Oxidant stress mechanism of homocysteine potentiating Con A-induced proliferation in murine splenic T lymphocytes

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Abstract

**Objective:** An elevated plasma homocysteine (Hcy) level is considered an independent risk factor for atherosclerosis. However, the mechanisms by which hyperhomocysteinemia induces atherosclerosis are only partially understood. The effect of Hcy on T lymphocyte proliferation and its mechanisms were examined in normal and hyperhomocysteinemia ApoE-knockout mice.

**Methods:** The mouse splenic T-cells were treated with Hcy, related compounds and/or antioxidants in the presence or absence of Concanavalin A (Con A). DNA synthesis, cell apoptosis, interleukin-2 level and production of reactive oxygen species (ROS) were measured.

**Results:** Hcy (0.3–3.0 mM) and related compounds with thiol (–SH), such as cysteine and glutathione significantly potentiated Con A-induced proliferation and partially inhibited apoptosis in T lymphocytes, but it had no direct effect on resting T lymphocyte. ApoE-knockout mice with hyperhomocysteinemia (the level of plasma Hcy was 20.3\(\pm\)2.9 vs. 2.6\(\pm\)0.6 mM in control group, \(P<0.05\)) had a significant promotion of T-cell proliferation in response to Con A. Hcy (0.3–3.0 mM) also increased the intracellular ROS. Radical scavengers reduced Hcy effect.

**Conclusions:** These data indicate that ROS generated by thiol (–SH) of Hcy auto-oxidation are involved in Hcy effect on Con A-induced T lymphocyte proliferation. These findings suggest a novel mechanism may be involved in chronic inflammatory progression of atherosclerosis with hyperhomocysteinemia.

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**Keywords:** Apoptosis; Atherosclerosis; Free radicals; Immunology; Infection/Inflammation; Leukocytes

1. Introduction

Homocysteine (Hcy) is the sulfur-containing amino acid formed during the metabolism of methionine. Hyperhomocysteinemia has been found in 20–30% of patients with premature atherosclerosis involving carotid, coronary and peripheral arteries [1]. Elevated plasma Hcy levels have been defined as an independent risk factor for coronary heart disease [2]. Yet the mechanism by which homocysteinemia induces atherosclerosis is still unclear.

Recent studies demonstrate that Hcy enhances endothelial dysfunction [3] and promotes the proliferation of vascular smooth muscle cells [4]. Substantial evidence suggests that it involves cellular immune system in atherogenesis. Atherosclerosis fulfills many criteria of a chronic inflammatory process. It is recognized that T lymphocytes accumulate in the lesions during the earlier stages of atherosclerosis, perhaps even preceding monocytes. In the advanced atherosclerotic plaque, T lymphocytes represent up to 20% of the cells, 10% of which are in an activated state [5]. However, the effects of Hcy on T lymphocyte function have not been investigated in normal and ApoE-knockout mouse with elevated Hcy.

The thiol (–SH) group of Hcy is readily oxidized and during oxidation, superoxide anion radical (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)) and hydroxyl radical (\(OH^-\)) are generated [6]. These oxygen-derived molecules are believed to account for Hcy-induced endothelial cytotoxicity and...
vascular smooth muscle cell proliferation [3,7]. Recently, it has been reported that the increase of reactive oxygen species (ROS) following various external stimuli at low concentration may function as cellular signaling intermediators and be associated with cellular proliferation [8]. Therefore, we hypothesized that there might be a relationship between Hcy stimulation, T lymphocyte proliferation and ROS production. The present study suggests that the ROS generated by thiol (–SH) auto-oxidation of Hcy might function as second messenger to promote Con A-induced proliferation in cultured mouse splenic T lymphocytes. The T-cell proliferation in response to Con A in ApoE-knockout mice with hyperhomocysteinemia is greatly increased. The hyperhomocysteinemia might enhance chronic inflammatory progression of atherosclerosis in concert with other stimuli.

2. Methods

2.1. Animals

The treatment of the laboratory animals and experimental protocols of the present study adhered to the guidelines of Peking University and were approved by the Institution Authority for Laboratory Animal Care. All experiments were carried out in healthy, male Balb/C mice (6–8 weeks old, 18–22 g). Animals were obtained from the animal laboratory of the Health Science Center of Peking University. They were housed in wire-mesh cages at 22 °C ambient temperature and maintained on food and water ad libitum with a 12-h light/12-h dark cycle for 1–2 weeks prior to all experiments. The ApoE-knockout mice were fed with a diet of methionine (400 mg/kg per day) for 6 weeks by which the hyperhomocysteinemia was induced as previously reported [9].

2.2. Preparation of T lymphocytes

Cells were obtained from spleen of male Balb/C mouse by techniques previously described [10]. T lymphocytes were purified by negative selection as described [11]. Briefly, single cell suspensions of spleen were centrifuged. After lysis of red blood cells, single cell suspensions were incubated for 2 h at 37 °C to allow adherence of macrophages. The non-adherent cells were collected and incubated in Petri dishes coated with mouse anti-IgG for 1 h at room temperature. The non-antibody-conjugated cells (T lymphocytes) were collected RPMI-1640 medium at 37 °C. The purity of the T-cells was shown to be 85% determined by the percentage of CD3⁺ cells in flow cytometry (Becton Dickinson). Cell viability was evaluated by trypan blue exclusion. Only cell preparations with a 95% viability or greater were used.

2.3. T-cell proliferation assay

Cell proliferation was determined by [³H]thymidine incorporation, an index of DNA synthesis. Cells were plated in triplicate samples in flat bottomed 96-well culture plates at a density of 2×10⁵ cells/well in RPMI-1640 medium containing 5% fetal calf serum. Cultures were treated for 72 h with Hcy (0.1–10 mM) in the presence or absence of 2 or 4 µg/ml Con A, or with related compounds including homoserine, homocysteine, cysteine, glutathione and methione, or 30-min pretreatment with antioxidants including catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX) and 1% DMSO before exposure to Hcy. [³H]Thymidine (0.2 µCi/well) was added during the last 6 h. The final total volume per well was 0.2 ml. The cells were then harvested onto glass fiber filters. After drying under 80 °C for 2 h, the radioactivity was determined by liquid scintillation counting (Beckman, USA) and presented as counts per minute. The data are expressed as the means±S.E.M. of the percentage of proliferation as compared to control group.

2.4. Measurement of plasma Hcy levels by EIA

Immediately following removal of blood of the ApoE-knockout mice with or without a diet of methionine, the samples were placed in EDTA-containing test tubes. Following centrifugation the plasma fractions were transferred to other polypropylene tubes and stored at −20 °C for not more than 2 months before analysis for Hcy levels by Hcy EIA reagent kit (Bio-Rad, CA, USA).

2.5. IL-2 assay

T-cells were incubated with Con A (2 µg/ml) and Hcy (1.0 mM) for different time periods. The culture supernatants were harvested and the interleukin-2 (IL-2) level was measured using an ELISA assay kit (Genzyme, Cambridge, MA). The assay was performed according to the manufacturer’s instructions.

2.6. Flow cytometric analysis

Cells (3×10⁶) were incubated with various concentrations of Hcy (0.1–10 mM) with or without Con A (2 µg/ml) for 60 h. The cells were collected and washed with PBS three times. Cells were incubated in the dark in a solution containing 0.1% citrate, 0.1% Triton X-100 and propidium iodide (PI) 50 µg/ml before measurement. The 488-nm line of an argon laser provided excitation of PI. The red fluorescence of PI was analyzed on a FACSScan (Becton Dickinson) using the CellOuest software. Results are shown as histograms of PI fluorescence. Forward and right angle scatter was used to define the cellular population. After excluding cellular debris, the hypodiploid
signals were determined as apoptotic cells. A total of 10,000 measurements were conducted per experiment.

2.7. TUNEL assay

Cells (1.0×10^6) were incubated with various concentrations of Hcy (0.1–10 mM) with or without Con A (2 or 4 μg/ml) for 60 h. Cells were then fixed in 4% methanol-free formaldehyde, permeabilized with 0.2% Triton X-100 in PBS, and labeled with fluorescein-tagged deoxyuridine triphosphate (dUTP) by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method according to TUNEL kits (Promega, Madison, WI). To quantify an apoptotic event, the percentage of TUNEL-positive cells to total cell population was calculated by counting all cells from five random microscopic fields at a magnification of ×100.

2.8. Measurement of intracellular ROS generation

Hcy (0.1–10 mM) was added to the cells together with 2′,7′-dichlorofluorescin diacetate (DCFH-DA) 15 μM for 25 min. The determination of intracellular oxidant production was based on the oxidation of DCFH-DA by intracellular ROS, resulting in the formation of the fluorescent compound 2′,7′-dichlorofluorescin (DCF) [12]. DCF fluorescence was monitored with a confocal laser-scanning microscope (Leica, Germany).

2.9. Measurement of LDH release

T lymphocytes were treated with Hcy (0.1–10 mM) in the presence of Con A (2 μg/ml) for 72 h. Cell suspensions were collected for determination of LDH and LDH activity was measured by spectrophotometric enzyme activity method [13]. LDH activity was expressed as units per liter medium.

2.10. Chemicals

D,L-Hcy, D,L-cysteine, D,L-homoserine, D,L-homocysteine, glutathione (reduced form), GPX and Con A were purchased from Sigma (St. Louis, MO). 2′,7′-Dichlorofluorescin diacetate was purchased from Molecular Probes (Eugene, OR). RPMI-1640 was purchased from Gibco (Grand island, NY). Mouse IL-2 ELISA kit was purchased from Genzyme (Cambridge, MA). TUNEL kit was purchased from Promega (Madison, WI). Catalase, SOD and other chemicals were purchased from Chinese Chemical, Beijing, China.

2.11. Statistical analysis

The results are expressed as the mean±S.E.M. The number of experimental animals used for each group is presented as n value in the figure legends. The data were analyzed using one-way ANOVA and further analyzed using the Student’s–Newman–Keuls test for multiple comparisons between treatment groups. A P-value of <0.05 indicates significant differences between treatment group means.

3. Results

3.1. Effects of Hcy on Con A-induced T lymphocyte proliferation

To test whether Hcy affected T lymphocyte proliferation, cells were cultured in 5% FBS RPMI-1640 with Hcy in the presence or absence of Con A 2 μg/ml for 72 h. As shown in Fig. 1A, Hcy (0.3–1.0 mM) caused a significant

![Fig. 1. Effect of Hcy on T lymphocyte proliferation. Cells were incubated with Hcy 0.1–10 mM in the presence (A) or absence (B) of Con A 2 μg/ml for 72 h. T lymphocyte proliferation was assayed by [3H]TdR incorporation. (A) The data represent the percentage of [3H]TdR incorporation of Con A alone. *P<0.05, compared with Con A alone; n=5 for each group. (B) The data are expressed as cpm (n=5).](https://academic.oup.com/cardiovascres/article-abstract/53/4/1035/412090)
potentiation of Con A-induced proliferation in T lymphocytes. Compared to Con A 2-μg/ml-induced \([^{3}H]TdT\) incorporation, it caused increases of 1204 and 2574%, respectively, and compared to Con A 4-μg/ml-induced \([^{3}H]TdT\) incorporation, increases of 1732 and 3361%, respectively. Hcy at 1.0 mM caused a maximum of T-cell proliferation in these cells. When the concentration of Hcy was in excess of 3.0 mM, potentiating effects of Hcy declined (Fig. 1A). In addition, Hcy (0.1–10 mM) had no direct effect on resting T lymphocyte proliferation (Fig. 1B).

### 3.2. Effect of Hcy on Con A-induced T lymphocyte apoptosis

To determine whether the increase of Con A-induced T lymphocyte proliferation by Hcy was produced by inhibiting its apoptosis, we tested cells after 24, 48, 60 and 72 h of stimulation for the presence of apoptotic cells and found that 60-h exposure to Hcy 1.0 mM began to significantly inhibit Con A-induced T lymphocyte apoptosis (data not shown). T-cells were then cultured with Hcy (0.3–10 mM) for 60 h in the presence or absence of Con A. As seen in Fig. 2A, Hcy (0.3–1.0 mM) could inhibit Con A-induced T lymphocyte apoptosis from 48.5 to 37.8 and 36.1%, respectively, as observed by flow cytometry. However, when the concentration of Hcy exceeded 3.0 mM, it could not further inhibit the Con A-induced apoptosis in the T lymphocytes. The Hcy had no direct effect on resting T lymphocyte apoptosis (data not shown). Hcy (0.3–1.0 mM) could also inhibit Con A 2-μg/ml-induced TUNEL-positive cells from 36.1 to 26.3 and to 20.9%, respectively. However, increasing the concentration of Hcy to 3.0 and 10 mM could not further inhibit the Con A-induced TUNEL-positive cells (Fig. 2B). The Hcy had no direct effect on resting T lymphocyte apoptosis (data not shown).

### 3.3. T lymphocyte proliferation or apoptosis in ApoE-knockout mice with hyperhomocysteinemia

To further investigate the effect of Hcy on T lymphocyte proliferation in vivo, hyperhomocysteinemia was induced after 6 weeks with a diet of methionine of 400 mg/kg per day. The level of plasma Hcy was 20.3±2.9 vs. 2.6±0.6 μM in control group \((P<0.05, n=4)\). Splenic T lymphocytes were incubated in the presence of Con A (2 or 4 μg/ml) for 72 h. As shown in Fig. 3A, Con A-induced proliferative response of T-cells obtained from ApoE-knockout mice with hyperhomocysteinemia was greatly enhanced as compared with control (no methionine treatment). In addition, Con A 4-μg/ml-, but not 2-μg/ml-, induced apoptotic response of T-cells obtained from ApoE-knockout mice with hyperhomocysteinemia was significantly inhibited as compared with control with no methionine treatment (Fig. 3B).

### 3.4. Effect of Hcy on Con A-induced IL-2 production in T lymphocytes

It is reported that the proliferation of T-cells in response to mitogens in vitro is largely dependent on the production of IL-2 [14]. To test whether IL-2 was involved in the potentiating effect of Hcy on Con A-induced T lymphocyte proliferation, the cells were incubated with Con A alone or plus Hcy 1.0 mM, which potentiated the Con A-induced T lymphocyte proliferation to a maximum at different time points. Con A 2 μg/ml caused a time-dependent increase of IL-2 production at 48, 60 and 72 h. However, at each...
Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structural formula</th>
<th>Cell proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine</td>
<td>HSCH₂CH₂(CH(NH₂)COOH</td>
<td>++</td>
</tr>
<tr>
<td>Homoserine</td>
<td>HOCH₂CH₂(CH(NH₂)COOH</td>
<td>–</td>
</tr>
<tr>
<td>Cysteine</td>
<td>HSCH₂(NH₂)COOH</td>
<td>+</td>
</tr>
<tr>
<td>Methionine</td>
<td>CH₃-S-CH₂CH₂(CH(NH₂)COOH</td>
<td>–</td>
</tr>
<tr>
<td>Homocystine</td>
<td>HOOC(NH₂)CH₂CH₂S-S-CH₂CH₂(CH(NH₂)COOH</td>
<td>–</td>
</tr>
<tr>
<td>Glutathione</td>
<td>γ-Glu-Cys-Gly</td>
<td>+</td>
</tr>
</tbody>
</table>

+, Capable of promoting Con A-induced T lymphocyte proliferation; –, not capable of promoting Con A-induced T lymphocyte proliferation.

Con A-induced proliferation to 778.3±127.3, 201.3±39.9 and 181±32.7%, respectively. In comparison with D,L-cysteine and glutathione, D,L-Hcy was three to four times more potent. However, other compounds without thiol (–SH) had no effects at all on Con A-induced proliferation in T lymphocytes.

3.6. Effect of Hcy on intracellular ROS levels

To determine whether Hcy induced more ROS production in Con A-stimulated T lymphocytes, we measured the ROS level using the redox sensitive fluorescent dye DCFH-DA, a process that has been demonstrated to depend on the intracellular production of ROS [15]. Our data demonstrated that the peak fluorescence intensity occurred within 25 min after adding the Hcy. T-cells were cultured with Hcy (0.3–10 mM) for 25 min in the presence of Con A. As shown in Fig. 4, Hcy (0.3–3.0 mM) could increase Con A-induced ROS production from 16.0±5.49 to 35.06±4.41 U with 1.0 mM Hcy. However, when the concentration of Hcy exceeded 3.0 mM, enhancement of intracellular ROS declined.

3.7. Inhibitory effect of antioxidants on Hcy-induced T lymphocyte proliferation

To test further the role of oxygen radicals in Con A- and/or Hcy-induced proliferation, cells were pretreated for 30 min with antioxidants such as catalase 250 µg/ml (a scavenger of H₂O₂), GPX 0.1 U/ml (a scavenger of H₂O₂), SOD 500 U/ml (a scavenger of O₂⁻) and 1% DMSO (a scavenger of OH⁻), respectively. The cells were then stimulated with Hcy 1.0 mM in the presence of Con A for 72 h. As seen in Fig. 5, these scavengers of ROS could significantly inhibit Con A 2-µg/ml- plus Hcy 1.0-mM-induced T lymphocyte proliferation from 593.1±87.6 to 260.8±40.5, 343.4±68.5, 101.7±29.3 and 234.7±90.4%, respectively. However, these antioxidants had no inhibitory effects on Con A alone-induced T lymphocyte proliferation.
4. Discussion

This study shows for the first time that Hcy potentiated Con A-induced proliferation and inhibited cellular apoptosis in mouse spleen T lymphocytes. ApoE-knockout mice with hyperhomocysteinemia had an enhanced susceptibility of mitogen-induced T lymphocyte proliferation comparing to control group. Related compounds with thiol (–SH), such as cysteine and glutathione, also promoted mitogen-induced T lymphocyte proliferation. Hcy increased the production of ROS from T lymphocytes. The potentiating effect of Hcy on Con A-induced T lymphocyte proliferation was significantly reduced by antioxidants. However, IL-2 was not involved in the action of Hcy. These data suggest that ROS generated by thiol (–SH) auto-oxidation is involved in T lymphocyte proliferation induced by Hcy. Hyperhomocysteinemia may be involved in the pathogenesis of atherosclerosis by enhancing T-cell

3.8. Effect of Hcy on LDH release from Con A-activated T lymphocytes

To determine the effect of Hcy on LDH release, the lymphocytes were incubated with Con A and/or Hcy at a concentration of 0.1–10 mM for 72 h. Hcy (3.0–10 mM) significantly caused a concentration-related increase in LDH release as compared with Con A alone (data not shown).

Fig. 4. Effect of Hcy on DCF formation in T lymphocytes. Cells were incubated with Con A 2 μg/ml and Hcy in the presence of DCFH-DA, a dye that allows the monitoring of intracellular ROS production. DCF fluorescence was monitored for 25 min. Results are means±S.E.M. *P<0.05, compared with Con A alone; n=4 for each group.

Fig. 5. Inhibitory effect of antioxidants on Hcy-induced proliferation in T lymphocytes. Cells were pretreated with antioxidants, catalase (Cata, 250 μg/ml), GPX 0.1 U/ml, SOD 500 U/ml or 1% DMSO for 30 min, and then stimulated by Hcy 1.0 mM in the presence of Con A for 72 h. Cell proliferation was assayed by [3H]Tdr incorporation. The data represent the percentage of [3H]Tdr incorporation in control (Con A alone). *P<0.05, compared with Con A plus Hcy; n=4 for each group.
susceptibility of T-cell proliferation and apoptosis in the presence of Con A. Therefore, these results suggest that Hcy acts only as a modulator to promote mitogen-induced proliferation in T lymphocytes.

It is demonstrated that apoptosis counterbalances the effect of cell proliferation by mitogenic stimulation [20,21]. Our results showed that elevated Hcy (0.3–1.0 mM), which potentiated Con A-induced T lymphocyte proliferation, could partially inhibit its apoptosis both in vitro and in vivo. This suggests that the potentiating effect of Hcy on Con A-induced T lymphocyte proliferation may be mediated in part through inhibition of cellular apoptosis. However, additional mechanisms may also be involved in Hcy-induced T lymphocyte proliferation since Hcy enhances proliferation more than it inhibits apoptosis in these cells.

It is reported that thiol (–SH) is involved in regulation of lymphocyte proliferation [22]. The present study demonstrated that compounds related to thiol (–SH), such as cysteine and glutathione, could promote Con A-induced proliferation in T lymphocytes, while other compounds without thiol (–SH) had no such effects. This suggests that thiol (–SH) plays a key role in the action of Hcy. In addition, we observed that the effect of Hcy was the strongest, compared with cysteine and glutathione. This difference might be due to its chemical property. The ammonium group of cysteine (HSCH_2CHNH_2COOH) exerts a strong electron withdrawing effect on the thiol group. However, with Hcy (HSCH_2CHNH_2COOH), because it has one more methylene group than cysteine, the withdrawing effect of ammonium group on the thiol group will be weaker. Therefore, the thiol of Hcy is more active and can be more easily auto-oxidated than that of cysteine.

There is now growing evidence that ROS at low concentrations play some physiological roles including activation and proliferation of lymphocytes [23,24]. To clarify the mechanism that ROS act as a mediator in the Con A-activated T lymphocytes proliferation potentiated by Hcy, we first assessed the production of intracellular ROS using the redox sensitive fluorescent dye DCFH-DA.

We found that 0.3–3.0 mM Hcy, the concentration which potentiated Con A-induced T lymphocyte proliferation, could increase the intracellular oxidation of DCFH, the latter being produced equally by the intracellular superoxide anion (O_2^-), hydroxyl ion (OH^-) or hydrogen peroxide (H_2O_2) [12]. However, when the concentration of Hcy exceeded 3.0 mM, it could not further increase the level of intracellular ROS. This might be due to its toxic injury of cells as determined by LDH release. In addition, the potentiating effect was prevented by pretreatment with different antioxidants that are well known as radical scavengers. For example, SOD catalyses the dismutation of superoxide anion radicals, catalase and GPX catalyse the reduction of hydrogen peroxide and 1% DMSO scavenges hydroxyl radicals (OH^-). However, these scavengers by themselves had no effect on Con A-induced T lymphocyte proliferation as previously reported [25]. These data suggest that the production of intracellular ROS induced by thiol (–SH) auto-oxidation is involved in the effect of Hcy-induced T lymphocyte proliferation. The types of ROS include super oxide anion (O_2^-), hydroxyl ion (OH^-) and hydrogen peroxide (H_2O_2). Other groups have also reported that Hcy-induced damage to the vascular endothelium and the promotion of vascular smooth muscle cell proliferation are secondary to oxygen radicals generated by oxidation of the thiol (–SH) group of Hcy [7,26].

IL-2 is a major growth factor of activated T lymphocytes during a developing immune response. We found that Con A at a concentration of 2 μg/ml caused time-dependent IL-2 production, which is consistent with the report of other groups [27]. However, at each time point, Hcy could not further increase Con A-induced IL-2 production. These data suggest that IL-2 is not involved in Hcy-potentiated T lymphocyte proliferation.

Atherosclerosis appears to be associated with Hcy concentrations of 0.2–0.25 mM and plasma concentrations up to 0.5 mM have been found in patients suffering from homocysteinuria [2]. In the present study, Hcy significantly increased Con A-induced T lymphocyte proliferation at 0.3 mM as threshold concentration, which is similar to those observed in clinical patients. In local plaque, the concentration of Hcy is still unknown; it may be higher than that of plasma. In atherosclerotic plaque, the activation of T-cells induces the secretion of cytokines, such as IFNy, IL-2, IL-6 and so on [28], which have profound effects on the progression of lesions, including oxLDL uptake, adhesion molecule expression and smooth muscle proliferation [29,30]. Our results suggest that Hcy may promote the function of activated T lymphocyte as a modulator and enhance susceptibility in T lymphocyte of ApoE-knockout mice, an animal model of atherosclerosis. The exact effect of Hcy on activated T lymphocyte in plaque of atherosclerosis in vivo is not yet known. The role of hyperhomocysteinemia in animals and patients with atherosclerosis is currently being investigated in our laboratory.

In conclusion, we demonstrated that Hcy significantly potentiated activated T lymphocyte proliferation by partially inhibiting its apoptosis in mouse splenic T lymphocytes. ApoE-knockout mice with hyperhomocysteinemia showed a significant enhancement of T lymphocyte proliferation in response to T-cell mitogen. The thiol (–SH) of Hcy and its oxidative products are involved in the action of Hcy. Elucidation of Hcy effect on T lymphocyte function might be helpful to explain the mechanism of hyperhomocysteinemia patients with atherosclerosis.

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