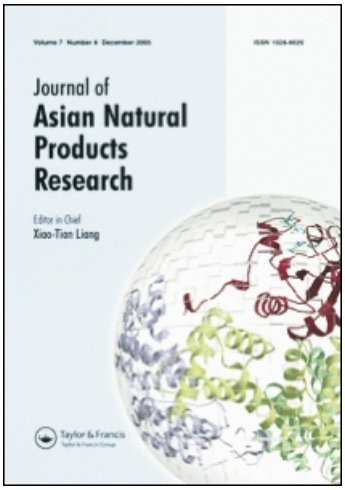

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3,4,5,6-Tetrahydroxyxanthone preserves intercellular communication by reduction of the endogenous nitric oxide synthase inhibitor level

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3,4,5,6-Tetrahydroxyxanthone preserves intercellular communication by reduction of the endogenous nitric oxide synthase inhibitor level

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To observe the direct effects of 3,4,5,6-tetrahydroxyxanthone on connexin43 (Cx43) expression in cultured endothelial cells, cells were treated with lysophosphatidylcholine (LPC, 10 mg/l) for 24 h in the presence or absence of different concentrations of 3,4,5,6-tetrahydroxyxanthone (1, 3, or 10 μmol l^{-1}). The reactive oxygen species (ROS) production, cell viability, asymmetric dimethylarginine (ADMA) levels, and Cx43 expression were detected. 3,4,5,6-Tetrahydroxyxanthone significantly inhibited the increase in ROS production and ADMA level, increased cell viability and up-regulated Cx43 mRNA and protein expression induced by LPC. 3,4,5,6-Tetrahydroxyxanthone has protective effect in LPC-induced atherosclerotic lesions, which is at least partly related to the reduction of ADMA level and downregulation of Cx43 expression.

Keywords: 3,4,5,6-tetrahydroxyxanthone; asymmetric dimethylarginine; connexin 43

1. Introduction

Gap junction intercellular communications (GJIC) are cytoplasmic exchange of ions and small metabolites between neighboring cells via gap junctions formed by the connexin (Cx) protein family [1]. GJIC has been demonstrated to be involved in the regulation of a variety of endothelial activities, such as homeostasis, morphogenesis, cell differentiation, and senescence [2]. Three Cx subtypes are expressed in the endothelium of blood vessels: Cx37, Cx40, and Cx43 [3,4]. Several *in vitro* studies showed that Cx-mediated endothelial GJIC was damaged by various pro-atherosclerotic factors such as high glucose, low-density lipoprotein (LDL), or nicotine. In addition, genetically modified Cx expression altered the course of atherosclerosis (AS) in mice [5,6]. These studies imply that endothelial gap junctions are involved in the atherogenesis, and atherogenic factors are potential regulators of endothelial gap junctions.

LDL is oxidatively modified into oxidized-LDL (ox-LDL), which is suggested to be a key event in early atherogenesis. It was reported that LDL-induced inhibition of GJIC contributes greatly to its atherogenic effects [5]. LDL inhibited GJIC in human vascular endothelial cells and smooth muscle cells, which could be diminished by high density lipoprotein or ascorbic acid in a dose-dependent manner [5,7]. This disruption of intercellular communication may also contribute to the pathogenesis of atherosclerotic lesions, like plaque [8]. In *in vivo* study, endothelial gap junctions and Cxs have been shown to

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be down regulated during long-term hyperlipidemia, which can be recovered by short-term treatment with simvastatin [9]. Moreover, down-regulated myocardial Cx43 and suppressed contractility in rabbits are subjected to a cholesterol-enriched diet [10]. Our recent study has displayed that lysophosphatidylcholine (LPC), a major component of ox-LDL, inhibited GJIC by down regulating Cx43 expression in cultured endothelial cells, which further confirmed the above studies [11].

Asymmetric dimethylarginine (ADMA), a major endogenous inhibitor of NO synthase (NOS), could reduce nitric oxide (NO) production and damage endothelial function [12]. The burgeoning clinical data showed that the elevated ADMA plasma levels are closely related to endothelial dysfunction in some cardiovascular abnormalities like hypercholesterolemia and can independently predict cardiovascular mortality and future occurrence in many patient populations such as coronary artery disease, suggesting that ADMA may be a novel and independent cardiovascular risk factor [13]. Previous observations and our works have shown that ox-LDL and its major component LPC result in endothelial dysfunction concomitantly with an elevation of ADMA level [14], both *in vivo* and *in vitro*.

Xanthones, a main component extracted from *Swertia davidi Franch*, have extensive pharmacological actions [15]. It has been reported that some xanthones have antioxidant, anti-inflammatory, and cardioprotection effects, which is closely related to its effects on the reduction of ADMA level [16,17]. 3,4,5,6-Tetrahydroxyxanthone **(1)** is a new xanthone monomer synthesized by our group (Figure 1). Our recent research has suggested that **1** could protect cardiac damage induced by ischemia–reperfusion and protect endothelial cells from apoptosis induced by high glucose [18,19]. In the present study, the direct effects of **1** on Cx43 expression in cultured endothelial cells were observed.

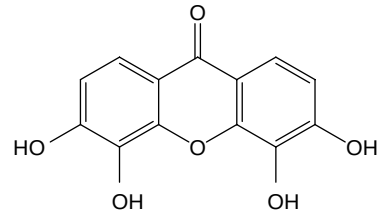

Figure 1. Chemical structure of compound **1**.

2. Results

2.1 The effects of 1 on cell viability and intracellular ROS production

Exposure of endothelial cells to LPC at the dose of 10 mg/l for 24 h significantly decreased cell viability and increased the intracellular production of reactive oxygen species (ROS). Pretreatment with **1** (3 or 10 $\mu mol\,l^{-1}$) attenuated such effects induced by LPC (Figures 2 and 3).

2.2 The effects of 1 on LPC-elevated concentration of ADMA

Figure 4 showed that the treatment with LPC (10 mg/l) for 24 h significantly elevated the level of ADMA in the medium. **1** (3 or 10 $\mu mol\,l^{-1}$) significantly inhibited the elevated concentration of ADMA induced by LPC. **1** or the vehicle itself had no effect on the level of ADMA.

2.3 The effects of 1 on Cx43 mRNA and protein expression

Figure 5(A and B) showed that the treatment with LPC (10 mg/l) for 24 h significantly decreased both the mRNA and protein levels of Cx43 in endothelial cells. **1** (3 or 10 $\mu mol\,l^{-1}$) significantly inhibited the reduction of Cx43 expression induced by LPC. **1** or vehicle itself had no effect on the mRNA and protein levels of Cx43 in endothelial cells.

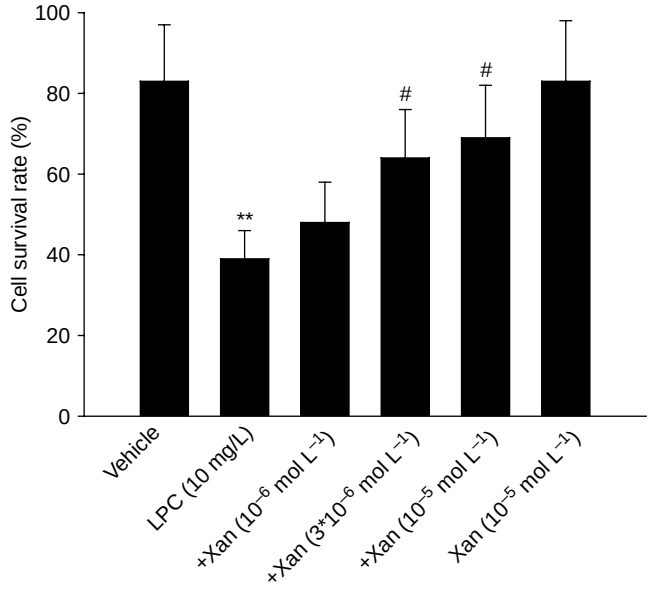

Figure 2. Effect of **1** on LPC-induced reduction in cell viability. Endothelial cells were treated with LPC (10 mg/l) for 24 h. $^{**}P < 0.01$, compared with vehicle; $^{\#}P < 0.05$, compared with LPC-treated group ($\bar{X} \pm$ SEM, $n = 3$). LPC: LPC (10 mg/l); + Xan (1, 3, or 10 μmol l^{-1}): LPC + **1**; Vehicle: Alcohol (1%); Xan: **1** (10 μmol l^{-1}).

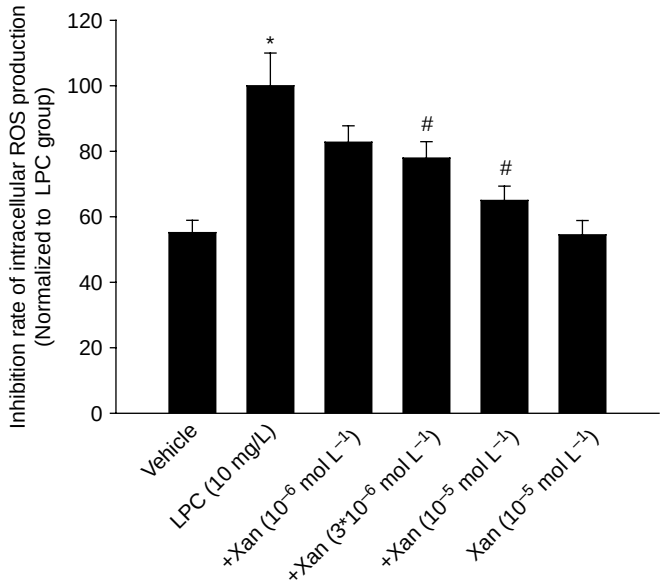

Figure 3. Effects of **1** on the intracellular ROS production cultured with LPC. Endothelial cells were treated with LPC (10 mg/l) for 24 h. $^{*}P < 0.05$, compared with vehicle; $^{\#}P < 0.05$, compared with LPC-treated group ($\bar{X} \pm$ SEM, $n = 3$). LPC: LPC (10 mg/l); + Xan (1, 3, or 10 μmol l^{-1}): LPC + **1**; Vehicle: Alcohol (1%); Xan: **1** (10 μmol l^{-1}).

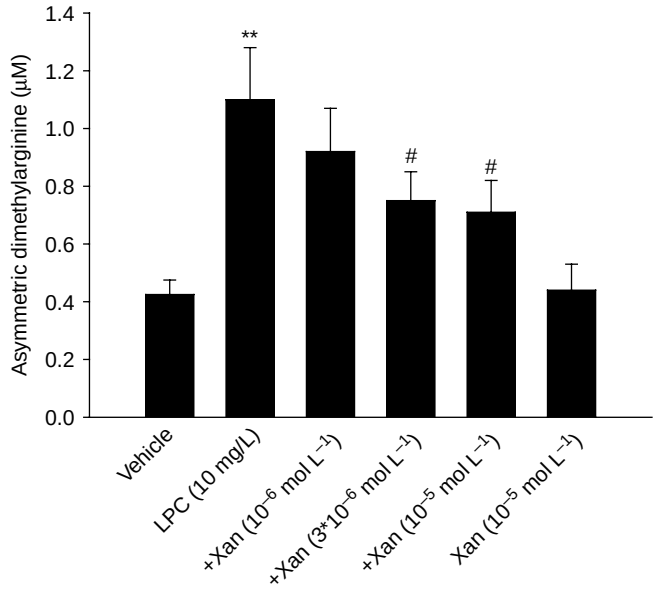

Figure 4. Effect of **1** on LPC-induced elevated concentration of ADMA in the conditioned medium. Endothelial cells were treated with LPC (10 mg/l) for 24 h. $^{**}P < 0.01$, compared with vehicle; $^{\#}P < 0.05$, compared with LPC-treated group ($\bar{X} \pm$ SEM, $n = 3$). LPC: LPC (10 mg/l); + Xan (1, 3, or 10 μmol l^{-1}): LPC + **1** Vehicle: Alcohol (1%); Xan: **1** (10 μmol l^{-1}).

3. Discussion

The main findings in this study are that **1** inhibited LPC-induced ROS production and decreased cell viability. **1** also upregulated Cx43 expression induced by LPC, which is closely related to the reduction of ADMA level. Our results demonstrate a novel mechanism that **1** inhibits the progression of AS.

As has been reported previously, ox-LDL or LPC plays a pivotal role in early atherogenesis and endothelial dysfunction. The present study confirmed the previous data that LPC treatment could decrease cell viability and increase the ROS production, which is closely related to the elevation of ADMA level [20].

Xanthone, a polyphenolic compound that commonly occurs in plants, has extensive pharmacological actions. Previous investigations have shown that some xanthones have a potent antioxidant activity and inhibit lipid peroxidation and block the oxidation of LDL *in vitro* [17]. Our previous work and others have found that some xanthones protect cardiac tissues and endothelial cells against oxidative damage, induced by various factors in animals, which is related to the reduction of ADMA generation [21]. Compound **1** is a newly synthesized xanthone monomer. Previous studies in our laboratory have shown that **1** can protect the isolated rat heart from ischemia–reperfusion damage, which is closely related to its antioxidative stress [18,19]. The results of the present study revealed that **1** significantly improved endothelial function by decreasing the ROS production concomitantly with a reduction of concentrations of ADMA.

Some recent studies have showed that Cx43 was of great importance in keeping the homeostasis of vascular cells and reducing the expression of Cx43 in the presence of the risk factors for AS such as hypercholesterolemia [6,22,23].

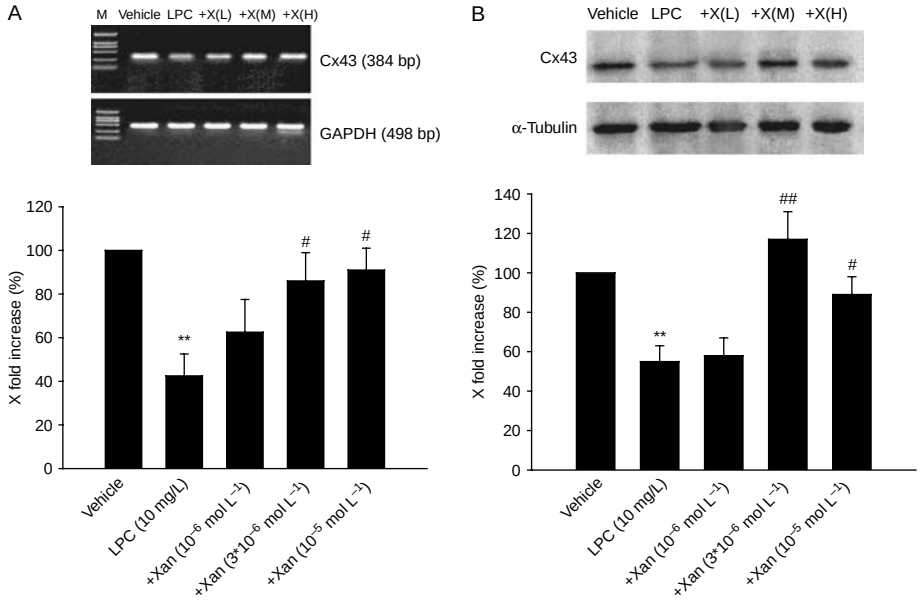

Figure 5. (A) Effect of **1** on Cx43 mRNA expression level in endothelial cells. (The upper panel shows the bands of PCR product of Cx43 and GAPDH; the below is change folds of Cx43 mRNA levels with statistical analysis.) Means ± SEM were plotted as percentages relative to the vehicle which was set to **1** (100%). $^{**}P < 0.01$, compared with vehicle; $^{\#}P < 0.05$, compared with LPC-treated group ($\bar{X} \pm$ SEM, $n = 3$). LPC: LPC (10 mg/l); + Xan (1, 3, or 10 μmol l^{-1}): LPC + **1**;Vehicle: LPC + Alcohol (1%). (B) Effect of **1** on Cx43 protein expression level in endothelial cells. (The upper panel shows western blot bands of Cx43 and α-tubulin; the below is the change folds of Cx43 protein levels with statistical analysis.) Means ± SEM were plotted as percentages relative to the vehicle which was set to **1** (100%). $^{**}P < 0.01$, compared with vehicle; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with LPC-treated group ($\bar{X} \pm$ SEM, $n = 3$). LPC: LPC (10 mg/l); + Xan (1, 3, or 10 μmol l^{-1}): LPC + **1**; Vehicle: Alcohol (1%).

Cx43 is the main subtype in cultured endothelial cells. The down regulation of endothelial Cx43 expression has been thought to contribute to the loss of functional or structural integrity of endothelium [24]. There is direct evidence showing that Cx43α1 gap junctions play an essential role in coronary vasculogenesis and angiogenesis [22]. These studies show that the loss of one Cx43α1 allele leads to a high incidence of coronary patterning defects. In the high-glucose condition, increased degradation of Cx43 and the reduction of GJIC may be of physiological importance by contributing to endothelial cell dysfunction, associated with the breakdown of the blood-retinal barrier in diabetic retinopathy [6]. In hypercholesterolemia rats, both fluorescent intensity and frequency of gap junction appear diminished [25]. In the present study, consistent with our previous study, we found that the LPC treatment down regulated Cx43 expression, suggesting that the atherogenic effects of LPC might be related to the inhibition of GJIC via down-regulated endothelial Cx43 expression. And **1** could inhibit the effects of LPC and up-regulated Cx43 expression, which may be related to its antioxidative effects.

NO, which is synthesized from L-arginine by NOS in endothelial cells, plays an important role in maintaining vascular structure and function, described as an 'endogenous anti-atherosclerotic

molecule.' Using exogenous NOS inhibitor N (G)-nitro-L-arginine methyl ester (L-NAME), the inhibition of NO has been shown to down regulate Cx43 expression. ADMA, a major endogenous inhibitor of NOS, could reduce NO production and damage endothelial function [12]. In L-NAME-induced hypertensive rats, endothelial Cx43 is reduced by 35%. Furthermore, the endothelial Cx43 expression is upregulated by carvedilol, independent of its antioxidant activity. In the present study, we confirmed that LPC treatment induced elevation of ADMA level, which was inhibited by **1**. Thus, we postulate that **1** exerts its effects via down regulation of ADMA.

In conclusion, LPC damages endothelial GJIC function via down regulating Cx43 expression, which is inhibited by **1**. Our results demonstrate a novel mechanism of xanthone for its therapeutic role in AS.

4. Materials and methods

4.1 Antibodies and reagents

Compound **1** (purity: 99.0%) was obtained from the School of Pharmaceutical Sciences, Central South University, China. The rabbit polyclonal antibody, LPC, ADMA standard, and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Carlsbad, CA, USA). Western blot kit was purchased from KPL (Gaithersburg, MD, USA). ROS detection kit was purchased from Beyotime Company (Jiangsu, China).

4.2 Cell culture

Human umbilical vein endothelial cells-12 were cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were passaged into 6-well culture dishes or 24-well culture dishes and were serum starved for 24 h in DMEM containing 1% FBS, when the cells had reached subconfluence. Then, cells were treated with LPC (10 mg/l) for 24 h in the presence or absence of different concentrations of **1** (1, 3, or 10 μmol l^{-1}).

4.3 Cell viability assay

Cell viability was determined by the tetrazolium salt MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The protocols were conducted as previously [20].

4.4 Determination of intracellular ROS generation

The intracellular ROS production was determined by measuring the oxidative conversion of cell-permeable 2′,7′-dichlorofluorescein diacetate to fluorescent dichlorofluorescein in DTX880 Multimode detector (Beckman Coulter, Brea, CA, USA).

4.5 Determination of ADMA concentration

ADMA level was measured by high performance liquid chromatography as previously [20].

4.6 Western blot

Western blot was carried out as routine. Cells were incubated with the primary antibodies [rabbit anti-Cx43 polyclonal antibody (1:1000)] overnight, followed by incubation for 1 h with corresponding peroxidase-conjugated secondary antibody IgG (1:2000).

4.7 RT-PCR

RNA was simultaneously determined by semi-quantitative RT-PCR using the forward primer 5′-TTAAGGATCGTGTGAAGGGAAAGAG-3′ and the reverse primer 5′-CTAGATCTCTAGGTCATCAGGCCG-3′ for amplification of Cx43, and

the forward primer 5′-AAGCCCATCAC-CATCTTCCA-3′ and the reverse primer 5′-CCTGCCTCACCACCTTCTTG-3′ for amplification of GAPDH. Electrophoresis on a 1.5% agarose gel stained with ethidium bromide was done for the products. Densitometry was performed at non-saturating exposures, and the Cx43/GAPDH ratios were determined.

4.8 Statistical analysis

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

Acknowledgement

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