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## Relationship between Protective Effect of Xanthone on Endothelial Cells and Endogenous Nitric Oxide Synthase Inhibitors

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**Abstract**—1,3,5,6-tetrahydroxyxanthone was synthesized. The relationship between protective effect of xanthone on endothelial cells and endogenous nitric oxide synthase inhibitors was investigated. Endothelial cells were treated with ox-LDL (100 µg/mL) for 48 h. Adhesion of monocytes to endothelial cells and release of lactate dehydrogenase (LDH) was determined. Levels of tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), nitric oxide (NO) and asymmetric dimethylarginine (ADMA, an endogenous inhibitor of nitric oxide synthase) in conditioned medium and activity of dimethylarginine dimethylaminohydrolase (DDAH) in endothelial cells were measured. Incubation of endothelial cells with ox-LDL (100 µg/mL) for 48 h markedly enhanced the adhesion of monocytes to endothelial cells, increased the release of LDH, the levels of TNF-α, MCP-1 and ADMA, and decreased the content of NO and the activity of DDAH. Xanthone (1,3,5,6-tetrahydroxyxanthone) (1, 3 or 10 µmol/L) significantly inhibited the increased adhesion of monocytes to endothelial cells and attenuated the increased levels of LDH, MCP-1 and ADMA induced by ox-LDL. Xanthone (1,3,5,6-tetrahydroxyxanthone) (3 or 10 µmol/L) significantly attenuated the increased level of TNF-α and decreased level of NO and activity of DDAH by ox-LDL. The present results suggest that xanthone (1,3,5,6-tetrahydroxyxanthone) preserves endothelial cells and inhibits the increased adhesion of monocytes to endothelial cells induced by ox-LDL, and that the protective effect of xanthone (1,3,5,6-tetrahydroxyxanthone) on endothelial cells is related to reduction of ADMA concentration via increase of DDAH activity.

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### Introduction

Xanthenes, a kind of polyphenolic compounds that commonly occur in plants, have widely been synthesized. A great deal of information has been demonstrated that xanthenes and xanthone derivatives have extensive pharmacological activities such as antioxidation, antihypertension and inhibition of platelet aggregation.<sup>1–3</sup> Recently, it was reported that some synthetic xanthenes show the strong property of anti-inflammation, which significantly inhibited the increased expression of intercellular adhesion molecule-1 (ICAM-1) induced by tumor necrosis factor-α (TNF-α), an inflammatory cytokine, in cultured human endothelial cells.<sup>4</sup> Our recent work has shown that some

xanthone compounds, extracted from *Swertia davidi* Franch (Gentianaceae), protect the myocardium against ischemia-reperfusion injury and attenuate the inhibition of vascular endothelium-dependent relaxation induced by lysophosphatidylcholine (LPC).<sup>5–7</sup>

There is growing evidence that endothelial dysfunction and later adhesion of circulating monocytes to endothelium play an important role in early events of atherogenesis.<sup>8</sup> It is known that endothelium-derived nitric oxide (NO), which regulates endothelial function, is synthesized from L-arginine by NO synthase (NOS) in endothelial cells. Recently, it has been found that endogenous inhibitors of NOS such as asymmetric dimethylarginine (ADMA), which inhibits NO synthesis, are significantly increased in animals and patients with atherosclerosis, and ADMA has been thought as a key factor contributing endothelial dysfunction.<sup>9–12</sup> It has been reported that oxidized low-density lipoprotein (ox-LDL)

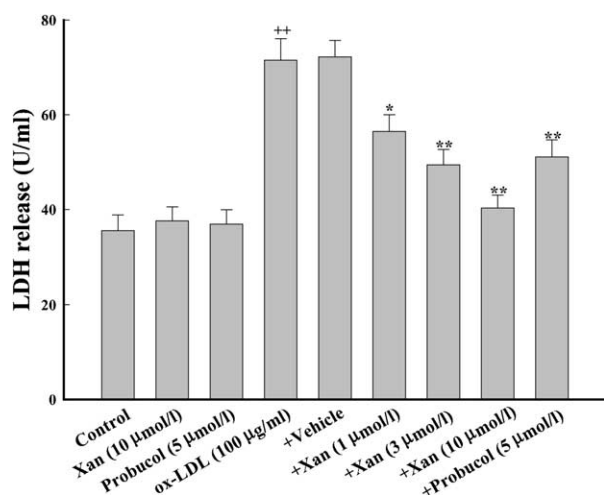
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or TNF- $\alpha$  increases the level of ADMA in cultured human endothelial cells.<sup>13</sup> Since some inflammatory cytokines induces production of ADMA and some xanthenes have anti-inflammation properties, we examined the relationship between the protection of xanthone (1,3,5,6-tetrahydroxyxanthone) of endothelial cells and reduction of ADMA in cultured endothelial cells treated with ox-LDL.

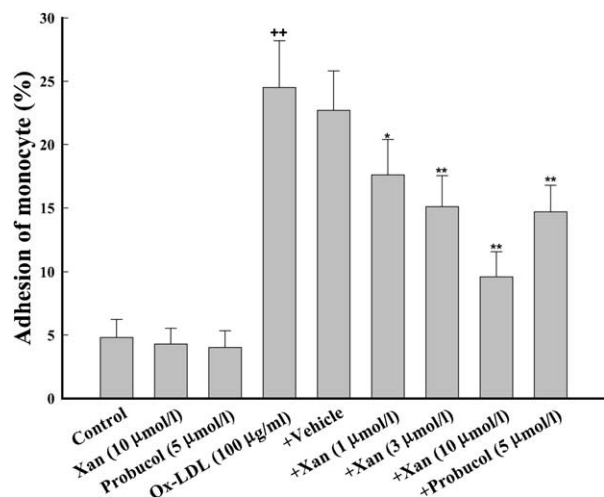
## Results

### Activity of lactate dehydrogenase (LDH)

Treatment with ox-LDL (100  $\mu\text{g/mL}$ ) for 48 h significantly increased the activity of LDH in the conditioned medium. Xanthone (1, 3 or 10  $\mu\text{mol/L}$ ) significantly inhibited the elevated activity of LDH by ox-LDL. The increased activity of LDH by ox-LDL was also inhibited by treatment with probucol (5  $\mu\text{mol/L}$ ).



**Figure 1.** Effect of xanthone on activity of LDH in the conditioned medium. Xan: 1,3,5,6-tetrahydroxyxanthone. Means  $\pm$  SEM,  $n=6$ . <sup>++</sup>  $p<0.01$  versus control, <sup>\*</sup>  $p<0.05$ , <sup>\*\*</sup>  $p<0.01$  versus ox-LDL.



**Figure 2.** Effect of xanthone on adhesion of monocytes to endothelial cells. Xan: 1,3,5,6-tetrahydroxyxanthone. Means  $\pm$  SEM,  $n=6$ . <sup>++</sup>  $p<0.01$  versus control, <sup>\*</sup>  $p<0.05$ , <sup>\*\*</sup>  $p<0.01$  versus ox-LDL.

L). However, Xanthone or probucol itself had no effect on the activity of LDH (Fig. 1).

### Adhesion of the monocytes to endothelial cells

Treatment with ox-LDL (100  $\mu\text{g/mL}$ ) for 48 h caused a significant increase in the adhesion of monocytes to endothelial cells. Xanthone (1, 3 or 10  $\mu\text{mol/L}$ ) significantly attenuated the increase in adhesion of monocytes by ox-LDL. The increased adhesion of monocytes by ox-LDL was also attenuated by treatment with probucol (5  $\mu\text{mol/L}$ ). Xanthone or probucol itself had no effect on the adhesion of monocytes to endothelial cells (Fig. 2).

### Concentrations of TNF- $\alpha$

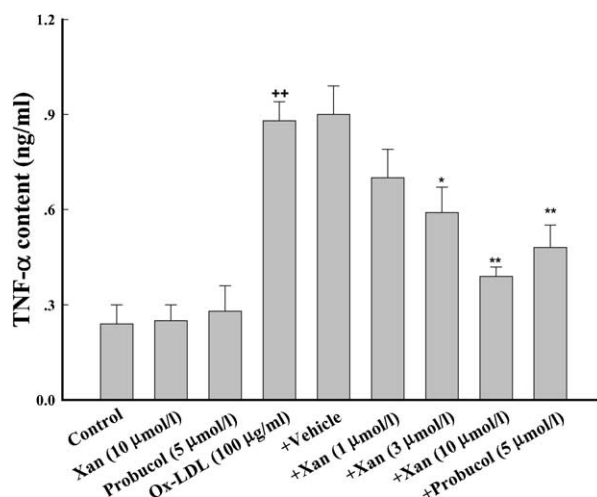
Treatment with ox-LDL (100  $\mu\text{g/mL}$ ) for 48 h caused a significant increase in concentrations of TNF- $\alpha$ . Xanthone (3 or 10  $\mu\text{mol/L}$ ) significantly inhibited the elevated concentration of TNF- $\alpha$  by ox-LDL (100  $\mu\text{g/mL}$ ). Probucol (5  $\mu\text{mol/L}$ ) also markedly inhibited the elevated concentration of TNF- $\alpha$  by ox-LDL. However, Xanthone or probucol itself had no effect on the concentration of TNF- $\alpha$  (Fig. 3).

### Concentrations of monocyte chemoattractant protein-1 (MCP-1)

Treatment with ox-LDL (100  $\mu\text{g/mL}$ ) for 48 h caused a significant decrease in concentrations of MCP-1 in the medium. Xanthone (1, 3 or 10  $\mu\text{mol/L}$ ) significantly attenuated the increased level of MCP-1 by ox-LDL. The increased level of MCP-1 by ox-LDL was also attenuated by treatment with probucol (5  $\mu\text{mol/L}$ ). Xanthone or probucol itself had no effect on the concentration of MCP-1 (Fig. 4).

### Concentrations of nitrite/nitrate

Treatment with ox-LDL (100  $\mu\text{g/mL}$ ) for 48 h caused a significant decrease in concentrations of nitrite/nitrate

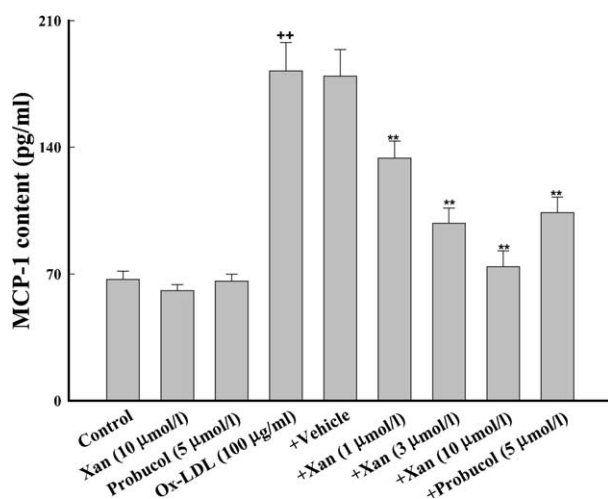


**Figure 3.** Effect of xanthone on levels of TNF- $\alpha$  in the conditioned medium. Xan: 1,3,5,6-tetrahydroxyxanthone. Means  $\pm$  SEM,  $n=6$ . <sup>++</sup>  $p<0.01$  versus control, <sup>\*</sup>  $p<0.05$ , <sup>\*\*</sup>  $p<0.01$  versus ox-LDL.

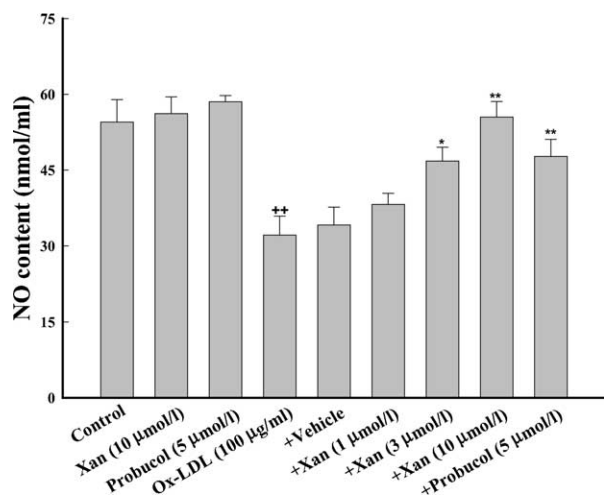
in the medium. Xanthone (3 or 10  $\mu\text{mol/L}$ ) significantly attenuated the decreased level of nitrite/nitrate by ox-LDL. The decreased level of nitrite/nitrate by ox-LDL was also attenuated by treatment with probucol (5  $\mu\text{mol/L}$ ). Xanthone or probucol itself had no effect on the concentration of nitrite/nitrate (Fig. 5).

### Concentrations of ADMA

The basal level of ADMA in the medium was  $0.45 \pm 0.07 \mu\text{mol/l}$ . Treatment with ox-LDL (100  $\mu\text{g/mL}$ ) for 48 h significantly increased concentrations of ADMA ( $1.35 \pm 0.13 \mu\text{mol/l}$ ,  $p < 0.01$  vs control). Xanthone (1, 3 or 10  $\mu\text{mol/L}$ ) significantly inhibited the elevated concentration of ADMA by ox-LDL. Probucol (5  $\mu\text{mol/L}$ ) also markedly inhibited the elevated concentration of ADMA by ox-LDL (100  $\mu\text{g/mL}$ ). However, Xanthone or probucol itself had no effect on the concentration of ADMA (Fig. 6).



**Figure 4.** Effect of xanthone on levels of MCP-1 in the conditioned medium. Xan: 1,3,5,6-tetrahydroxyxanthone. Means  $\pm$  SEM,  $n = 6$ .  $^{++}p < 0.01$  versus control,  $^{*}p < 0.05$ ,  $^{**}p < 0.01$  vs ox-LDL.



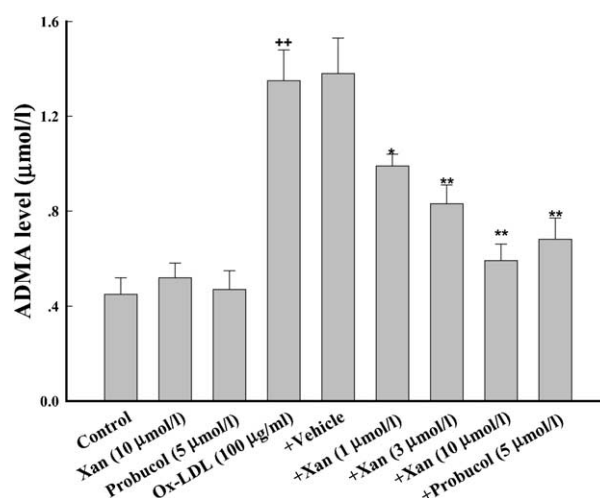
**Figure 5.** Effect of xanthone on concentrations of nitrite/nitrate in the conditioned medium. Xan: 1,3,5,6-tetrahydroxyxanthone. Means  $\pm$  SEM,  $n = 6$ .  $^{++}p < 0.01$  vs control,  $^{*}p < 0.05$ ,  $^{**}p < 0.01$  vs ox-LDL.

### Activity of dimethylarginine dimethylaminohydrolase (DDAH)

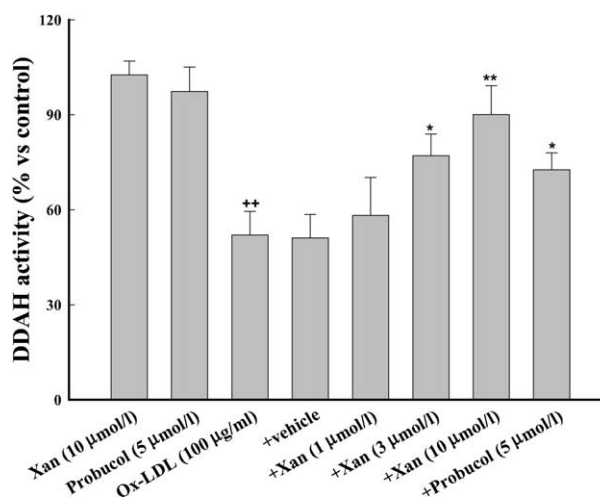
DDAH activity was significantly decreased in the endothelial cells treated with ox-LDL (100  $\mu\text{g/mL}$ ) for 48 h ( $52.0 \pm 7.5\%$  vs control,  $p < 0.01$ ). Xanthone (3 or 10  $\mu\text{mol/L}$ ) significantly attenuated inhibition of endothelial DDAH activity by ox-LDL. Probucol (5  $\mu\text{mol/L}$ ) also markedly attenuated inhibition of endothelial DDAH activity by ox-LDL. However, Xanthone or probucol itself had no effect on the activity of DDAH (Fig. 7).

### Discussion

NO, an important local regulator factor in cardiovascular tissues, is synthesized from L-arginine by NOS in endothelial cells. It possesses complex cardiovascular actions such as protecting endothelial cells, decreasing the endothelial adhesiveness and inhibiting the adhesion



**Figure 6.** Effect of xanthone on levels of ADMA in the conditioned medium. Xan: 1,3,5,6-tetrahydroxyxanthone. Means  $\pm$  SEM,  $n = 6$ .  $^{++}p < 0.01$  versus control,  $^{*}p < 0.05$ ,  $^{**}p < 0.01$  versus ox-LDL.



**Figure 7.** Effect of xanthone on activity of endothelial DDAH. Xan: 1,3,5,6-tetrahydroxyxanthone. Means  $\pm$  SEM,  $n = 6$ .  $^{++}p < 0.01$  versus control,  $^{*}p < 0.05$ ,  $^{**}p < 0.01$  versus ox-LDL.

of monocytes to endothelial cells.<sup>14–16</sup> Recently, it has been found that L-arginine analogues such as ADMA, which is present in blood of both humans and animals, can inhibit NOS in vivo and in vitro.<sup>17,18</sup> There is growing evidence that endothelial dysfunction in some cardiovascular diseases such as hypercholesterolemia, heart failure and hypertension is associated with elevation of ADMA, and endogenous inhibitors of NOS have been thought as a novel predictor of endothelial dysfunction.<sup>19</sup> ADMA is synthesized by protein arginine methyltransferases (PRMTs), which utilizes S-adenosylmethionine methyl group donor, and degraded by dimethylarginine dimethylaminohydrolase (DDAH), which hydrolyzes ADMA to L-citrulline and dimethylamine asymmetric dimethylarginine.<sup>13,20</sup> ADMA and DDAH are widely distributed in tissues including endothelial cells.<sup>21</sup> There is evidence that lipid-induced dysregulation of DDAH may be an important factor contributing the elevation of ADMA in hypercholesterolaemia and hyperhomocyst(e)inemia.<sup>9,22</sup> Others have been reported that the elevated content of ADMA is also related the decreased activity of DDAH in cultured endothelial cells treated with LDL or ox-LDL.<sup>13</sup>

There is growing evidence that ox-LDL plays a key role in the development of atherosclerotic lesions.<sup>23</sup> Ox-LDL exhibits numerous biological effects, including endothelial dysfunction, activation of endothelial adhesiveness, monocyte differentiation and adhesion.<sup>24,25</sup> As has been reported previously,<sup>13</sup> in the present study ox-LDL significantly increased the release of LDH which is positive related to the extent of cell injury, and decreased the activity of DDAH and increases the level of ADMA in the cultured endothelial cells. It has been shown that the level of ADMA is associated with an increase in adhesiveness of monocyte in patients with hypercholesterolemia.<sup>26</sup> In the present study, treatment with ox-LDL increased the expression of MCP-1 and the adhesion of monocytes to endothelial cells concomitantly with an increase level of ADMA. Others have found that ADMA enhances adhesion of monocytes in cultured endothelial cells,<sup>27</sup> in further support of the hypothesis that the increased adhesion of monocytes to endothelial cells induced by ox-LDL is related to elevation of ADMA level.

It has been suggested that inflammatory responses are involved in the development of atherosclerosis.<sup>8</sup> It was reported that inflammatory cytokines such as TNF- $\alpha$  were significantly increased in animals and patients with hypercholesterolaemia,<sup>28,29</sup> and that TNF- $\alpha$  upregulated the expression of adhesion molecules and increased the adhesion of monocyte to endothelium,<sup>30</sup> suggesting to play an initial factor in a series of inflammatory response in the atherosclerotic lesions. TNF- $\alpha$  is present in various cells including macrophages and endothelial cells. Recently, it has been shown that treatment with TNF- $\alpha$  significantly elevated the level of ADMA concomitantly with a decrease of DDAH activity in cultured human endothelial cells.<sup>13</sup> The present results confirmed previous observations that treatment with ox-LDL significantly increased the levels of TNF- $\alpha$  and ADMA and decreased the activity of

DDAH in cultured endothelial cells.<sup>13,31</sup> These results suggest the increased level of ADMA induced by ox-LDL may be related to decrease of DDAH activity by elevation of TNF- $\alpha$  production.

Xanthones, a kind of polyphenolic compound, have extensive pharmacological actions including antioxidation, anti-inflammation and cardioprotection.<sup>1,5,6</sup> Recently, it was reported that some synthetic xanthones significantly inhibited the increased expression of ICAM-1 induced by TNF- $\alpha$  in cultured human endothelial cells.<sup>4</sup> Our recent work has also shown that daviditin A, a xanthone compound extracted from *S. davidi* Franch (Gentianaceae), protects the endothelium against damage induced by LPC concomitantly with a decrease of ADMA level.<sup>7</sup> The results of the present study revealed that xanthone significantly inhibited the release of LDH, decreased the level of MCP-1 and attenuated the increased adhesion of monocytes to endothelial cells in cultured endothelial cells induced by ox-LDL. As mentioned above, there are interactions of ADMA with cytokines in the development of atherosclerosis. In the present study xanthone also significantly attenuated the elevations of TNF- $\alpha$  and ADMA level and decrease of DDAH activity in cultured endothelial cells treated with ox-LDL, suggesting that the protective effect of xanthone on endothelial cells may be related to reduction of ADMA level.

## Conclusion

The present results suggest that xanthone preserves the damage of endothelial cells and inhibits the addition of monocytes to endothelial cells induced by ox-LDL, and that the protective effect of xanthone on the endothelial cells is related to decrease of ADMA concentration via increasing DDAH activity.

## Experimental

### Materials

Probuco, asymmetric dimethylarginine (ADMA) was obtained from Sigma. DMEM was obtained from Gibco. Lactate dehydrogenase (LDH) and nitric oxide (NO) assay kits were obtained from Ju-Li Biological Medical Engineering Institute (Nanjing, P. R. China). TNF- $\alpha$  and monocyte chemotactic protein-1 (MCP-1) assay kits were obtained from Sen-xiong science and technology industrial Co. Ltd (Shanghai, China).

### Synthesis of xanthone

1,3,5,6-Tetrahydroxyxanthone was synthesized by the Friedel-Crafts acylation of 1,3,5-trimethoxybenzene with 2,3,4-trimethoxybenzoyl chloride to yield a mixture of 2-hydroxy-2',3,4,4',6'-pentamethoxybenzophenone and 6'-hydroxy-2,2',3,4,4'-pentamethoxybenzophenone which cyclised to give 1,3,5,6-tetra-hydroxyxanthone, followed by demethylation (Fig. 8).<sup>32</sup> <sup>1</sup>NMR:  $\delta$  6.20 (d,

$J=2.5$  Hz, 1H, H-2), 6.46 (d,  $J=2.5$  Hz, 1H, H-4), 6.91 (d,  $J=8.5$  Hz, 1H, H-7), 7.57 (d,  $J=8.5$  Hz, 1H, H-8); literature data:  $^1\text{NMR}$ :  $\delta$  6.28 (d,  $J=2.5$  Hz, 1H, H-2), 6.54 (d,  $J=2.5$  Hz, 1H, H-4), 6.99 (d,  $J=8.5$  Hz, 1H, H-7), 7.96 (d,  $J=8.5$  Hz, 1H, H-8) Xanthone was initially dissolved in ethanol and further diluted in conditioned medium to proper final concentration.

### Preparation and oxidation of LDL

Native LDL (nLDL) was isolated from freshly prepared normal human plasma through sequential density gradient ultracentrifugation in sodium bromide density solutions in the density range 1.019–1.063 g/mL as previously described.<sup>33</sup> Then LDL was dialyzed against 0.01 M PBS (0.01 M phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) containing 0.01% EDTA. Protein concentration was measured by previously described methods.<sup>34</sup>

Oxidation of nLDL was induced by adding 10  $\mu\text{M}$   $\text{CuSO}_4$  for 24 h at 37 °C. The amount of thiobarbituric acid reactive substance (TBARS), reflecting the extent of LDL oxidation, was measured by previously described methods.<sup>35</sup> The amount of TBARS was  $6.80 \pm 0.92$  and  $24.19 \pm 4.79$  nmol/mg protein for nLDL and oxLDL, respectively ( $n=3$ ,  $p<0.01$ ).

### Cell culture and treatment

ECV304 cells, a human umbilical vein endothelial cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100  $\mu\text{g/mL}$  streptomycin. When the cells had reached subconfluence, cells were passaged into 24-well culture dishes and the culture medium was replaced the serum-free medium. Cell numbers were  $6.1 \pm 0.3 \times 10^6$  cells/well ( $n=6$ ). Cells were counted by trypan blue exclusion and showed >95% viability. Cells were treated with ox-LDL. For xanthone and probucol which was as a positive control, endothelial cells were exposed to xanthone (1, 3 or 10  $\mu\text{mol/L}$ ) or probucol (5  $\mu\text{mol/L}$ ) for 1 h, and then exposed to ox-LDL for 48 h in the presence of xanthone or probucol.

### Determination of LDH activity

The activity of LDH in the conditioned medium, as an indicator of cell cytotoxicity, was measured spectrophotometrically using a commercially available assay kit.

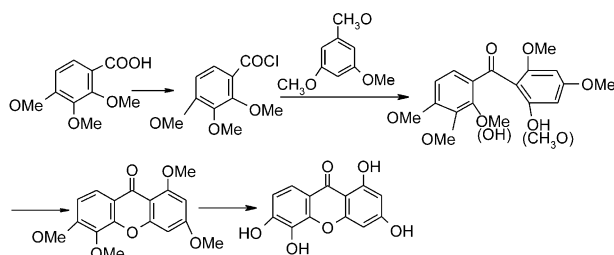


Figure 8. The synthesis of 1,3,5,6-tetrahydroxyxanthone.

### Monocyte-endothelial cell adhesion assay

Monocytes were obtained from fresh peripheral blood of healthy volunteers. Monocytes were isolated by the Ficoll–Paque density centrifugation method as previously described.<sup>36</sup> The collected monocytes were washed twice in Hanks' balanced salt solution (HBSS) and resuspended in DMEM containing 10% (v/v) FBS, 100 U/mL penicillin and 100  $\mu\text{g/mL}$  streptomycin. This preparation method routinely harvested >95% of monocytes and maintained >95% viability as assessed by trypan blue exclusion.

The adhesion of the monocytes to endothelial cells was evaluated by protein content as previously described methods.<sup>37</sup> The monolayers of endothelial cells treated were washed three times with HBSS before addition of monocytes. Monocytes cells were diluted to a final concentration of  $10^6$  cells/mL and were added to each well (1 mL) of endothelial cells. An additional 1 mL of the monocyte suspension was also obtained for protein determination. The monocytes were allowed to incubate with the endothelial cells monolayer at room temperature for 30 min on a rocking platform and nonadherent monocytes were carefully removed by two washes with HBSS. ECV304 cells and adherent monocytes were then lysed with 0.5 M NaOH for protein analysis as previously described.<sup>34</sup> The adhesion of monocytes was estimated by comparing the amount of protein in wells containing endothelial cells and monocytes minus the amount of protein in wells containing ECV304 cells alone, divided by the amount of protein in 1 mL of monocytes suspension.

### Determination of TNF- $\alpha$ concentration

TNF- $\alpha$  level in culture medium was assayed by enzyme-linked immunosorbent assay (ELISA). The values were measured at 405 nm by a microplate reader (Biotek). The standard curve for TNF- $\alpha$  measured by this ELISA was linear from 78 pg/mL to 10 ng/mL; the detection limit was 100 pg/mL.

### Determination of MCP-1 concentration

MCP-1 in culture medium was measured by ELISA using with recombinant human MCP-1 as a standard. The values were measured at 492 nm by a microplate reader (Biotek). The standard curve for MCP-1 measured by this ELISA was linear from 8 to 500 pg/mL; the detection limit was 10 pg/mL.

### Determination of nitrite/nitrate concentration

The level of nitric oxide in the conditioned medium was determined indirectly as the content of nitrite and nitrate. Level of nitrite/nitrate in the cells conditioned medium was measured as previously described.<sup>38</sup>

### Determination of ADMA concentration

The protein in the conditioned medium was removed using 5-sulfosalicylic acid (5-SSA). The content of

ADMA was measured high-performance liquid chromatography (HPLC) as described previously with some modification.<sup>39</sup> HPLC was carried out using a Shimadzu LC-6A liquid chromatograph with Shimadzu SCL-6A system controller and Shimadzu SIC-6A auto-sampler. *O*-Phthaldialdehyde adducts of methylated amino acids and internal standard ADMA produced by precolumn mixing were monitored using a model RF 530 fluorescence detector set at  $\lambda^{\text{ex}} = 338$  and  $\lambda^{\text{em}} = 425$  nm on a Resolve C<sub>18</sub> column. Samples were eluted from the column using a linear gradient containing mobile phase A composed of 0.05 mol/l (pH 6.8) sodium acetate–methanol–tetrahydrofuran (81:18:1 v:v:v) and mobile phase B composed of 0.05  $\mu$ mol sodium acetate–methanol–tetrahydrofuran (22:77:1 v:v:v) at a flow-rate of 1 mL/min.

### DDAH activity assay

The activity of DDAH in endothelial cells was estimated by directly measuring the amount of ADMA metabolized by the enzyme.<sup>40</sup> In an ice bath, cell lysates were divided into two groups, and ADMA was added (final concentration 500  $\mu$ mol/L). To inactivate DDAH, 30% sulfurosallyclic acid was immediately added to 1 experimental group. This group provided a baseline of 0% DDAH activity. The other lysate was incubated at 37 °C for 2 h before the addition of 30% sulfurosallyclic acid. The ADMA level in each group was measured by HPLC as described above. The difference in ADMA concentration between two groups reflected the DDAH activity. For every experiment, DDAH activity of ECV304 cells exposed to normal conditioned medium is defined as 100%, and DDAH activity in other conditions were expressed as percentages of the ADMA metabolized compared with the control.

### Statistic analysis

Results are expressed as means  $\pm$  SEM. Data were analysed by ANOVA followed by the unpaired Student's *t*-test for multiple comparisons. The significance level was chosen as  $p < 0.05$ .

### Acknowledgements

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