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Original Article

Antioxidant and cytoprotective effects of L-Serine on human endothelial cells

M. Naderi Maralani[1], A. Movahedian[1,*], Sh. Haghjooy Javanmard[2]

[1]Department of Clinical Biochemistry and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R.Iran.
[2]Applied Physiology Research Center, Department of Physiology, Isfahan University of Medical Sciences, Isfahan, I.R.Iran.

Abstract

Oxidative stress has been implicated as a prominent determinant in the development of several diseases such as atherosclerosis. Anti atherosclerotic effects of L-serine have been shown previously but its responsible mechanisms remained unidentified .This study aimed to investigate the antioxidant and cytoprotecrtive effects of L-serine and its possible mechanisms. For this purpose ,cell viability analysis and nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) activity, heme oxygenase-1 (HO-1) concentration, total Nitric Oxide (NOx) production were evaluated in oxidative stress-induced Human Umbilical Vein Endothelial Cells (HUVECs) pretreated by L-serine. Cytoprotective effects of L-serine was measured through 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Nrf2 activity and HO-1 concentration were determined in the cell lysate by commercial immunoassay methods. NOx was assayed in the supernatant of culture medium through colorimetric Griess method. Pretreatment with L-serine (0.1-3.2 mM) protected endothelial cells from hydrogen peroxide-mediated cell cytotoxicity (H_2O_2, 0.5 mM) and lead to significant induction of Nrf2 activity, HO-1 expresssion and NOx production. These findings demonstrated that L-serine has antioxidant and cytoprotective effects through the elevation of some crucial antioxidant factors such as Nrf2, HO-1 and NO.

Keywords:Nrf2; Heme oxygenase-1; Nitric oxide; MTT; L-serine; Antioxidant

INTRODUCTION

Oxidative stress causes endothelial dysfunction and has a prominent role in the pathogenesis of atherosclerosis and cardiovascular diseases (1,2). Antioxidant small molecules such as some amino acids protect endothelium against oxidative injuries through several mechanisms (3). Some amino acids have been proposed to greatly decline endothelial cell injury caused by reactive oxygen species (ROS) (4). Antiatherogenic properties of the amino acid L-serine in hypercholesterolemic rabbits has been already reported by our group (5). The exact underlying mechanism for the antiatherogenic effects of L-serine is not yet fully understood. A possible mechanism might be the induction of antioxidant mediators and protein genes which protect cells from oxidative stress injury. In recent years, extensive studies have fulfilled the pivotal role of nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) as an antioxidant and anti inflammatory mediator (6). Nrf2 is a redox-sensitive transcription factor, which promotes the production of many phase 2 antioxidant enzymes such as heme oxygenase-1 (HO-1) through regulating of antioxidant response elements (ARE) that encodes phase 2 detoxification enzymes, thereby protects the endothelium against cytotoxic electrophiles or ROS (7). Also it has been demonstrated that Nrf2 protects cells/tissues from inflammatory injuries (6,8). Increased HO-1 activity has been reported as a possible mechanism through which the antiatrogenic properties of some antioxidant amino acids such as L-alanine and L-methionine have been explained (9,10). HO-1 is an inducible enzyme that degrades

*****Corresponding author: A. Movahedian, this paper is extracted from the MSc thesis No. 389175
Tel. 0098 311 7922593, Fax. 0098 311 6680011
Email: movahedian@pharm.mui.ac.ir

heme. Degradation of heme leads to the elevation of bilirubin which has strong antioxidant effects at physiological levels. Bilirubin is inversely related to atherogenic risk factors and protects endothelium against oxidative stress (11-13). Carbon monoxide (CO) is another product of heme degradation that exerts a vasoerelaxant effect through regulation of K^+ channels in smooth muscle cells as well as lead to vasodilation by guanylate cyclase induction (11). Nitric oxide (NO) is an important agent through which some antioxidant molecules prevent endothelial cells from pro-inflammatory responses that lead to the endothelial cells death (14). NO has a crucial role in regulation of vascular function as a potent vasorelaxant, and vascular muscle cell migration and proliferation inhibitor. Furthermore, there is a significant relationship between Nrf2, HO-1, heme degradation products and NO bioavailability (15). One possible mechanism through which L-serine exerts its antiatherogenic effects might be through the elevation of Nrf2, HO-1 and NO. In the present study, we aimed to evaluate the possible antioxidant effects of L-serine through increasing of Nrf2 activity, HO-1 concentration, and total nitric oxide (NOx) levels in human umbilical vein endothelial cells (HUVECs) induced oxidative stress mediated by H_2O_2.

MATERIALS AND METHODS

Cell culture

HUVEC line was purchased from National Cell Bank of Iran (Pasteur Institute, Iran). The cells were cultured in 25 and/or 75 cm^2 culture flasks in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing 10% fetal bovine serum (Gibco-Invitrigen), penicillin-streptomycin mixture (100 U/ml Penicillin and 100 µg/ml streptomycin,Gibco) and incubated in circumstance of 5% CO_2 and 95% air at 37°C incubator. The medium of cultured cells renewed every 48 h.

Evaluation of cell viability

In order to determine the protective effect of L-serine on H_2O_2-oxidative cell injury, cytotoxicity assay was performed using 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (16). Briefly cell suspension (2×10^4 cells/180 µl medium) was transferred to 96-well microtiter plates. The medium also contained 10% fetal bovine serum. After 24 h incubation at 37°C, the cells were treated by L-serine (Sigma) in the concentration range of 0.1 to 3.2 and 10 mM (final concentration in wells) for an additional 24 h. Then, the cells were washed out with phosphate buffered saline (PBS, PH 7.4), new medium and hydrogen peroxide (Merck KGaA Co.) at the concentration of 0.5 mM was added to the wells and incubated for 2 h. Afterward, catalase (0.1 mg/ml) was added as an oxidative stress stopper. The cells were then washed out with PBS and renewed by medium. 20 µl MTT (0.5 mg/ml; Sigma) was later added per well and incubated for 3 h in 37°C. Finally MTT reacted with living cells and converted by mitochondrial enzyme to foramazan crystals with dark purple color which is an insoluble component that dissolved by dimethyl sulfoxide (DMSO) and its optical density (OD) measured in 550 nm wavelength by microplate reader (BioTek Instruments, EL x 800 TM, USA). The wells just containing cell suspension without any treatment with L-serine or exposing through H_2O_2 were assumed as a negative control. DMEM was used as the blank. The percentage of cell viability in the negative control was considered as 100. Cell viability determined through a relevant known formula (17):

Cell viability (%)=(OD test-OD blank/ OD negative control-OD blank)*100

For obtaining appropriate concentration and time for inducing oxidative stress through H_2O_2, half maximal inhibitory concentration (IC_{50}) experiments were performed .The cytotoxicity effect of H_2O_2 was stopped by the addition of catalase (0.1 mg/ml, Sigma) for removing excess hydrogen peroxide in wells as described previously (18).

Nrf2 activity assay

To determine Nrf2 activity, the HUVECs was pretreated with L-serine (0.1 to 3.2 and 10 mM) and exposed to the oxidative stress by 0.5 mM H_2O_2. At first, the media was removed

from dish and the cells were washed out three times with a mixture of PBS and phosphatase inhibitor which then gently scraped from dish by cell lifter/scraper without trypsynizing or enzymatic scraping. The nuclear extract was prepared using the nuclear extract kit (Trans AM^{TM} Nrf2, Active Motif, Carlsbad, USA, Cat No:40010). The activity of Nrf2 in the nuclear extract was assayed using a commercial Nrf2 activity kit (Active Motif, Carlsbad, USA, Cat No:50296) (19) which is based on the DNA-binding Enzyme-Linked Immunosorbent Assay (ELISA). According to the manual of the kit, for providing quantitative results, the detection range of this kit was 0.15 to 2.5 µg/ml of nuclear extract per well. Equal and distinguished concentrations of nuclear extracts that were diluted in lysis buffer (1.25 µg/ml) added per well. For this, nuclear extract protein concentrations were evaluated through Bradford based protein quantification assay kit (Bioo Scientific, Max Discovery, USA, Cat No: 3440-01) according to the instruction provided by the manufacturer.

HO-1 concentration assay

The amount of HO-1 in cell lysates was measured using a human HO-1 ELISA Kit (Assay Designs, Stressgen, Enzo Life Sciences, UK, Cat No: ADI-EKS-800). For this purpose, the cultured cells that pretreated with L-serine (0.1 to 3.2 and 10 mM) and exposed to the oxidative stress through H_2O_2 (0.5 mM) were scraped by cell lifter and then centrifuged. The cell pellet was washed three times with cold PBS. For every 1×10^6 cells, 1 ml of lysis buffer of the kit was applied to prepare cell lysate. HO-1 in cell lysate was measured according to the instruction provided by the manufacturer. The intra-assay and inter-assay precision of this kit were <10% (20).

Total NO assay

The amount of NOx production in the cultured cells that pretreated with L-serine (0.1 to 3.2 and 10 mM) and then exposed to oxidative stress by 0.5 mM H_2O_2, was assessed through determination of NOx level in medium or supernatant of harvested cells using a commercial kit (Cayman Chemicals Company, Ann Arbor, MI,USA, Cat No:780001) based on the Griss reaction (21,22) according to the instructions of its manufacturer. The intra-assay and inter-assay coefficient of variations for the assay were 2.7% and 3.4%, respectively.

Sample's OD in all experiments was detected using an absorbance microplate reader and all of the cells used in this study had been passaged 3 to 5 times.

Statistical analysis

All data were analyzed by SPSS software version 16.0. Statistical analysis were accomplished through One-way ANOVA for determining differences between multiple experimental groups (different concentration of L-serine treatment), followed by LSD or bonferoni post hoc test for comparing two groups. In this study, the results were determined and expressed as mean ± standard deviation (SD) in triplicate independent, experiments. Statistical significance was considered $P<0.05$.

RESULTS

Cytoprotective effects of L-serine against oxidative stress induced cell injury on HUVECs

HUVECs exposed to various concentration of H_2O_2 (0.0625-2mM) for three different time periods (1,2 and 4 h) remarkably reduced cell viability (51%) at 0.5 mM concentration for 2 h (IC_{50}). Through this experiment, appropriate time and concentration of H_2O_2 for inducing oxidative stress on HUVECs was achieved. (Fig. 1 A).The addition of L-serine (0.1 to 10 mM) to the cultured HUVECs markedly decreased cell death caused by the exposure to hydrogen peroxide and 10 mM concentration of L-serine did not have any meaningful result rather than 3.2 mM (Fig .1 B). Half maximal effective concentration (EC_{50}) of L-serine for this experiment was 0.45 mM. Furthermore, gallic acid, an organic and phenolic acid, which is a known high potent antioxidant and its cytoprotective and antioxidant effects on HUVECs described previously (23), was applied for controlling the cell viability assessment in this study. Gallic acid significantly increased cell viability (Data not shown).

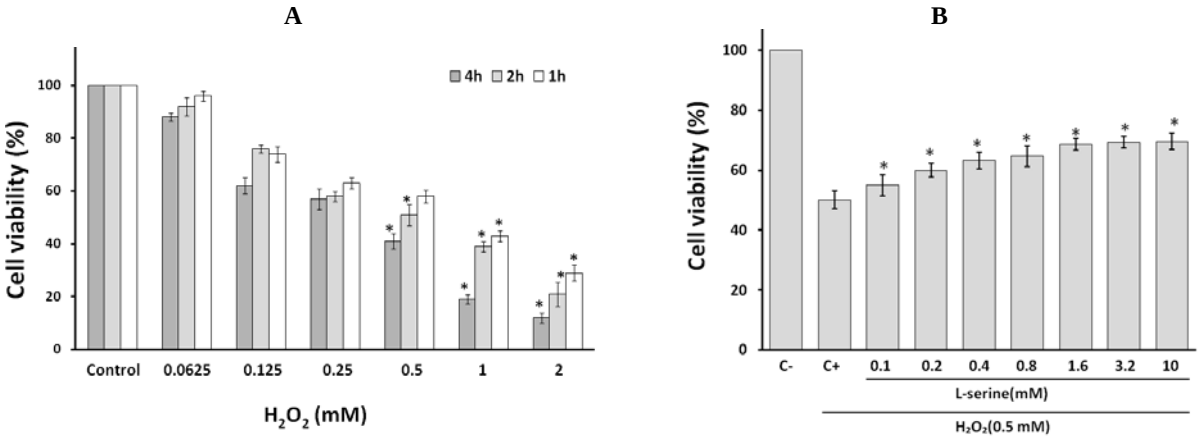

Fig. 1. Effect of L-serine on hydrogen peroxide mediated cytotoxicity in HUVECs. **A)** The viability of HUVECs against various concentration of H_2O_2 (0.0625-2 mM) for 1, 2 and 4 h. * indicates significant differences compared with negative control for expressing half maximal cytoxicity of H_2O_2 at $P<0.05$ **B)** The protective effects of L-serine evaluated at 7 different concentrations. C^- and C^+ are negative and positive controls, respectively. Values are expressed as mean ± SD. * indicates significant differences as compared with positive control at $P<0.05$.

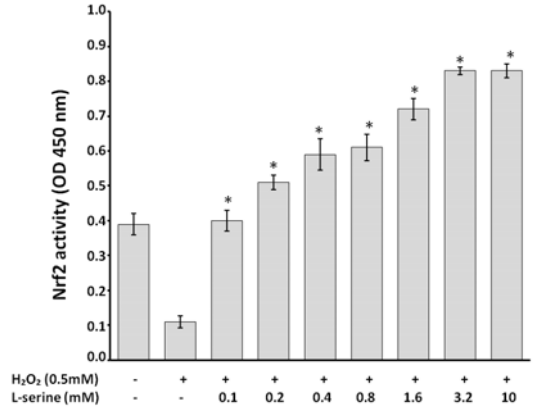

Fig. 2. L-serine pretreatment increased Nrf2 activation in HUVECs that exposed to oxidative stress through H_2O_2 as determined in the nuclear extract of the cultured cells. * indicates significant differences as compared with positive control at $P<0.05$.

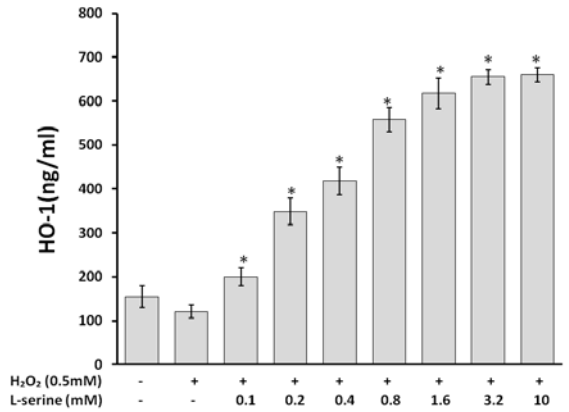

Fig. 3. Effect of L-serine on HO-1 concentration in HUVECs induced cell injury by H_2O_2. L-serine, in the range of 0.1 to 3.2 mM had an increased effect on HO-1 induction in the cells. * indicates significant differences as compared with positive control at $P<0.05$.

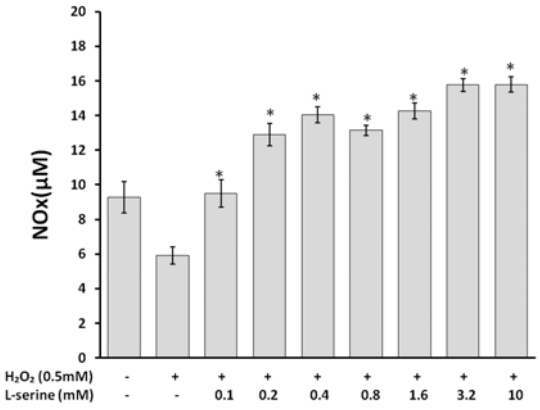

Fig. 4. NOx production in the medium of HUVECs pretreated with different levels of L-serine. * indicates significant differences as compared with positive control at $P<0.05$

L-serine pretreatment effect on Nrf2 activity in HUVECs under oxidative stress condition

L-serine (0.1 to 10 mM) caused an elevation in Nrf2 activity in HUVECs as determined by measuring the activity of Nrf2 in the nuclear extract of the cultured cells (Fig. 2). Nrf2 activity of the nuclear extract samples are expressed as the amount of their ODs at 450 nm. EC_{50} of L-serine for induction Nrf2 activity was 0.2 mM.

HO-1 concentration influenced by L-serine on HUVECs under oxidative stress circumstances

HO-1 induction by L-serine occurred at various protein levels determined by ELISA (Fig. 3). As illustrated in the figure, incubation of cells with L-serine (0.1 to 10 mmol/L) followed by exposure to the H_2O_2 (0.5 mM, 2 h) lead to a significant elevation of HO-1 protein expression (Fig. 3). EC_{50} of L-serine for HO-1 expression was found to be 0.55 mM. The effect after 3.2 mM of L-serine became constant.

Evaluation of NOx production on HUVECs pretreated with L-serine under oxidative stress condition

The levels of nitrate and nitrite (the end products of NO) in the culture medium of cells pretreated with various concentrations of L-serine (0.1 to 10 mM) for 24 h under oxidative stress for 2 h under H_2O_2 (0.5 mM) were measured (Fig. 4). L-serine at concentration of 3.2 mM exerted the greatest effects on NO production in the cultured cells and EC_{50} of L-serine for NOx production was found to be 0.25 mM.

DISCUSSION

The results of the present study showed that the amino acid L-serine has cytoprotective and antioxidant effects against oxidative stress induced in HUVECs with H_2O_2, especially at concentration range of 0.1 to 3.2 mM with no more activity at concentration above 3.2 mM (10 mM). The cytoprotective and antioxidant effects may be brought about through the elevation of antioxidant agents such as Nrf2, HO-1and NO.

The antioxidant properties of some other amino acids such as L-alanine (10), L-arginine (24), L-tryptophan (25), L-methionine (9) and L-cystiene (26) have been evaluated and the underlying antioxidant mechanisms are addressed. Antinerotoxic effects of L-serine, *in vitro* and *in vivo,* have been previously demonstrated (27). Antiatherogenic feature of L-serine has been demonstrated in hypercholestrelemic rabbits in our previous study but its responsible mechanisms and mediators have not been determined (5). However, we hypothesized that the antioxidant and anti-inflammatory features of L-serine may, in part, account for this property. L-serine is a simple and neutral amino acid with no active metabolites such as 3-hydroxy antranilic acid or NO and agmatine as seen with L-tryptophan or L-arginine, respectively. Especial group like thiol group as present in L-cystiene is absent in the chemical structure of L-serine. We, therefore, decided to conduct more experiments to disclose the possible underlying antioxidant mechanisms of L-serine.

It has been demonstrated that Nrf2 has antioxidant activity as a transcriptional factor which induces HO-1 expression and NO production. Althogh there is a causal link between Nrf2 activation and NO levels and bioavailibility (28,29), it is well established that induction of HO-1 leads to anti-inflammatory, anti-atherogenic and cytoprotective effects. HO-1 through the degradation of the pro-oxidant heme, produces bilirubin and CO that are important antioxidants (15, 30-32). The importance of NO in endothelium and cardiovascular system integrity has been emphasized in many studies (14,33). The effects of L-serine on the elevation of Nrf2 activity, HO-1 expression and NO production observed in the present study, may be responsible for the antioxidant effects of the amino acid as determined through its cytoprotective effect. On the other hand, the cytoprotective effects of some amino acids such as L-alanine and L-glycine and structurally similar amino acids against free radical-induced injury that has been reported by some studies (4, 34-35) are in consistence with our results.

CONCLUSION

In summary, our study for the first time demonstrated that L-serine exerts antioxidant and cytoprotective effects on HUVECs through induction of other antioxidant factors such as Nrf2, HO-1 and NO. Therefore, L-serine may have beneficial effects on endothelium homeostasis.

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