ABSTRACT We showed previously that homocysteine thiolactone (HcyT) is a potent inducer of apoptosis in HL-60 cells. In the present study, the role of some radical scavengers (N-acetylcysteine, vitamin C, vitamin E and folate) on the reduction of HcyT-induced apoptosis was investigated. Preincubation of HcyT-treated HL-60 cells with vitamin C (Vit C; 100 μmol/L) or vitamin E (Vit E; 100 μmol/L) for 2 h significantly reduced the proportion of apoptotic cells with hypodiploid DNA contents or with membrane phosphatidylserine exposure, and attenuated the apoptotic DNA fragmentation. Preincubation of cells with N-acetylcysteine (NAC; 5 mmol/L) for 2 h significantly reduced HcyT-promoted apoptosis measured by membrane phosphatidylserine exposure only. The reduction of HcyT-induced apoptosis by NAC, Vit C or Vit E occurred simultaneously with a significant decrease in intracellular H₂O₂ levels and reduced caspase-3 enzymatic activity. In contrast, folate had no H₂O₂ scavenging capacity and did not suppress caspase-3 activity 6 h after HcyT treatment, although folate exhibited antioxidant behavior toward superoxide anions, hydroxyl radicals and peroxynitrite. Preincubation of cells with folate (10 μmol/L) for 3 d did not affect the extent of HcyT-promoted apoptotic damage. Taken together, our findings suggest that antioxidant pretreatment with NAC, Vit C or Vit E exerts more beneficial effects than folate on reducing apoptotic cell damage induced by homocysteine thiolactone.

KEY WORDS: • homocysteine thiolactone • antioxidant • folate • apoptosis • HL-60 cells

Homocysteine thiolactone (HcyT) is a derivative of homocysteine; it is a sulfated amino acid product of the demethylation of methionine. HcyT and homocysteine are metabolically interchangeable in human cells (1,2) and serum (3). Increased levels of homocysteine as well as HcyT are considered to be atherosclerotic and carcinogenic (4–6). The molecular mechanisms of these homocysteine derivatives to cause cellular injury have been investigated extensively. It was suggested that both homocysteine and thiolactone derivatives could elicit oxidative stress in cells and animals (7–10). The resulting oxidative damage is evidenced by enhanced lipid peroxidation (11–13), elevated 8-hydroxy-2′-deoxyguanosine levels in DNA (14) and impaired antioxidant enzymatic function (15). Homocysteine-induced oxidative injury can be prevented by a variety of antioxidants, suggesting that reactive oxygen species (ROS) act as mediators (16,17).

Apoptosis, a programmed cell death, can be activated in response to oxidative stress conditions such as during ROS generation (18,19). Apoptosis in cells is characterized by chromatin condensation, nucleosomal DNA fragmentation and membrane disruption due to phosphatidylserine (PS) exposure (20–22). As cells are stimulated to undergo intra- or extranuclear apoptotic events, the common signaling pathways for initiation and execution of apoptosis are involved in the activation of caspases, a family of aspartate-specific cysteine proteases (23,24). One of the signals for apoptosis execution involves caspase-3, which is an important apoptotic effector for DNA damage or membrane disorder (25–27). Although the mechanisms by which ROS induce apoptosis are not fully defined, it has been suggested that the activation of caspases is involved. Evidence has shown that apoptosis induced by ROS was accompanied by caspase-3 activation, whereas inhibition of caspase-3 blocked apoptosis (28). After hydrogen peroxide (H₂O₂) treatment, caspase-3 was activated during apoptosis in HL-60 cells (29).

HcyT was shown previously to induce apoptotic damage in vascular endothelial cells (30). We demonstrated that HcyT-induced apoptosis is mediated by H₂O₂ generation and caspase-3 activation in HL-60 cells (31). Because oxidative apoptotic damage may play a pivotal role in the etiology of malignancies and diseases (32), it is of interest to study the protective effects of some antioxidants on HcyT-induced apoptosis. N-Acetylcysteine (NAC) is a precursor of reduced glutathione that has the ability to quench hydroperoxides. Vitamin C (Vit C) is an effective scavenger of hydroxyl radicals, and vitamin E (Vit E) is a lipid radical chain breaker that scavenges oxygen radicals and alkyl radicals (33). The ability of NAC, Vit C or Vit E to reduce cell damage elicited by various apoptotic stimuli has also been well studied (34–37). Folate has recently been...
The annexin-V-Fluos kit was used to measure the apoptotic cells with PS folate for 3 d before HcyT treatment. Cells grew normally under our antioxidant treatment to suppress HcyT-induced cytotoxicity were previously tested (39,40). The concentration selected to work in this experiment compared with control values and this persisted for 6 h of HcyT incubation. It increased to 4.3-fold of control at 6 h, and the proportion of apoptotic cells (measured by membrane PS exposure) increased by 50% after 3 h of HcyT incubation. It showed the kinetic profile of apoptotic and ROS generation in HcyT-treated cells during a 24-h incubation. Compared with the HcyT untreated control cells at the corresponding time, the proportion of apoptotic cells (measured by membrane PS exposure) increased by 50% after 3 h of HcyT incubation. It increased to 4.3-fold of control at 6 h, and declined to 1.3-fold at 12 and 24 h of HcyT incubation. Maximal apoptotic damage occurred at 6 h of HcyT treatment. Caspase-3 activity was assayed by 3'-fluorometric assay using DEVD-pNA. Samples were incubated at 37°C for 6 h in a 96-well flat-bottomed microplate. The fluorescence was read using an MRX microplate reader (Dynatech Laboratories, West Sussex, UK) at 405 nm wavelength.

Time-dependent effect of HcyT treatment on apoptosis, caspase 3 activation and ROS generation in HL-60 cells. Figure 1 shows the kinetic profile of apoptotic and ROS generation in HcyT-treated cells during a 24-h incubation. Compared with the HcyT untreated control cells at the corresponding time, the proportion of apoptotic cells (measured by membrane PS exposure) increased by 50% after 3 h of HcyT incubation. It increased to 4.3-fold of control at 6 h, and declined to 1.3-fold at 12 and 24 h of HcyT incubation. Maximal apoptotic damage occurred at 6 h of HcyT treatment. Caspase-3 activity was assayed by 3'-fluorometric assay using DEVD-pNA. Samples were incubated at 37°C for 6 h in a 96-well flat-bottomed microplate. The fluorescence was read using an MRX microplate reader (Dynatech Laboratories, West Sussex, UK) at 405 nm wavelength.

RESULTS

Time-dependent effect of HcyT treatment on apoptosis, caspase 3 activation and ROS generation in HL-60 cells. Figure 1 shows the kinetic profile of apoptotic and ROS generation in HcyT-treated cells during a 24-h incubation. Compared with the HcyT untreated control cells at the corresponding time, the proportion of apoptotic cells (measured by membrane PS exposure) increased by 50% after 3 h of HcyT incubation. It increased to 4.3-fold of control at 6 h, and declined to 1.3-fold at 12 and 24 h of HcyT incubation. Maximal apoptotic damage occurred at 6 h of HcyT treatment. Caspase-3 activity was assayed by 3'-fluorometric assay using DEVD-pNA. Samples were incubated at 37°C for 6 h in a 96-well flat-bottomed microplate. The fluorescence was read using an MRX microplate reader (Dynatech Laboratories, West Sussex, UK) at 405 nm wavelength.

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profile of DCF intensity, although the magnitude of changes was substantially lower for HE intensity.

**Effects of antioxidants on ROS scavenging capacity during HcyT-induced apoptosis.** Preincubation of cells with catalase (10^6 u/L) for 2 h completely diminished the rise of intracellular DCF intensity induced by 3- and 6-h HcyT treatment, suggesting that the ROS in these cells were mostly H2O2 species (Table 1). Preincubation of cells with NAC for 2 h significantly reduced the increased H2O2 generation at 3 and 6 h HcyT treatment. Because our experiments revealed that the percentage of apoptotic cells was maximal after 6 h of HcyT treatment (Fig. 1), this time point was chosen to examine the effects of antioxidants on apoptosis reduction. Preincubation of HL-60 cells with NAC, Vit C, or Vit E significantly reduced the percentage of cells with membrane PS exposure treated with HcyT for 6 h (Fig. 2). Preincubation with folate did not protect cells from apoptotic membrane damage, whereas catalase preincubation completely protected HcyT-treated cells.

The percentages of apoptotic cells with hypodiploid DNA content were significantly reduced when HcyT-treated cells were preincubated with Vit C and Vit E for 2 h (Fig. 3). These two antioxidants markedly reduced the percentage of cells with hypodiploid DNA content to nearly half of that of HcyT-treated cells. Preincubation of cells with NAC and folate did not protect HcyT-treated cells from DNA breakage. Catalase preincubation completely protected HcyT-treated cells from chromosome breakage.

### Table 1

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Control</th>
<th>HcyT + NAC</th>
<th>HcyT + Vit C</th>
<th>HcyT + Vit E</th>
<th>HcyT + Folate</th>
<th>HcyT + Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCF fluorescent intensity, % of control</td>
<td>100 ± 13c</td>
<td>391 ± 14a</td>
<td>258 ± 16b</td>
<td>390 ± 46a</td>
<td>432 ± 22a</td>
<td>377 ± 60a</td>
</tr>
<tr>
<td>3 h</td>
<td>100 ± 8c</td>
<td>351 ± 54a</td>
<td>215 ± 9b</td>
<td>212 ± 55b</td>
<td>198 ± 10b</td>
<td>400 ± 47a</td>
</tr>
<tr>
<td>6 h</td>
<td>101 ± 11b</td>
<td>162 ± 19a</td>
<td>103 ± 10b</td>
<td>80 ± 13b</td>
<td>75 ± 9b</td>
<td>83 ± 16b</td>
</tr>
<tr>
<td>HE fluorescent intensity, % of control</td>
<td>101 ± 13ab</td>
<td>123 ± 10a</td>
<td>123 ± 32a</td>
<td>73 ± 12b</td>
<td>88 ± 14b</td>
<td>73 ± 5b</td>
</tr>
<tr>
<td>3 h</td>
<td>101 ± 14c</td>
<td>292 ± 10a</td>
<td>212 ± 10b</td>
<td>224 ± 11b</td>
<td>254 ± 15b</td>
<td>208 ± 5b</td>
</tr>
<tr>
<td>6 h</td>
<td>102 ± 20c</td>
<td>394 ± 61a</td>
<td>164 ± 31b</td>
<td>203 ± 31b</td>
<td>198 ± 7b</td>
<td>431 ± 46b</td>
</tr>
</tbody>
</table>

1. HL-60 cells were preincubated with NAC (5 mmol/L), Vit C (100 μmol/L), Vit E (100 μmol/L) or catalase (1 × 106 u/L) for 2 h, or preincubated with folate (10 μmol/L) for 3 d before 1000 μmol/L HcyT treatment. At 3 or 6 h after HcyT treatment, HcyT-untreated cells (Control), HcyT-treated only (HcyT) and HcyT-treated cells with antioxidant preincubation were harvested, and the following parameters were measured: dichlorofluorescein (DCF) fluorescent intensity (for hydrogen peroxide and nitric oxide), hydroethidium (HE) fluorescent intensity (for superoxide anions, hydroxyl radicals and peroxy nitrite) and caspase-3 activity.

2. Data are mean ± so, n = 3 (three independent experiments in triplicate). Values in a row without a common letter differ, P < 0.05.

3. Data are not available.
The inhibitory effect of the four antioxidants on apoptotic DNA damage was further confirmed by electrophoretic analysis of DNA laddering (Fig. 4). Preincubation with Vit C or Vit E inhibited the DNA fragmentation induced by 6 h HcyT treatment (lanes 2, 5). NAC or folate did not diminish HcyT-induced DNA laddering (lanes 1, 3), whereas catalase (lane 4) or the combination of NAC, Vit C and Vit E pretreatment (lane 6) completely protected HcyT-treated cells from DNA fragmentation.

FIGURE 4 Effects of antioxidants on apoptotic DNA laddering during homocysteine thiolactone (HcyT) treatment. Cells were preincubated with N-acetylcysteine (NAC; 5 mmol/L), vitamin C (Vit C; 100 μmol/L), vitamin E (Vit E; 100 μmol/L), combined antioxidants (5 mmol/L NAC + 100 μmol/L Vit C + 100 μmol/L Vit E) or catalase (1 × 10⁶ U/L) for 2 h, or with folate (10 μmol/L) for 3 d before 1000 μmol/L HcyT treatment. At 6 h after HcyT treatment, cells were harvested and agarose gel electrophoresis of DNA extract from each group was performed. Lane M: DNA markers of molecular size at 100 base pair multimers. Lane C: HcyT-untreated control. Lane 1: HcyT + NAC. Lane 2: HcyT + Vit C. Lane 3: HcyT + folate. Lane 4: HcyT + catalase. Lane 5: HcyT + Vit E. Lane 6: HcyT + NAC + Vit C + Vit E.

DISCUSSION

The data in this study confirmed our previous finding that HcyT-induced apoptosis was rapid, and further established that ROS generation and caspase-3 activation preceded the maximal apoptotic damage in HcyT-treated cells (Fig. 1). Catalase pretreatment fully quenched the rise of HcyT-induced DCF intensity (Table 1) and completely prevented HL-60 cells from caspase-3 activation as well as apoptotic damage (Table 1, Figs. 2–4). In contrast, the increase of HE intensity in HL-60 cells was substantially lower than the rise of DCF during HcyT treatment (Fig. 1, Table 1), and the addition of superoxide dismutase did not protect cells from caspase-3 activation or from apoptotic DNA damage (31). These observations suggest that the generation of superoxide anions is not the mediator of apoptosis induced by HcyT, whereas H₂O₂ generation serves as a necessary messenger in the signaling transmission of HcyT-induced apoptosis. Similarly, the ROS-elicted damage due to homocysteine treatment was associated with DNA strand breaks and apoptosis in rat hippocampal neurons (44). The prevention of homocysteine-induced toxicity by catalase indicates that H₂O₂ acts as the mediator of oxidative injury (6,14,16,17). Our findings with those of others suggest that ROS generation, particularly H₂O₂, plays a key role in the apoptotic damage induced by homocysteine derivatives.

Our results demonstrated that the NAC, Vit C or Vit E pretreatment significantly inhibited HcyT-induced apoptosis. The strong antioxidant effect on H₂O₂ scavenging may largely account for their inhibition of HcyT-mediated cytotoxicity. The capacity of Vit C and Vit E to suppress caspase-3 activation coincided with their efficiency in inhibiting HcyT-induced apoptotic events including membrane PS exposure, DNA loss and DNA fragmentation. It was reported that Vit E can restore mitochondria function to inhibit cytochrome c release (45), which in turn modulates caspase-3 activation to affect apoptotic end point damage (46). NAC was also a potent inhibitor of hydrogen peroxide production and
caspase-3 activation in HcyT-treated cells. However, NAC pretreatment reduced only HcyT–promoted apoptotic membrane PS exposure, not DNA breakage. The reasons are presently unknown. The antioxidant activity of NAC completely prevented the induction of various DNA alterations in rat lung cells (47). Conversely, it was reported that NAC treatment induced DNA damage in HL-60 cells (48). The authors proposed that radicals other than hydrogen peroxide might be elicited during NAC treatment in the presence of metal ions, which might promote additional DNA damage (48). This possibility is further supported by our finding that preincubation of HcyT-treated cells with the combination of NAC, Vit C and Vit E can offset the DNA-damaging effect of NAC under HcyT–treated conditions, and completely prevent cells from apoptotic DNA fragmentation (Fig. 4).

In the present study, preincubation of HcyT-treated cells with folate did not ameliorate the apoptotic damage. The failure of folate to protect cells from apoptotic DNA fragmentation or membrane dysfunction may result from its inability to scavenge HcyT–elicited intracellular H2O2, and subsequently to suppress caspase-3 activation (Figs. 2–4). This finding is in agreement with that of Duthie and Hawdon (49) who showed that the folate supply had no effect on the reduction of oxidative DNA damage as indicated by oxidized pyrimidine levels in lymphocytes. Although folate had no H2O2 scavenging capacity and did not suppress caspase-3 activity, it demonstrated antioxidant behavior. Our data provided the direct evidence to support the hypothesis that folate is an effective free radical scavenger, as demonstrated by Joshi et al. (38) in an in vitro radical reaction system. Our data also confirmed the finding of Doshi et al. (50) that folate exhibited an antioxidant effect and reduced intracellular superoxide. The concentrations of folate necessary to reduce intracellular ROS was reported by Doshi et al. (50) to be in the 500 micromolar range; we reported 10 μM/L in this present study, whereas normal human plasma folate levels are at the nanomolar range (12,51). At micromolar concentrations, folate (10 μM/L), Vit C (100 μM/L) and Vit E (100 μM/L) had similar antioxidant capacities to scavenge superoxide, hydroxyl radicals and peroxynitrite. To our knowledge, this phenomenon has not been observed previously in intact cells. In circumstances in which ROS such as superoxide anions, hydroxyl radicals or peroxynitrite are the major contributors toward the etiology of diseases, folate supplementation may be protective. Further studies are required to elucidate folate’s antioxidant capabilities under various physiologic and pathologic conditions.

In conclusion, the present work demonstrated that preincubation of HL-60 cells with NAC, Vit C or Vit E significantly attenuated HcyT–induced apoptotic damage. The capacity of NAC, Vit C and Vit E to scavenge HcyT–induced hydrogen peroxide and to suppress caspase-3 activation correlates well with their efficiency to protect against HcyT–promoted apoptotic damage. Folate had no hydrogen peroxide-scavenging capacity and did not counteract HcyT–induced apoptosis, although it did exhibit antioxidant behavior toward superoxide anions, hydroxyl radicals and peroxynitrite.

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