsequent average daily doses determined from these measurements (Table 1). The study was approved by the Animal Ethics Committee of Monash University, Melbourne, Australia.

4.2 | Blood sampling

After 7 days of adaptation, blood samples were taken from the tail vein to establish the initial baseline plasma lipid parameters. Plasma lipid analyses were also performed at week 16 (the end of the normal diet administration or ‘induction phase’) and at the end of week 24 (following the 8 weeks on the high fat, high cholesterol challenge diet or ‘challenge phase’). At this time, animals were killed by CO<sub>2</sub> asphyxiation and blood was collected from the right ventricle.

4.3 | Plasma lipid measurements

For plasma lipid measurements, total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) levels, in 250 μL of plasma was independently analysed (Gribbles Veterinary Pathology, Melbourne, Australia) using enzymatic in vitro tests for direct quantitative determination of lipids in plasma on automated clinical chemistry analysers (Roche/Hitachi 912/917). Plasma lipid levels were measured at baseline, after the induction phase and at the end of the experiment using enzymatic assays.

4.4 | Quantification of atherosclerotic plaques

In each group atherosclerotic lesions were measured using en face staining with oil red O in the longitudinally cut aorta as previously described. ⁴⁰ Imaging was performed using the Moticam-480P Digital Camera and quantitated using the appropriate software (Motic Images Plus 2.0). Lesion area was expressed as a percentage of the total luminal area of the vessel.

4.5 | Localization of superoxide by dihydroethidium staining

Superoxide measurements were obtained in a subset of n = 7 mice/group to examine the influence of TA and the approximate tocopherol equivalent (TE) TPM dose (ie 200 mg/kg chow) on oxidative stress. Dihydroethidium (DHE; 2 μmol L<sup>−1</sup>) was used as previously described⁴⁰ to detect superoxide in frozen aortic sections (30 μm thick) of the thoracic aorta. DHE is oxidized to ethidium by O<sub>2</sub><sup>−</sup>, which can be excited at 488 nm with an emission spectrum of 610 nm. Images were obtained with an Olympus Fluoview 500 confocal microscope equipped with a krypton/argon laser. Laser settings were identical for each image acquired. The intensity of fluorescence was quantified in two whole aortic sections from each mouse at two optical levels approximately 3 μm apart, using analySIS software (Soft Imaging System, Singapore) with identical measurement settings.

4.6 | Plasma biomarker measurements

Plasma levels for known pro-atherogenic biomarkers including interleukin (IL)-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), were measured using a Bio-Plex cytokine assay (Mouse cytokine 5-plex panel, Bio-Rad, Australia). The detection range was 0.5–32 000 pg/mL and assays were performed according to the manufacturer’s instructions.

4.7 | Statistical analysis

All results are expressed as mean ± SEM. Statistical comparisons were done by one-way analysis of variance (ANOVA) with Bonferroni corrections where appropriate using Graphpad Prism version 5 (Graphpad Software, San Diego CA, USA).

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REFERENCES


