

Ellagic acid suppresses oxidised low-density lipoprotein-induced aortic smooth muscle cell proliferation: studies on the activation of extracellular signal-regulated kinase 1/2 and proliferating cell nuclear antigen expression

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Proliferation of intimal vascular smooth muscle cells is an important component in the development of atherosclerosis. Ellagic acid is a phenolic compound present in fruits (raspberries, blueberries, strawberries) and walnuts. The present study investigated the effect of ellagic acid on the oxidised LDL (ox-LDL)-induced proliferation of rat aortic smooth muscle cells (RASMC). The study found that ellagic acid significantly inhibited ox-LDL-induced proliferation of RASMC and phosphorylation of extracellular signal-regulated kinase (ERK) 1/2. Furthermore, ellagic acid also blocked the ox-LDL-induced (inducible) cell-cycle progression and down regulation of the expression of proliferating cell nuclear antigen (PCNA) in RASMC. Therefore, ellagic acid reduced the amount of ox-LDL-induced proliferation of RASMC via inactivation of the ERK pathway and suppression of PCNA expression. These results may significantly advance the understanding of the role that antioxidants play in the prevention of atherosclerosis.

Ellagic acid: Oxidised low-density lipoprotein: Rat aortic smooth muscle cell proliferation: Extracellular signal-regulated kinase 1/2: Proliferating cell nuclear antigen

Elevated plasma LDL has been implicated in the pathogenesis of atherosclerosis⁽¹⁾. Oxidised LDL (ox-LDL) stimulates the proliferation of aortic smooth muscle cells and the up regulation of extracellular signal-regulated kinases (ERK)⁽²⁾. Therefore, the ox-LDL-induced proliferation of vascular smooth muscle cells (VSMC) in the intima of the arterial wall is believed to be a critical event in the development of atherosclerotic plaque.

Several studies have indicated that naturally occurring polyphenols can inhibit migration and proliferation of vascular cells^(3,4). Polyphenols found in green tea have been shown to inhibit LDL-induced human VSMC proliferation and to arrest endothelial cells in the G1 phase of the cell cycle⁽⁵⁾. Red wine polyphenols have also been found to decrease the proliferation and migration of VSMC by down regulating cyclin A expression and inactivating of the ERK pathway^(3,6).

The protein proliferating cell nuclear antigen (PCNA) has been implicated in a number of cellular processes, including DNA replication, DNA repair, and cell-cycle regulation^(7–9). It has also been reported that PCNA synthesis is strictly regulated during the cell cycle, and that there is a clear increase in PCNA protein during the G1/S transition. It is evident that PCNA represents a key protein necessary for the transition of cells from quiescence to the S phase⁽¹⁰⁾.

Ellagic acid is a phenolic compound present in berries and nuts^(11–13). It has been found to have antioxidative properties

and to inhibit LDL oxidation⁽¹⁴⁾. Our recent study also indicated that ellagic acid lowers oxidative stress and reduces atherosclerosis in hyperlipidaemic rabbits⁽¹⁵⁾.

We investigated the effect of ellagic acid on ox-LDL-induced proliferation of rat aortic smooth muscle cells (RASMC). We hypothesised that this effect is due to the activation of ERK 1/2 and the expression of cell-cycle regulatory protein PCNA.

Materials and methods

Isolation and preparation of oxidised low-density lipoprotein

LDL was isolated from normal human plasma by ultracentrifugation in the presence of EDTA (1 mmol/l) in a KBr gradient at 200 000 g for 14 h at 4°C. The LDL fraction was decanted and dialysed against three changes of 1500 ml of NaCl (150 mmol/l) and EDTA (0.25 mmol/l) at pH 7.4. LDL was sterilised using 0.22 µm Millipore filters and stored in the dark at 4°C. Concentrations of LDL are given as µg LDL protein. LDL (1–2 mg/ml) was mildly oxidised at 37°C in the presence of CuSO₄ at a final concentration of 5 µmol/l for 24 h. The oxidation was terminated by the addition of EDTA (1.0 mmol/l). Ox-LDL fractions were dialysed against PBS with EDTA (0.1 mmol/l). The purity

Abbreviations: ERK, extracellular signal-regulated kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium; ox-LDL, oxidised LDL; PCNA, proliferating cell nuclear antigen; RASMC, rat aortic smooth muscle cell; VSMC, vascular smooth muscle cell.

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and the extent of the oxidation of LDL were determined by the formation of thiobarbituric acid reactive products according to the method of Ohkawa *et al.* with minor modifications⁽¹⁶⁾.

Cell culture and measurement of cell viability

Rat thoracic aortic smooth muscle cells (RASMC) were obtained from the National Health Research Institute, and cultured in Dulbecco's modified Eagle medium (Gibco, Carlsbad, CA, USA) at 37°C in a humidified incubator with an atmosphere of 95% air and 5% CO₂. Cells were subcultured into a ninety-six well plate with 1 × 10⁴ cells per well in 100 µl RPMI-1640 medium (Sigma, St Louis, MO, USA) and then treated with ox-LDL (100 µg/ml), with or without ellagic acid (50 µmol/l) for 24 h. All subcultures were done in triplicate. At the end of the incubation period, the cells were harvested and washed with PBS. Then, 10 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-terazolium (MTT) (Sigma) were added to the cells and incubated for 3 h at 37°C. The cells were then incubated overnight with 100 µl solution buffer (10% SDS; HCl (0.01 mol/l)). The absorbance was measured on an ELISA reader (Multiskan EX; Labsystems, Helsinki, Finland) at a test wavelength of 570 nm, and a reference wavelength of 650 nm⁽¹⁷⁾.

Cell-cycle analysis

Cellular DNA contents were measured by flow cytometry as described previously⁽¹⁸⁾ in order to estimate the proportion of RASMC in different phases of the cell cycle affected by ellagic acid. Approximately 5 × 10⁵ cells/well of subcultured cells, which had been treated with or without ellagic acid, were harvested by centrifugation. They were fixed with 70% ethanol on ice before being stored at 4°C overnight. The next day, the cells were finally re-suspended in PBS containing propidium iodide (40 µg/ml) and RNase (0.1 mg/ml; Sigma, St Louis, MO, USA), and 5% triton X-100 (Amresco, Inc., Solon, OH, USA) for 30 min at 37°C. Cells were then analysed in a flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an Ar ion laser at a wavelength of 488 nm in order to determine the cell cycle.

Western blot analysis of extracellular signal-regulated kinase 1/2 and proliferating cell nuclear antigen expression

The expression of ERK 1/2 and PCNA was measured by Western blot analysis with a commercially available kit using rabbit polyclonal anti-p-ERK 1/2 mitogen-activated protein kinases and anti-PCNA primary antibodies (Abcam, Cambridge, Cambs, UK)^(5,19). RASMC were stimulated with ox-LDL (100 µg/ml) for 10 min after pretreatment with ellagic acid (50 µmol/l) for 24 h. The experiments were terminated by rinsing the cells with PBS. After lysis of the cells, β-actin, phosphorylated ERK 1/2 and PCNA were extracted according to the manufacturer's instructions. The blots were subsequently incubated with the desired primary antibody. A Zero-D scanTM (Scanalytics, Inc. (BD Biosciences Bioimaging), Rockville, MD, USA) was used for densitometric

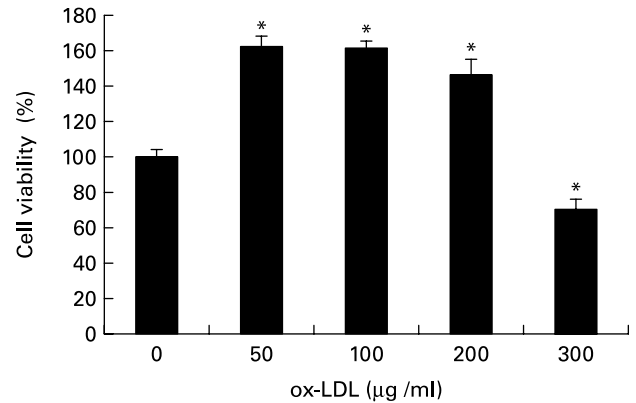


Fig. 1. Effects of different concentrations of oxidised LDL (ox-LDL) on rat aortic smooth muscle cells (RASMC) viability assayed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-terazolium test. RASMC were treated with increasing concentrations (50–300 µg/ml) of ox-LDL for 24 h in 1% fetal bovine serum–Dulbecco's modified Eagle medium. Results were from three experiments each and each was performed in triplicate. Values are means, with their standard errors represented by vertical bars. * Mean value was significantly different to that of the control group ($P < 0.05$).

quantification. The β-actin was used for normalisation of protein expression.

Statistical analysis

All data are expressed as mean values with their standard errors. Comparisons between the four groups were made by one-way ANOVA. Tukey's *post hoc* test was used to analyse significant effects. A P value of 0.05 was taken as the threshold for statistical significance.

Results

The effect of ellagic acid on the viability of rat aortic smooth muscle cells

Cell viability was assayed by the MTT test. After 24 h incubation with 0.1, 10, 50, and 100 µM-ellagic acid, cell

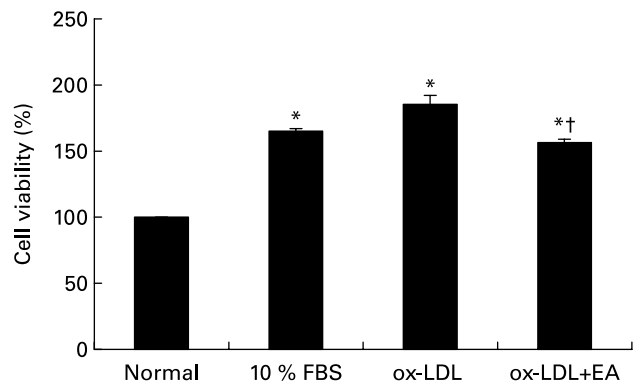


Fig. 2. Effect of ellagic acid (EA) on oxidised LDL (ox-LDL)-induced proliferation of rat aortic smooth muscle cells (RASMC). RASMC were incubated with ox-LDL (100 µg/ml) alone or with 50 µM-ellagic acid for 24 h in 0.1% fetal bovine serum (FBS)–Dulbecco's modified Eagle medium. Values are means ($n = 3$), with their standard errors represented by vertical bars. * Mean value was significantly different to that of the normal group ($P < 0.05$). † Mean value was significantly different to that of the ox-LDL group ($P < 0.05$).

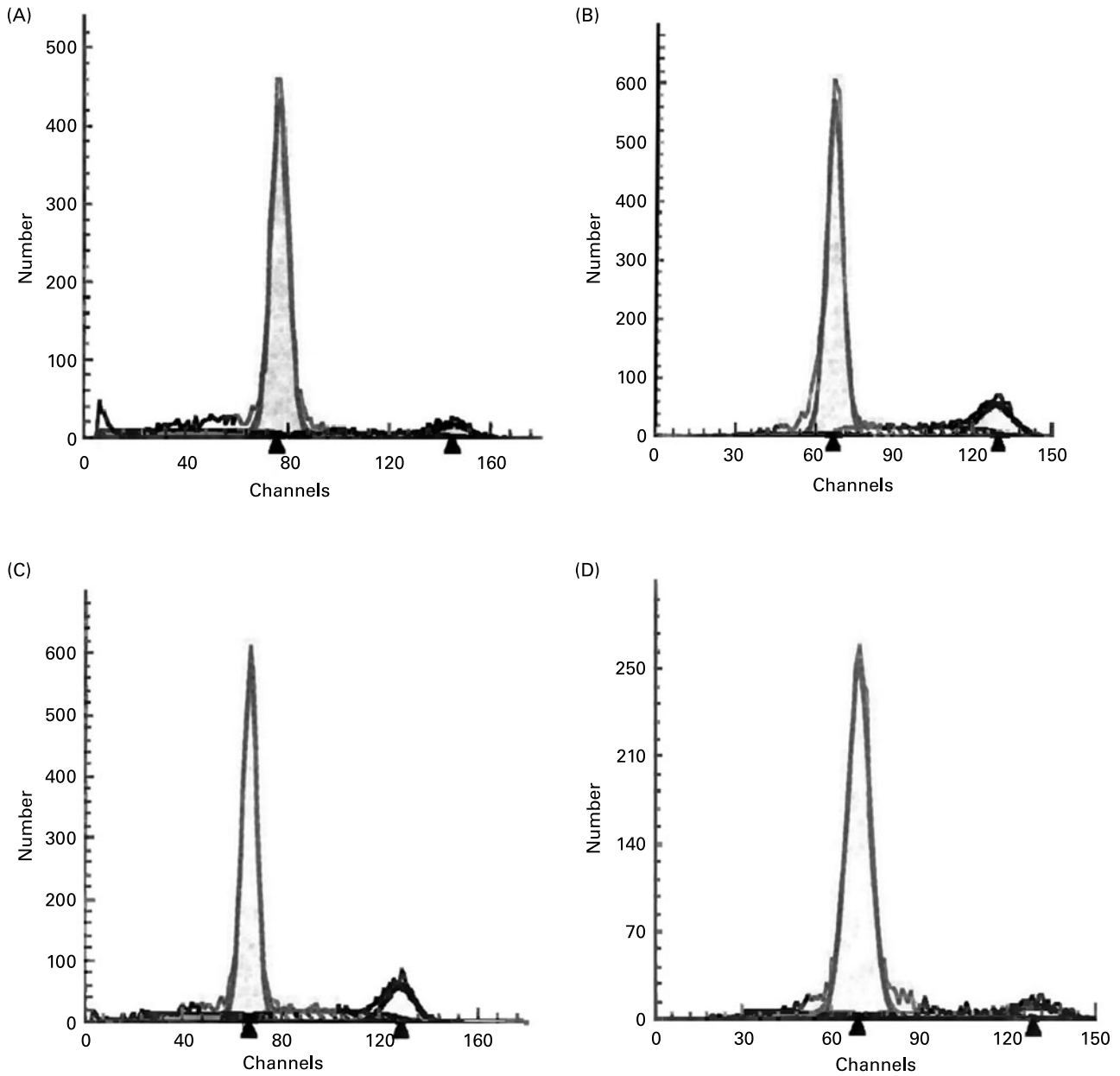


Fig. 3. Effect of ellagic acid (EA) on rat aortic smooth muscle cells (RASMC) cell cycle induced by oxidised LDL (ox-LDL). RASMC were incubated with ox-LDL (100 $\mu\text{g/ml}$) alone or with 50 μM -EA for 24 h in 1% fetal bovine serum (FBS)–Dulbecco's modified Eagle medium. (A) Normal cells – G0/G1, 86.86%; G2/M, 6.38%; S, 7.76%. (B) 10% FBS-treated cells – G0/G1, 75.91%; G2/M, 12.32%; S, 11.7%. (C) ox-LDL-treated cells – G0/G1, 71.01%; G2/M, 13.57%; S, 15.42%. (D) Cells treated with ox-LDL and EA – G0/G1, 83.2%; G2/M, 7.32%; S, 9.4%.

viability was, respectively, 106 (SEM 2.2), 106.4 (SEM 2.8), 93.8 (SEM 2.8) and 39.4 (SEM 2.9) % of control levels. Clearly, 100 μM -ellagic acid causes a significant reduction in cell viability.

Oxidised low-density lipoprotein-induced rat aortic smooth muscle cell proliferation

The effect of ox-LDL on the proliferation of RASMC was detected by MTT assay. As shown in Fig. 1, treatment with ox-LDL (50 to 200 $\mu\text{g/ml}$) for 24 h resulted in increased cell viability; however, doses that exceeded 200 $\mu\text{g/ml}$ had a cytotoxic effect.

Effect of ellagic acid on oxidised low-density lipoprotein-induced proliferation of rat aortic smooth muscle cells

As shown in Fig. 2, stimulation of RASMC with ox-LDL (100 $\mu\text{g/ml}$) for 10 min resulted in increased cell viability. Pre-incubation of ox-LDL with ellagic acid (50 $\mu\text{mol/l}$), however, significantly inhibited ox-LDL-induced cell viability.

Effect of ellagic acid on the rat aortic smooth muscle cell cycle and proliferating cell nuclear antigen expression

To clarify the effect of ellagic acid on cell-cycle regulation, we analysed the phases of cell cycle using flow cytometry. As shown in Fig. 3, the percentages of G2/M and S phase increased

after stimulation of RASMC with ox-LDL and decreased after pre-treatment of ox-LDL with ellagic acid. Using Western blotting, we examined whether the effect of ellagic acid on the cell cycle was associated with the expression of PCNA (Fig. 4). The gene expression patterns were consistent with the cell-cycle analysis data. Cells that had been treated with ox-LDL only increased in number in the S phase; this increase was accompanied by increasing the expression of PCNA. However, cells pre-treatment with ox-LDL and ellagic acid decreased in number with the S phase.

Effect of ellagic acid on extracellular signal-regulated kinase 1/2 phosphorylation

Activation of ERK 1/2 was accompanied by phosphorylation of the Tyr204 residue. RASMC were stimulated with ox-LDL (100 µg/ml) for different periods of time; enhanced phosphorylation of ERK 1/2 occurred after cells had been exposed to ox-LDL for 5 min, but then began to decline (Figs. 5 (A) and (B)). Compared with the control group (1% fetal bovine serum), exposure of RASMC to ox-LDL caused a marked increase in the phosphorylation of ERK 1/2. The ox-LDL-stimulated phosphorylation of ERK 1/2 was inhibited by ellagic acid (Fig. 6).

Discussion

The present study demonstrates that ellagic acid significantly inhibited ox-LDL-induced proliferation of RASMC perhaps by inactivating the ERK pathways and blocking cell-cycle

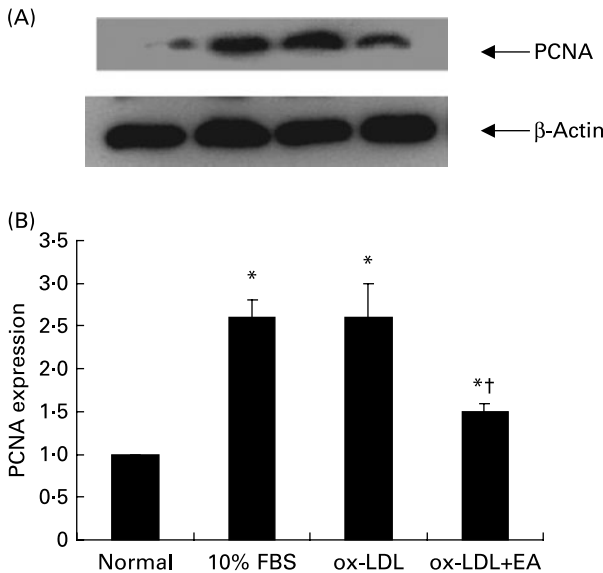


Fig. 4. Effects of ellagic acid (EA) on oxidised LDL (ox-LDL)-induced protein-proliferating cell nuclear antigen (PCNA) expression. (A) Western blot of PCNA. (B) PCNA expression as a ratio to the control. Rat aortic smooth muscle cells were incubated with ox-LDL (100 µg/ml) with or without 50 µM-EA for 24 h in 1% fetal bovine serum (FBS)–Dulbecco’s modified Eagle medium. Proteins were extracted from the cells and analysed by Western blot analysis. The immunoblots shown are representative of three independent experiments. Values are means (*n* 3), with their standard errors represented by vertical bars. * Mean value was significantly different to that of the normal group ($P < 0.05$). † Mean value was significantly different to that of the ox-LDL group ($P < 0.05$).

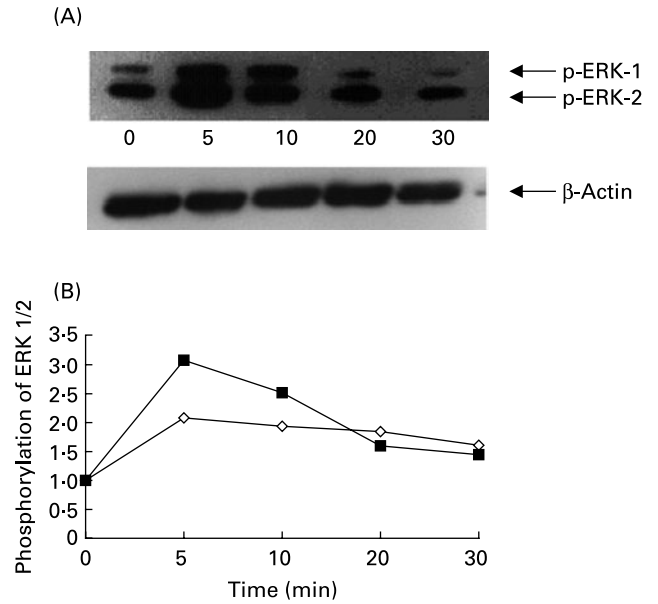


Fig. 5. Time course of oxidised LDL (ox-LDL)-induced phosphorylation of extracellular signal-regulated kinases (ERK) 1 and 2 in rat aortic smooth muscle cells (RASMC). (A) Western blot of phosphorylated ERK (p-ERK) 1 and 2. (B) Phosphorylation of ERK 1 (◇) and ERK 2 (■) expressed as a ratio to the control. Cells were cultured in twelve-well plates until confluence and then transferred to medium with serum-free medium for 24 h. After 24 h cultivation, the RASMC were stimulated with ox-LDL (100 µg/ml) for the indicated time periods (0 min to 30 min).

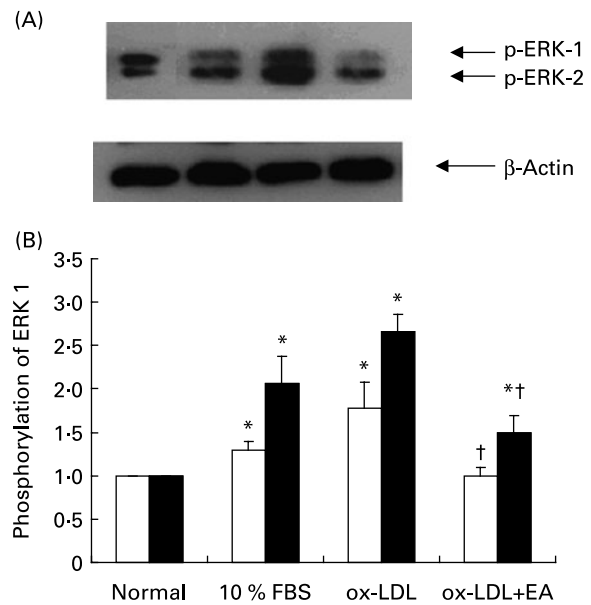


Fig. 6. Effects of ellagic acid (EA) on oxidised LDL (ox-LDL)-induced extracellular signal-regulated kinases (ERK) 1 and 2 phosphorylation. (A) Western blot of phosphorylated ERK (p-ERK) 1 and 2. (B) Phosphorylation of ERK 1 (□) and ERK 2 (■) expressed as a ratio to the control. Rat aortic smooth muscle cells were stimulated with ox-LDL (100 µg/ml) for 10 min after pre-treatment with 50 µM-EA for 24 h in 1% fetal bovine serum (FBS)–Dulbecco’s modified Eagle medium. Proteins were extracted from the cells and analysed by Western blot analysis. The immunoblots shown are representative of three independent experiments. Values are means (*n* 3), with their standard errors represented by vertical bars. * Mean value was significantly different to that of the normal group ($P < 0.05$). † Mean value was significantly different to that of the ox-LDL group ($P < 0.05$).

progression. Ellagic acid is a phenolic compound found in fruits including grape juice (10.2 mg/100 g), grape wine (5.6 mg/100 g), blueberries (0.9 mg/100 g), blackberries (42.4 mg/100 g), raspberries (17.9 mg/100 g) and strawberries (19.8 mg/100 g)⁽²⁰⁾. The typical dietary intake of ellagic acid in humans is approximately 40–80 mg/d if 200 g strawberries or blackberries are eaten⁽²⁰⁾. In the present study, we found that 50 μ M-ellagic acid treatment effectively reduced ox-LDL-induced RASMC proliferation. Previous studies also indicated that other polyphenols, such as vitamin E (50 μ mol/l) and resveratrol (30–50 μ mol/l) also inhibited proliferation of VSMC^(6,21). This dose (50 μ mol/l) of ellagic acid is equivalent to the dietary intake of approximate 200 g of berries subject to absorption^(20–23).

Increased proliferation of intimal VSMC is an important component in the development of atherosclerosis, and ox-LDL is a mitogen for VSMC⁽²⁾. In the present study, we found that ox-LDL induced RASMC proliferation. Ox-LDL at low concentrations (0–100 μ g/ml) increased cell viability in a dose-dependent manner; however, ox-LDL at doses of 200 μ g/ml began to elicit a cytotoxic effect. This finding of ox-LDL exhibiting a biphasic effect on VSMC is similar to that reported by other researchers; low concentrations (20–120 μ g/ml) had a proliferative effect while high doses (> 200 μ g/ml) had a cytotoxic effect^(6,24,25). The increased mitogenic effect of ox-LDL is attributed to the chemical changes brought about by the process of oxidation of LDL components such as the generation of H₂O₂, and the conversion of phosphatidylcholine to lysophosphatidylcholine and 4-hydroxy-2-nonenal^(26,27). In addition, certain oxidation products of unsaturated NEFA have been shown to stimulate mitogenic signalling pathways in VSMC⁽²⁸⁾.

Previous studies have indicated that ox-LDL stimulates VSMC proliferation via activation of the ERK pathway⁽²⁾. It is known that the ERK pathway is activated by oxidative stress. Liu *et al.* postulated that ox-LDL might induce a response to oxidative stress by stimulating the generation of intracellular reactive oxygen species⁽⁶⁾. Previous study also indicated that reactive oxygen species are able to induce tyrosine phosphorylation of mitogen-activated protein kinases and cell growth⁽²⁹⁾. The present results indicated that treatment with ox-LDL significantly increased in cell proliferation and activation of ERK 1/2.

Our previous study found that ellagic acid exhibits free-radical scavenging activities and reduces oxidative stress in cell and animal models^(15,30). We proposed that the inhibitory effect of ellagic acid on ox-LDL-induced cell proliferation might be due to its antioxidative ability. It might decrease reactive oxygen species production induced by ox-LDL and therefore inhibit ERK 1/2 expression.

PCNA synthesis is strictly regulated during the cell cycle and its protein is necessary for the transition of cells from quiescence to the S phase⁽¹⁰⁾. In the present study, we found that the amount of PCNA in the G2/M and S phases increased after treatment of ox-LDL but decreased after prior addition of ellagic acid to the ox-LDL (Fig. 3). The amount of PCNA protein was consistent with the cell-cycle analysis data (Fig. 4). It is therefore likely that ellagic acid reduced ox-LDL-induced RASMC proliferation by suppressing PCNA transcription.

In conclusion, cell proliferation stimulated by ox-LDL was inhibited by ellagic acid according to the MTT test and G0/G1

cell-cycle arrest in smooth muscle cells. The expressions of ERK 1/2 and PCNA were also suppressed by ellagic acid. Therefore, ellagic acid inhibits ox-LDL-induced aortic smooth muscle cell proliferation by inactivating the ERK 1/2 pathway and by suppressing the expression of PCNA. These results may advance the understanding of the role of antioxidants in the prevention of atherosclerosis.

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