Oxidised plant sterols as well as oxycholesterol increase the proportion of severe atherosclerotic lesions in female LDL receptor\(^{+/−}\) mice

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Abstract

Oxysterols (oxidised cholesterol) may play a role in the pathogenesis of CVD. Similar to cholesterol, plant sterols are susceptible to oxidation. However, less is known about the potential atherogenicity of oxidised plant sterols (oxyphytosterols). In the present study, the atherogenicity of a mixture of oxyphytosterols was examined by feeding female LDL receptor-deficient (LDLR\(^{+/−}\)) mice for 35 weeks a control diet (atherogenic high-fat diet; \(n\) 9), an oxysterol diet (control diet + 0·025 % (w/w) oxysterols; \(n\) 12) or an oxyphytosterol diet (control diet + 0·025 % (w/w) oxyphytosterols; \(n\) 12). In the LDLR\(^{+/−}\) mice, serum levels of cholesterol, lipoprotein profiles, cholesterol exposure and inflammatory markers at the end of the experiment were comparable between the three diet groups. Nevertheless, the proportion of severe atherosclerotic lesions was significantly higher after oxysterol (41 %; \(P = 0.004\)) and oxyphytosterol (34 %; \(P = 0.011\)) diet consumption than after control diet consumption (26 %). Oxyphytosterol levels in the lesions were the highest in the oxyphytosterol group. Here, we show that not only dietary oxysterols but also dietary oxyphytosterols increase the proportion of severe atherosclerotic lesions. This suggests that plant sterols when oxidised may increase atherosclerotic lesion severity instead of lowering the size and severity of lesions when fed in their non-oxidised form. Therefore, this finding might give an indication as to where to find the answer in the current hot debate about the potential atherogenicity of plant sterols. However, to what extent these results can be extrapolated to the human situation warrants further investigation.

Key words: Diets: Oxysterols: Oxyphytosterols: Lipoproteins: Atherosclerosis

Several lines of evidence suggest that oxysterols (oxidised cholesterol) are atherogenic and play a role in the pathogenesis of CVD. Plant sterols are structurally related to cholesterol\(^{(1)}\), and the presence of one or more unsaturated bonds also makes the plant sterols susceptible to oxidation. Only small amounts of oxidised plant sterols (oxyphytosterols) can be found in the diet\(^{(2,3)}\). Nevertheless, relatively high concentrations of oxyphytosterols are present in the serum of sitosterolaemic patients\(^{(4)}\) and smaller amounts in the plasma of healthy individuals\(^{(5,6)}\). The fact that oxyphytosterol concentrations are high in sitosterolaemic patients, who also have severely elevated plasma plant sterol concentrations, may suggest that higher plasma plant sterol concentrations translate into higher plasma oxyphytosterol concentrations. Indeed, Husche et al\(^{(6)}\) have recently shown that consumption of a plant sterol-enriched margarine for 4 weeks increases serum \(7β\)-OH-campesterol concentrations in healthy individuals. Factors that are related to the oxidative behaviour of plant sterols are unknown. For cholesterol, however, it has been suggested that patients characterised by oxidative stress such as type 2 diabetics\(^{(7)}\) and patients with stable coronary artery disease\(^{(8)}\) have increased oxysterol concentrations.

If anything, endogenous oxidation of plant sterols is probably very low. This, in combination with the low serum plant sterol concentrations, makes it difficult to experimentally show the effects of endogenously formed oxyphytosterols on lesion formation. Except for studying the \(in \text{vitro}\) effects of oxyphytosterols, feeding oxyphytosterols, as has been done for oxysterols\(^{(9)}\), is another approach. Animal models have shown that oxyphytosterols are absorbed from the diet and

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**Abbreviation:** LDLR\(^{+/−}\), LDL receptor-deficient.

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transported to the lymph and accumulate in the serum, liver and aorta\(^{10}\). Furthermore, oxyphytosterols exhibit cytotoxic effects in cultured macrophages that are similar to those of oxysterols\(^{11}\). So far, there have been only limited data regarding the effects of an oxyphytosterol-enriched diet on atherogenesis\(^{10,12}\). However, from these animal studies, it is still unclear whether oxyphytosterols are atherogenic, as has been suggested for oxysterols. We, therefore, decided to investigate the effects of oxysterols and oxyphytosterols simultaneously on serum lipoproteins, inflammatory markers and atherosclerotic lesion development using heterozygous female LDL receptor-deficient (LDLR\(^{-/-}\)) mice.

**Methods**

**Animal study**

LDLR\(^{-/-}\) mice were bred in our laboratory by mating LDLR\(^{-/-}\) males with C57BL6/J (Charles River Laboratories) females. For the experiment, thirty-six female LDLR\(^{-/-}\) littermates were used. LDLR\(^{-/-}\) mice were chosen to simulate a human model for mild hypercholesterolaemia. Female mice were used because they are more responsive to the development of diet-induced atherosclerosis. At the age of 5 weeks, these mice were divided over twelve cages, with three mice per cage. The mice were housed under standard conditions in wire-topped Macrolon type-I cages with a layer of maize fibres as bedding. All diets and water were given ad libitum. Until the start of the intervention study, the mice were fed a regular mouse diet (Hope Farms). The study was conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Maastricht University.

At the age of 8 weeks, a run-in period of 2 weeks started, during which all the mice received an atherogenic control diet. This diet contained, per 100 g, 17·2 g fat with a Western diet-like fatty acid profile (4·5 g palm oil, 1·7 g coconut oil, 4·0 g soya oil, 2·2 g olive oil and 4·8 g cacao oil), 20·0 g protein (casein), 0·25 g cholesterol, 0·25 g cholic acid, 0·20 g methionine, 4·85 g mineral mixture and 0·25 g vitamin mixture. After the run-in period, the mice in the twelve cages were randomly allocated to one of the three semi-synthetic test diet groups. For the next 35 weeks, the first group (n = 12) was given the same atherogenic control diet, the second group (n = 12) received the same atherogenic control diet except that 10% of the added cholesterol was replaced by oxyysterols (0·025 g/100 g diet). The third group (n = 12) was given the same atherogenic diet, but now with 0·025 g oxyphytosterols (replacing cholesterol). For both oxysterols and oxyphytosterols, this means an approximate intake of 0·03 mg oxysterol or oxyphytosterol/g body weight per d. Oxysterols and oxyphytosterols were prepared by heating cholesterol or plant sterols at 180\(\degree\)C for 3 h (Raisio Group; Benecol Limited). In the oxysterol mixture and the oxyphytosterol mixture, 41 and 64% of the sterols identified were still present in the non-oxidised form, respectively (Table 1). As has been indicated, there is a difference in the composition of the oxysterol and oxyphytosterol mixtures, which may in theory – if there is a difference in the potential atherogenicity of different oxy(phyto)sterol forms – have had a slight influence on the results of the study.

**Sampling of blood and tissues and determination of body weight**

Fasting blood was sampled by retro-orbital bleeding under halothane anaesthesia at weeks 2, 7, 17, 27 and 37. Before sampling, the mice were weighed. Blood was collected into plastic tubes (Eppendorf AG) filled with glass beads to prepare serum. After the last blood sampling (week 37), all the mice were euthanised.

The entire heart (with aorta) of each mouse was disected, sectioned perpendicularly to the heart axis just below the atrial tips and directed put into aluminium cryotubes (Omnilabo International) placed in dry ice-cooled isopentane. The cryotubes were stored at −80\(\degree\)C until use.

**Analysis of serum cholesterol and lipoprotein profiles**

Directly after sampling, blood samples were analysed for serum total cholesterol concentrations using a commercially available enzymatic kit (GHOD/PAP method; Roche Diagnostics). The AUC was calculated for each mouse as an indicator of overall cholesterol exposure of the aorta during the 37-week experiment\(^{13}\). Before the analysis, the sera of mice housed in the same cage were pooled.

**Quantification of inflammatory marker concentrations**

The concentrations of a panel of inflammatory markers (interferon-\(\gamma\), IL-6, IL-10, IL-12, monocyte chemoattractant protein-1 (MCP-1) and TNF-\(\alpha\)) were determined using the mouse inflammation cytoketometric bead array (Becton Dickinson Biosciences) according to the manufacturer’s protocol. Briefly, 5\(\mu\)L of the sample or the cytokine standard mixture were mixed with 5\(\mu\)L of the mixed capture beads and 5\(\mu\)L of the detection antibody-phycocyrtin reagent and incubated at room temperature for 2 h in the dark. Then, a two-colour flow cytometric analysis

<table>
<thead>
<tr>
<th></th>
<th>Oxysterol mixture</th>
<th>Oxyphytosterol mixture</th>
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</thead>
<tbody>
<tr>
<td>7-Keto (% of total)</td>
<td>40·3</td>
<td>28·2</td>
</tr>
<tr>
<td>Epoxides (% of total)</td>
<td>35·7</td>
<td>20·0</td>
</tr>
<tr>
<td>5α,6α-Epoxy-</td>
<td>30·3</td>
<td>7·2</td>
</tr>
<tr>
<td>5β,6β-Epoxy-</td>
<td>5·4</td>
<td>12·7</td>
</tr>
<tr>
<td>Hydroxides (% of total)</td>
<td>24·0</td>
<td>51·8</td>
</tr>
<tr>
<td>7α-Hydroxy-</td>
<td>4·0</td>
<td>19·8</td>
</tr>
<tr>
<td>7β-Hydroxy-</td>
<td>13·8</td>
<td>32·0</td>
</tr>
<tr>
<td>Others</td>
<td>6·1</td>
<td>0·0</td>
</tr>
</tbody>
</table>

### Table 1. Composition of the oxysterol and oxyphytosterol mixtures
was performed using a FACScan® flow cytometer (Becton Dickinson Immunocytometry Systems). Before the analysis, equal volumes of sera from mice housed in the same cage were pooled. Data were acquired and analysed using the Becton Dickinson Cytometric Bead Array software (Becton Dickinson Immunocytometry Systems). Before the analyses, equal volumes of sera from mice housed in the same cage were pooled.

Quantification of atherosclerotic lesion size and severity and oxyphytosterol concentrations

After placing the frozen hearts on an object holder in the microtome/cryostat with the sectioned plane facing up, they were embedded in Tissue Tek (Sakura Finetek Europe BV). Subsequently, the frozen hearts were sectioned towards the aortic axis. Upon reaching the aortic root, serial cross-sections (7 µm thick) were made until the aortic valves disappeared. These sections were mounted onto microscope slides and air-dried on silica for 24 h. The slides were kept frozen (−80°C) until staining. Atherosclerotic lesion size was analysed for each mouse using four serial sections with a 42 µm interval. The collected sections were stained with toluidine blue and digitally photographed (Nikon DXM1200) and quantified using the digital image software (Adobe Photoshop CS3). The same colour settings and range selection was applied to measure the collagen content per lesion area. For each mouse, one section was used. Only those TUNEL-positive nuclei that displayed morphological features of apoptosis, including cell shrinkage, aggregation of chromatin into dense mass and nuclear formation, were counted.

Quantification of collagen content and apoptosis in atherosclerotic lesions

Sirius red staining was performed to visualise the collagen content per lesion area. For each mouse, one section was used. All the stained sections were photographed under the same conditions with a digital microscope camera (Nikon DXM1200). To quantify the proportion of collagen within the lesions, the lesion area was selected manually and a colour range selection was applied to measure the collagen content (Adobe Photoshop CS3). The same colour settings were used for all the sections. For the quantification of macrophages, lesions from the aortic root were fixed in acetone and incubated with antibodies against macrophages (MOMA-2, a gift from G. Kraal) as described by Goossens et al. The number of apoptotic cells per lesion area was quantified using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay (TUNEL) technique. For each mouse, one section was used. Only those TUNEL-positive nuclei that displayed morphological features of apoptosis, including cell shrinkage, aggregation of chromatin into dense mass and nuclear formation, were counted.

Table 2. Effects of oxysterols and oxyphytosterols on body weight, serum cholesterol and inflammatory marker concentrations, cholesterol exposure, and collagen and apoptotic cell contents at the end of the study

<table>
<thead>
<tr>
<th></th>
<th>Control group (n 9)</th>
<th>Oxysterol group (n 12)</th>
<th>Oxyphytosterol group (n 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>23·9</td>
<td>22·5–24·2</td>
<td>23·4</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/l)</td>
<td>11·8</td>
<td>9·6–16·1</td>
<td>11·0</td>
</tr>
<tr>
<td>Cholesterol exposure (mmol/l × week)</td>
<td>450</td>
<td>341–485</td>
<td>405</td>
</tr>
<tr>
<td>Collagen content (× 10⁴ µm²)</td>
<td>117</td>
<td>22–194</td>
<td>149</td>
</tr>
<tr>
<td>Collagen content (%) of lesion</td>
<td>52</td>
<td>32–71</td>
<td>55</td>
</tr>
<tr>
<td>Macrophage:collagen ratio</td>
<td>0·39</td>
<td>0·08–0·74</td>
<td>0·41</td>
</tr>
<tr>
<td>Apoptotic cells (× 10⁶ µm²)</td>
<td>12·0</td>
<td>4·8–15·5</td>
<td>6·1</td>
</tr>
<tr>
<td>Serum inflammatory markers*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>167</td>
<td>90–230</td>
<td>98</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>39</td>
<td>28–54</td>
<td>20</td>
</tr>
</tbody>
</table>

* Before the analyses, equal volumes of sera from mice housed in the same cage were pooled.
Effects of oxy(phyto)sterols on atherosclerosis

Fig. 2. Effects of oxysterol- and oxyphytosterol-enriched diets on atherosclerotic lesion severity in female LDL receptor \(^{+/−}\) mice. Values are presented as medians. Lesions were categorised as mild (□), moderate (□) and severe (□). Differences in atherosclerotic lesion severity were tested using the \(\chi^2\) test. * Median values were significantly different from those of the control group \((P<0.017)\).

Statistical analyses

Differences between the three diet groups were tested for statistical significance using the non-parametric Kruskal–Wallis test. When a significant diet effect was found, pairwise comparisons were made using the non-parametric Mann–Whitney test. Values are presented as medians (with ranges). Differences in atherosclerotic lesion severity were tested using the \(\chi^2\) test. Differences between the groups were considered as statistically significant at \(P<0.017\). Statistical analyses were performed using SPSS 11.0 (version 11.0.3; SPSS, Inc.) for MacIntosh OS X (version 10.3.9; Apple Inc.).

Results

Effects of oxysterol and oxyphytosterol consumption in the LDL receptor-deficient \(^{+/−}\) mice

Body weight and cholesterol and lipoprotein profiles. At the end of the experiment, body weight \((P=0.867;\) Table 2) and serum cholesterol concentrations \((P=0.843)\) of the LDLR \(^{+/−}\) mice were similar in the three diet groups. In addition, no differences were found in the cholesterol AUC values \((P=0.163)\), indicating that the overall cholesterol exposure of the aorta was similar for all the diet groups. Lipoprotein profiles also did not differ (data not shown).

During the experiment, changes in serum total cholesterol concentrations did not differ between the three diet groups, except at weeks 7 and 17. Compared with the control diet, the oxysterol diet significantly lowered serum total cholesterol levels at week 7 by 13% \((P=0.006)\) and at week 17 by 12% \((P=0.001)\). Serum total cholesterol concentrations in the oxyphytosterol group followed the same pattern, but no significant differences were found when compared with the control group.

Atherosclerotic lesion size and severity. Although at the end of the experiment, lesion size seemed to be more pronounced in the oxysterol and oxyphytosterol groups than in the control group, differences between the three diet groups did not reach statistical significance \((P=0.420;\) Fig. 1). The proportion of severe atherosclerotic lesions, however, was significantly higher after oxysterol \((P=0.004)\) and oxyphytosterol \((P=0.011)\) diet consumption than after control diet consumption \((P=0.25)\). Proportions between the oxysterol and oxyphytosterol groups did not differ significantly \((P=0.125)\).

Oxyphytosterol amounts within the atherosclerotic lesions. Within the atherosclerotic lesions, the amounts of oxysterols in the mice fed the oxysterol diet were higher \((P<0.017)\) than those in both the oxysterol and control groups \((P=0.125)\). All oxyphytosterols examined \((7\beta\text{-OH}-\text{sitosterol} and 7\beta\text{-OH}-\text{campesterol}, 7\text{-keto-sitosterol} and 7\text{-keto-campesterol}, and 7α\text{-OH}-\text{sitosterol} and 7α\text{-OH}-\text{campesterol})\) exhibited essentially the same pattern.

Collagen content and macrophages and apoptotic cell number in the atherosclerotic lesions. Collagen content and relative collagen content (expressed as percentage of lesion size) were comparable \((P=0.675\) and 0.377, respectively) between the three diet groups (Table 2). No significant differences were found in the macrophage-collagen ratio and in the number of apoptotic cells in the atherosclerotic lesions between the diet groups \((P=0.301)\).

Inflammatory marker concentrations. At the end of the experimental period, serum concentrations of MCP-1 \((P=0.472)\) and TNF-α \((P=0.232)\) were similar between the diet groups \((P=0.25)\). Those of interferon-γ, IL-6, IL-10 and IL-12 were below the detection limits, and therefore they are not reported.

Discussion

Oxysterols are present in human plasma and are increased in type 2 diabetics\(^{57}\) and patients with stable coronary artery disease\(^{58}\). Likewise, oxyphytosterols are found in human plasma\(^{60}\), although at much lower concentrations. Less is

Fig. 3. Effects of oxysterol- and oxyphytosterol-enriched diets on oxysterol concentrations (ng) in the atherosclerotic lesions of female LDL receptor \(^{+/−}\) mice. * Median values were significantly different from those of the control and oxyphytosterol groups \((P<0.017)\). Amounts are reported in ng/μm-thick section. Control group; oxysterol group; oxyphytosterol group.
known, however, about the potential adverse health effects of oxyphytosterols. We found that consumption of oxyphytosterols—similar to that of oxysterols—increased the proportion of severe atherosclerotic lesions in female LDLR−/− mice. Interestingly, without claiming causality, the concentrations of oxyphytosterols in the lesions of the oxyphytosterol diet-fed group were higher than those in the lesions of the oxysterol diet- and control diet-fed groups.

So far, only a few animal studies have examined the atherosclerotic potential of oxysterols or oxyphytosterols. Staprans et al. (9) found significant increases in lesion size after 4 months in apoE−/− mice fed an oxysterol-enriched diet when compared with those fed a cholesterol-enriched diet. These effects were not strain dependent, as in the LDLR−/− mice, an oxysterol diet also significantly increased the lesion size after a 7-month intervention. On the other hand, Ando et al. (15) observed no significant differences in lesion size after 8 weeks of feeding a control, cholesterol or oxysterol diet to apoE−/− mice, which may have been related to the relatively short duration of the study. So far, only two studies have examined the potential atherogenicity of oxyphytosterols (10,12). Tomoyori et al. (10) found no significant differences in lesion size after 9 weeks of feeding a plant sterol or an oxysterol diet to apoE−/− mice. It should be noted, however, that the effects of oxysterols were compared with those of phytosterols and not with those of an atherogenic control diet, making it difficult to compare these results with our findings. In addition, the duration of that study was substantially shorter than that of the present study. Recently, Liang et al. (12) have shown in hamsters that diets enriched with the oxidation products of sitosterol or stigmastanol did not lower serum total cholesterol and LDL-cholesterol concentrations when compared with their non-oxidised counterparts. The oxidised products also did not affect atherosclerotic plaque size and cholesterol accumulation in the aorta when compared with those of the group that received the non-oxidised plant sterols.

There are several potential mechanisms by which oxy(phyto)sterols may promote the development of atherosclerosis. Among others, these components may modulate lipid homeostasis, trigger cell death or activate oxidation and inflammation. Concerning the effects on lipid homeostasis, this cannot explain the present results, since cholesterol exposure time did not differ between the three groups. In vitro experiments have shown that oxysterols trigger cell death in many different cell types, such as endothelial cells (16), macrophages (17), smooth muscle cells (18) and lymphocytes (19). Oxysterols have also been shown to exhibit cytotoxic effects in cultured macrophages (11). Besides apoptosis, oxysterols can also induce necrosis in various cell types, especially at higher concentrations (20). Furthermore, it has been suggested that the cytotoxic characteristics of an oxysterol mixture depend on the oxysterols in the mixture, their levels and their relative proportions (20). Since it is unknown how these in vitro findings translate to the in vivo situation, this may explain the lack of the effect on the apoptotic cell content in the present animal study. Furthermore, both in vitro and animal studies have shown that oxysterols activate oxidative processes and inflammation. In our cell experiments using HCAEC also, oxysterol consumption increased the production of MCP-1, a marker of endothelial activation. However, serum levels of TNF-α and MCP-1 were not significantly changed in the present study. Clearly, more research is needed to elucidate the potential atherogenic mechanism of oxy(phyto)sterols in vivo.

In the present study, the increased proportion of severe atherosclerotic lesions did not parallel the differences in serum total cholesterol levels, total cholesterol exposure or lipoprotein profiles. Consumption of oxysterolos, however, resulted in a significant reduction in serum total cholesterol concentrations at weeks 7 and 17. Although oxysterol consumption resulted in the same pattern, differences did not reach statistical significance. Staprans et al. (9) also observed no significant differences in serum cholesterol concentrations between the apoE−/− mice fed a control diet and those fed an oxysterol diet. However, in the LDLR−/− mice, serum total cholesterol concentrations were significantly reduced by the oxysterol diet when compared with the control diet. The study of Ando et al. (15) also suggested that dietary oxysterols and cholesterol had comparable effects on serum total cholesterol concentrations. Furthermore, Tomoyori et al. (10) found no significant differences in serum total cholesterol concentrations after 9 weeks of feeding a phytostanol or an oxysterol diet. Thus, it is not clear whether oxysterols and oxyphytosterols affect serum total cholesterol concentrations.

It is well known that sterols, in general, are present in their oxidised form in Western diets. Various food products, such as dairy products, eggs, meat and fish, may contain some oxy-sterols (21). Food processing, especially heat treatment, drying and storage, can also induce oxidation. Oxysterols have been identified in coffee beans (22), French fries (23), heated vegetable oils (24), infant milk formulas (25), parental nutrition (4) and potato chips (25). The dietary intake of oxysterols from phytosterol-enriched foods or supplements is estimated to be relatively low (<1-7 mg/d). Results from animal studies have suggested that an increased dietary intake of oxysterols elevates serum concentrations. Data regarding the relative absorption of oxysterols are limited. Tomoyori et al. (10) observed a greater total lymph recovery of both oxidised campesterol (15-9%) and β-sitosterol (9-1%) than that of campesterol (5-5%) and β-sitosterol (2-2%) in lymph duct-cannulated rats fed diets containing cholesterol, plant sterols or their oxidised derivatives. There are, however, no data from human intervention studies that have quantified the absorption of dietary oxysterols. Except from dietary sources, oxysterols can also be formed endogenously (26), and this may also be true for oxysterols. An important question is whether in vitro-formed oxysterols and oxysterosterols have atherogenic potential the same as that of their diet-derived counterparts that we used in the present study. To what extent oxysterols are formed, metabolised and excreted in the human body has not been studied.

In the present study, oxysterol and oxyphytosterol mixtures were used. Whether all isoforms have similar effects is not clear.
known, but this certainly needs to be addressed in future studies. In this respect, recent initiatives to specifically synthesise individual oxysterols from sitosterol and campesterol\(^\text{27}\) are a major step forward.

In summary, the present results indicate that not only dietary oxysterols but also dietary oxysterols increase the proportion of severe atherosclerotic lesions. This suggests that plant sterols when oxidised may increase atherosclerotic lesion severity instead of lowering the size and severity of lesions when fed in their non-oxidised form. Therefore, this novel finding might give an indication as to where to find the answer in the current hot debate about the potential atherogenicity of plant sterols. However, to what extent these results can be extrapolated to the human situation warrants further investigation.

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