Laboratory Research

Effects of antioxidants on homocysteine thiolactone-induced apoptosis in human umbilical vein endothelial cells

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Background and objectives Hyperhomocysteinemia is an independent risk factor for cardiovascular disease. Homocysteine thiolactone (HcyT), one of the homocysteine metabolites in vivo, is toxic both in vivo and in vitro. The aim of this study was to investigate the effect of HcyT on apoptotic damage in human umbilical vein endothelial cells (HUVECs) and the role of antioxidants in the reduction of HcyT-induced apoptosis. Methods HUVECs were cultured in DMEM supplemented with 20% heat inactivated fetal bovine serum cell cultures were maintained in a humidified 5% CO₂ atmosphere at 37°C. Cytotoxicity was determined by MTT assay, which consists of hypodiploid cells with propidium iodide labeling and intracellular reactive oxygen species levels using 2',7'-dihlorofluorescein diacetate as the probe by flow cytometry. Results HcyT (250-2000μM) induced HUVECs apoptosis in a time- and concentration-dependent manner. Reactive oxygen species levels rose in response to increasing HcyT concentrations at 24-h incubation. The reduction of cell apoptosis by N-acetylcysteine, vitamin E, or pyrrolidine dithiocarbamate, occurred simultaneously with a significant decrease in intracellular reactive oxygen species levels. Conclusion HcyT exerts its cytotoxic effects on endothelial cells through an apoptotic mechanism involving cellular reactive oxygen species production. The capacity of N-acetylcysteine, vitamin E, and pyrrolidine dithiocarbamate to scavenge HcyT-induced cellular reactive oxygen species correlates well with their efficiency to protect against HcyT-promoted apoptotic damage. The protective effect of pyrrolidine dithiocarbamate on cell apoptosis indicates HcyT-generated hydrogen peroxide may provoke cell apoptosis via activating nuclear factor-kappa binding protein. (J Geriat Cardiol 2006; 3(2):107-12.)

Key Words homocysteine thiolactone; apoptosis; antioxidant; reactive oxygen species; endothelial cell

Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid product of the demethylation of methionine. Clinical studies have shown that elevated serum Hcy is an independent risk factor for cardiovascular disease in humans.¹ Although the exact mechanism of Hcy toxicity remains largely unknown, it is believed that Hcy or its metabolite adversely affects vascular endothelium. It has been suggested that the homocysteine derivative of homocysteine thiolactone (HcyT), synthesized by methionine synthetase in cultured mammalian cells, may provide a plausible chemical mechanism in explaining Hcy toxicity to the human vascular endothelium; the damage that plays a central role in atherosclerosis. HcyT can be detected in normal human plasma, in which it presents at a level up to 30-fold lower than total Hcy. The extent of HcyT formation in human umbilical vein endothelial cells (HUVECs) depends on levels of Hcy, methionine, folate, and high density lipoprotein (HDL).

The extent of thiolactone formation in HUVECs is positively correlated with concentrations of Hcy, and negatively correlated with concentrations of methionine, folic acid, and HDL.² HcyT is toxic both in vivo and in vitro. Chronic infusions of baboons with HcyT causes atherosclerosis.³ It has been demonstrated that cell injury induced by homocysteine derivatives involves oxidative damage.⁴ Cell culture studies have suggested that HcyT induces HL-60 cells apoptosis in a caspase-dependent manner and increases intracellular reactive oxygen species (ROS) concurrently.⁵

Apoptosis is a form of programmed cell death that plays a significant role in the etiology of atherosclerosis. It is characterized by DNA damage including chromatin fragmentation into oligonucleosomal lengths as well as formation of apoptotic bodies with hypoploid DNA contents, and disruption of membrane asymmetry that leads to phosphatidylserine exposure on the membrane surface.⁶ ⁷ ⁸ The purpose of the present study was to examine the toxicity of HcyT to HUVECs through the apoptotic mechanism and to investigate the effect of antioxidants (N-acetyl cysteine or NAC, vitamin E or VitE, and pyrrolidine dithiocarbamate or PDTC) on the reduction of HcyT-induced apoptosis and intracellular ROS generation.
**Methods**

**Reagents**

L-HcyT (Homocysteine thiolactone hydrochloride salt), Vit E (α-tocopherol acetate), NAC, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), and propidium iodide (PI) were purchased from Sigma (U.S.A). MTT, DMEM medium and trypsin were obtained from Gibco Laboratories (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from HyClone Laboratories (U.S.A). Stock solutions of Vit E and L-HcyT were prepared in dimethyl sulfoxide (DMSO) at 30 and 100 mM respectively. Stock solutions of NAC and PDTC were prepared at 200 mM and 10 mM in the culture medium. A stock solution of DCFH-DA was prepared at 20 mM in ethanol. The final work concentrations of the reagents above were diluted with medium just before use. The DMSO or ethanol concentration in the culture medium has no effect on cell viability.

**Cell culture and cytotoxicity determination**

HUVEC cells were cultured in DMEM supplemented with 20% heat inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cell cultures were maintained in a humidified 5% CO₂ atmosphere at 37°C. Cytotoxicity was determined by MTT assay. Viable cells in various HcyT treatments were expressed as a percentage of control.

**Induction of apoptosis**

Confluent cells were treated with 1 mM HcyT for 0, 3, 6, 12, or 24 h and harvested for apoptosis assay. Besides, after being cultured for 24 h with 0.05, 0.25, 0.5, 1, and 2 mM HcyT, cells were harvested for apoptosis and intracellular ROS assay. The final work concentration of antioxidant in this study was 5 mM for NAC, and 100 mM for both PDTC and Vit E. Cells were preincubated with NAC and Vit E for 12 h, or preincubated with PDTC for 2 h before HcyT treatment. Cells grew normally under our preincubation conditions. Further incubation with antioxidants alone did not affect cellular viability. After 24 h of incubation, HcyT-untreated (control), HcyT-treated only (HcyT), and HcyT-treated cells with antioxidant preincubation were harvested for the following assays.

**Analysis of apoptotic cells with hypodiploid DNA contents**

Briefly, cells were harvested in 1.5 ml Eppendorf tubes and fixed in ice-cold 70% ethanol overnight. They were then incubated with PI (50 mg/L) for 20 min at 37°C. Cellular DNA (red fluorescence) in 10,000 cells was analyzed on a FACS Calibur flow cytometer with the System of CellQuest Software program (U.S.A). Hypodiploid cells, i.e., with sub-G₁ DNA contents, were defined as apoptotic cells.

**Agarose electrophoresis of DNA fragmentation**

Cells at each indicated time point were harvested and suspended in ice-cold lysis buffer (50 mM Tris-HCl PH 7.8, 10 mM ethylenediaminetetra-acetic acid (EDTA), 0.5% SDS and 0.5 mg/ml ribonuclease A) for 60 min in a 37°C water bath, then in a 50°C water bath for another 60 min with 200 μg/ml proteinase K. The phenol and chloroform/isoamyl alcohol (24:1) was used in succession to remove protein and extract DNA. DNA fragments were electrophoresed on a 1.8% agarose minigel at 100 V for 40 min and visualized with ethidium bromide staining under UV illumination. Multimers of 100 base pair DNA were used as DNA markers.

**Determination of intracellular ROS levels**

According to the method of Robinson et al., intracellular ROS were assayed using an oxidation-sensitive fluorescent probe DCFH-DA by flow cytometry. After incubated with 25 mM DCFH-DA and HcyT with or without antioxidants for 24 h, cells were harvested and washed; cells associated with fluorescence (excitation 488 nm, emission 525 nm) were monitored on a FACS Calibur flow cytometer. Data were analyzed using the System of CellQuest Software (San Jose, CA, USA). The intracellular fluorescence intensity is proportional to the amount of ROS produced by the cells. The fluorescence in control cells was designated as 100% and the fold of increase was calculated between treated cells and control cells.

**Statistical analysis**

Results were presented as means ± SD. One-way ANOVA was used for comparisons among groups. P value of smaller than 0.05 was considered to be significant.

**Results**

**Cytotoxic effect of HcyT on cellular viability**

HcyT induced HUVECs death in a dose- and time-dependent manner. (Fig.1) HcyT at 0.25 mM failed to induce cell death in a 72 h incubation. HcyT at 0.50 mM induced cell death significantly in a 48 h incubation. Obvious cell death was induced after incubation with 1 or 2 mM of HcyT for 24 h at HcyT 2 mM presented the most potent cytotoxic effect on HUVECs in a 72 h incubation.

![Fig. 1. Cytotoxic effect of HcyT on cellular viability](image-url)

**HcyT induced apoptosis in a dose-and time-dependent manner**

As shown in Fig. 2A, the proportion of apoptotic cells (measured by hypodiploid cells) became greater with increased concentrations of HcyT ranging from 0.25-1 mM for 24 h.
treated with 0.50, 1, or 2 mM HcyT exhibited a significant elevated percentage of apoptotic cells compared with the controls. This difference was not observed when incubated with 0.25 mM HcyT. Fig. 2B shows the kinetic profile of apoptotic progress in a 24-h incubation with 1 mM HcyT. Compared with HcyT-untreated control cells at the same corresponding time, the proportion of apoptotic cells increased to 2-fold after incubation with HcyT for 3 h; the difference was also significant at 12 and 24 h HcyT incubation. This apoptotic DNA damage was further confirmed by oligonucleosomal DNA fragmentation as shown in Figure 2C. The DNA ladder was visible at 24 h 0.5 and 1 mM HcyT treatment. Taken together, the data indicated that Hcy induced apoptosis with nuclear DNA damage in a time- and dose-dependent manner.

**HcyT affected intracellular ROS generation**

HUVECs treated with 0.25 mM HcyT for 24 h had significantly higher intracellular DCF-fluorescence than control cells(Fig. 3), which was observed before the appearance of apoptotic DNA fragmentation induced by HcyT treatment. The intracellular fluorescence intensity is proportional to the amount of ROS produced by the cells. As shown in Figure 3, intracellular ROS levels rose in response to increasing HcyT concentrations.

**Effects of antioxidants on HcyT-induced apoptosis**

Preincubation of cells with NAC for 12 h completely reduced the increase in percentage of apoptotic cells induced by 24-h HcyT treatment. The percentages of apoptotic cells were significantly reduced when HcyT-treated cells were preincubated with NF-κB inhibitor PDTC and Vit E for 2 and 12 h respectively (Table 1). These two agents reduced the percentage of hypodiploid cells to nearly half that of HcyT-treated only cells.

**Effects of antioxidants on ROS scavenging capacity during HcyT-induced apoptosis**

NAC and Vit E pretreatment completely diminished the increased cellular DCF fluorescence at 24-h HcyT incubation, indicating that the two antioxidants were potent ROS scavengers. Preincubation of cells with PDTC for 2 h significantly reduced cellular ROS. DCF fluorescent intensity in HcyT-treated cells preincubated with PDTC was reduced to 65% of HcyT-treated only cells (Table 1).

**Discussion**

The data in this study showed HcyT induced HUVECs apoptosis in a dose- and time-dependent manner (Fig. 2). HcyT had a significant cytotoxic effect on HUVECs accompanied by the occurrence of DNA ladder and hypodiploid cells, characteristics of apoptosis. The increased intracellular ROS induced by HcyT were in parallel with the appearance of HcyT-induced apoptosis (Fig. 2). NAC pretreatment fully quenched the rise of HcyT-induced DCF intensity (Table 1), and com-
Table 1. Effects of Vit E, NAC, and PDTC on cell apoptosis and ROS production induced by HcyT

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HcyT</th>
<th>HcyT + NAC</th>
<th>HcyT + VitE</th>
<th>HcyT + PDTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis (% of 10,000 cells counted)</td>
<td>1.35 ± 0.12</td>
<td>23.16 ± 4.27*</td>
<td>1.95 ± 0.23#</td>
<td>11.01 ± 2.62##</td>
<td>11.46 ± 1.81##</td>
</tr>
<tr>
<td>Fluorescent intensity (% of control)</td>
<td>104.35 ± 6.15</td>
<td>232.85 ± 4.03*</td>
<td>107.95 ± 3.47#</td>
<td>119.85 ± 13.36#</td>
<td>151.65 ± 6.86##</td>
</tr>
</tbody>
</table>

n=3 (three independent experiments in triplicate).

*p<0.05 compared with the HcyT-untreated control; #P<0.05 compared with the HcyT-treated only group

complete prevented HUVECs from apoptotic damage (Table 1), whereas another potent antioxidant Vit E could partially protect cells from apoptosis. The components of ROS such as superoxide anions, hydroxyl radicals or peroxynitrite are major contributors towards the etiology of diseases. NAC is a precursor of reduced glutathione which is a potent inhibitor of hydrogen peroxide production. Vit E is a lipid radical chain breaker that scavenges oxygen radicals and alkyl radicals.10 These observations suggest that the generation of hydrogen peroxide may serve as a necessary mediator of apoptosis induced by HcyT.

The hypothesis that HcyT might cause vascular disease was originally advanced by McCully, based on the observation that premature thromboembolism and atherosclerosis were features of homocystinuria. In a recent study, Jakubowski et al.11 showed that metabolic conversion of homocysteine to thiolactone and protein homocysteinylthiolactone by thiolactone might play a role in homocysteine-induced vascular damage. HcyT has been shown to directly damage endothelial cells and increase proliferation of smooth muscle cell in vitro.12,13 However, it is not clear why excess HcyT is harmful. Under physiological conditions of pH and temperature, HcyT easily reacts with proteins by forming isopeptide bonds with side chain amino groups of lysine residues.14,15 In human serum supplemented with HcyT, protein N-homocysteinylthiolactone occurs with a half-life of 1.5 h, N-homocysteinylthiolactone proteins become prone to aggregation, particularly cytochrome c,16 and can be physiologically detrimental; for instance, they can elicit an immune response, as shown by injecting rabbits with low density lipoprotein (LDL) modified with HcyT.17 In vitro tissue culture, Ferretti G et al.18 has revealed that N-homocysteinylated-LDL exerted a cytotoxic effect that is likely related to an increase in lipid peroxidation and oxidative damage of endothelial cell. HcyT induced apoptotic DNA damage mediated by increased intracellular hydrogen peroxide and caspase 3 activation in HL-60 cells.6 NF-κB is a major transcription factor that when activated plays a pivotal role in many cellular responses to environmental changes. NF-κB is activated by extracellular signals such as ROS, cytokines, tumor necrosis factor, and interleukin-1. It is ubiquitously expressed and serves as a critical regulator of the inducible expression of many genes.19 Hcy-induced nitric oxide production in vascular smooth-muscle cells is dependent on NF-κB transcriptional activation of Nos2.20 Studies proved NF-κB is one of the signaling molecules which mediate cell apoptosis. In this study, pretreatment with NF-κB inhibitor PDTC for 2 h was capable of preventing HcyT-treated cells from apoptosis by more than 50%. This inhibitory effect of apoptosis by PDTC preincubation may indicate the involvement of NF-κB in HcyT-induced apoptosis in HUVEC cells; NF-κB may be activated by HcyT-generated ROS.

In conclusion, the present work demonstrated that HcyT exerted its cytotoxic effects to HUVECs through an apoptotic mechanism involving cellular ROS production. The capacity of NAC and Vit E to scavenge HcyT-induced cellular ROS correlated well with their efficiency to protect against HcyT-promoted apoptotic damage. The protective effect of inhibitors of NF-κB activation PDTC on HcyT-induced apoptosis indicated HcyT-generated hydrogen peroxide may provoke cell apoptosis via activating NF-κB.

References


