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Short Communication

Homocysteine Induces DNA Synthesis and Proliferation of Vascular Smooth Muscle Cells by a Hydrogen Peroxide-Independent Mechanism

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ABSTRACT

Elevated plasma levels of homocysteine have been identified as an important and independent risk factor for cerebral, coronary, and peripheral atherosclerosis, although the mechanisms are unclear. Homocysteine has been shown to promote cell proliferation and induction of the gene transcription factor c-*fos* in vascular smooth muscle cells. Earlier reports have suggested that homocysteine exert its effect via hydrogen peroxide (H_2O_2) produced during its metabolism. To evaluate the contribution of homocysteine to the pathogenesis of vascular diseases, we examined whether the effect of homocysteine on vascular smooth muscle cell growth is mediated by H_2O_2. We observed that 1.0 m*M* homocysteine induces DNA synthesis by 1.5-fold and proliferation of vascular smooth muscle cells two-fold in the presence of peroxide scavenging enzyme, catalase (2,600 U/ml). Our results suggest that homocysteine induces smooth muscle cell growth by an H_2O_2-independent pathway and that the effects of homocysteine may sum together with the known initiating events produced by oxidative stress and accelerate the progression of atherosclerosis. Antiox. Redox Signal. 1, 365–369.

INTRODUCTION

HOMOCYSTEINE, A SULFUR-CONTAINING AMINO ACID, is an intermediate metabolite of methionine. Elevated plasma levels of homocysteine have been identified as an important and independent risk factor for atherosclerosis. Epidemiological studies in the general population have suggested associations between elevated circulating homocysteine levels and premature coronary, cerebral, and peripheral atherosclerosis (Clarke *et al.*, 1991; Taylor *et al.*, 1991; Nygard et al., 1995; Miner *et al.*, 1997; Stehouver *et al.*, 1998; Welch *et al.*, 1997). In baboons, it has been demonstrated that blood infusion of homocysteine results in human atheroma-like myointimal lesions (Harker *et al.*, 1983). In another study, homocysteine has been shown to induce atherosclerosis-like alterations of the aorta in normotensive and hypertensive rats (Matthias *et al.*, 1996). However, the mechanisms by which homocysteine promotes atherosclerosis formation are not clearly defined.

Because smooth muscle cell migration and proliferation are hallmarks of atherosclerosis, it was suggested that homocysteine may contribute to the clinical presentation of this vascular disease through such a process. Tsai *et al.* (1994) have shown that homocysteine can stimulate vascular smooth muscle cell growth and DNA synthesis and inhibit endothelial cell proliferation. In these experiments, homocysteine

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increased both cyclin D_1 and cyclin A mRNA expression. It has been reported that homocysteine stimulates aortic cyclin dependent kinase, and that, as a consequence, aortic cells would proliferate (Lubec *et al.*, 1996). On the other hand, homocysteine has recently been shown to act as an inducer of c-*fos* and c-*myc* (Dalton *et al.*, 1997). Earlier reports by Starkebaum and Harlan (1986) have suggested that high concentrations of homocysteine damage endothelial cells *in vitro* through the oxidative stress generated by hydrogen peroxide (H_2O_2) production during the oxidation of homocysteine to homocystine. However, the possible role of H_2O_2 in homocysteine signaling is yet unclear.

In the present study, to evaluate the pathological mechanisms involved in homocysteine-induced atherosclerosis, we have examined the effect of homocysteine on DNA synthesis and proliferation in vascular smooth muscle cell cultures. The results show that homocysteine induces smooth muscle cell proliferation by a mechanism that does not involve H_2O_2. On the other hand, the growth-promoting effect of homocysteine on vascular smooth muscle cells represents an important event that may, at least in part, explain homocysteine-induced atherosclerosis.

MATERIALS AND METHODS

Growth media and serum for cell culture were obtained from Gibco Laboratories. A7r5 rat aortic smooth muscle cells were obtained from the American Type Culture Collection (Rockville, MD). DL-homocysteine and catalase were from Sigma Chemical Co. (St. Louis, MO). [*Methyl*-^{3}H]thymidine (25 Ci/mmol) was purchased from Amersham International. All other chemicals used were of the purest grade commercially available.

Cell culture

A7r5 cells are maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS). Cells were cultured at 37°C in a humidified 5% CO_2 athmosphere. Cells in a subconfluent state are made quiescent by incubation in DMEM containing 0.2 % FCS for 48 hr. Then, cells are washed with phosphate-buffered saline (PBS) and treated as indicated in the figure legends. Viability is determined by the trypan blue dye method.

Determination of cell number (cell proliferation)

A7r5 cells made quiescent in six-well plates were restimulated to grow by addition of 10% FCS. DL-Homocysteine and/or catalase were added to cells at the indicated concentrations. Cell number was determined 48 hr later by using an hemocytometer.

Measurement of [^{3}H] thymidine incorporation (DNA synthesis)

The cells made quiescent in 12-well plates were restimulated to grow by addition of 10 % FCS, homocysteine, and catalase for 20 hr. Plates were pulsed with [^{3}H]thymidine (1 μCi/well) during the last 2 hr of incubation. After labeling, cells were washed twice with PBS, fixed for 20 min with ice-cold 5% trichloroacetic acid, and solubilized in 0.1 *M*

TABLE 1. EFFECT OF HOMOCYSTEINE ON VASCULAR SMOOTH MUSCLE CELL PROLIFERATION

Homocysteine concentrations	*Cell number* $\times$ 10^3/*ml*
10% FCS (control)	973.3 ± 190.5
10% FCS + 0.10 m*M* homocysteine	995.6 ± 212.3[a]
10% FCS + 0.25 m*M* homocysteine	1128.9 ± 230.2[a]
10% FCS + 0.50 m*M* homocysteine	975.0 ± 251.6[a]
10% FCS + 1.0 m*M* homocysteine	657.1 ± 381.8[b]

Compared to control [a]$p > 0.50$, [b]$p < 0.05$ but viability was <50%.
The values represented as mean ± SD of 10 separate experiments ($n = 10$).

TABLE 2. ROLE OF DIFFERENT CATALASE CONCENTRATIONS ON VASCULAR SMOOTH MUSCLE CELL PROLIFERATION

Catalase concentrations	*Cell number × 10^3/ml*
10% FCS (control)	510.0 ± 212.1
10% FCS + catalase 26 U/ml	490.0 ± 127.3
10% FCS + catalase 260 U/ml	475.0 ± 35.4
10% FCS + catalase 2600 U/ml	130.0 ± 42.4[a]

Compared to control [a]$p < 0.05$.
The values represented as mean ± SD of 10 separate experiments ($n = 10$).

NaOH/2% Na_2CO_3 /1% SDS. The radioactivity incorporated into the acid insoluble material was determined in a liquid scintillation analyzer. [^{3}H]Thymidine incorporation was expressed as cpm/mg protein. Protein concentration was measured by the method of Lowry *et al.* (1951).

Statistics

The values are expressed as means ± SD from three measurements. The significance of the difference between mean values was analyzed by Student's *t*-test.

RESULTS

To determine if homocysteine can induce vascular smooth muscle cell proliferation, the cells were serum starved for 48 hr and restimulated to growth with 10% FCS in the presence of increasing homocysteine concentrations. As shown in Table 1, increasing the homocysteine concentration from 0.1 m*M* to 1.0 m*M* resulted in no significant effect on vascular smooth muscle cell proliferation. However, the addition of homocysteine in 1.0 m*M* concentration produced a toxic effect on vascular smooth muscle cells, as assessed by trypan blue exclusion.

We estimated the effect of homocysteine on vascular smooth muscle cell proliferation in the presence of catalase. As the activity of added catalase was increased, cell proliferation was inhibited (Table 2.). In the presence of 2,600 U/ml catalase, a complete block of growth factor-induced rise in H_2O_2 and of cell growth stimulation by FCS were observed. Under these conditions, no other effects of catalase could be seen on vascular smooth muscle cells and especially on the viability as assessed by trypan blue exclusion. [^{3}H]Thymidine incorporation was studied as a measure of DNA synthesis. It was evaluated after 20 hr of incubation with FCS, a time-point that represents a peak in [^{3}H]thymidine incorporation (data not shown). FCS induced-[^{3}H]thymidine incorporation was reduced in the presence of increased amounts of catalase (Table 3).

To establish whether H_2O_2 has a role in homocysteine-initiated signal transduction, vascular smooth muscle cells were treated with

TABLE 3. EFFECT OF DIFFERENT CATALASE CONCENTRATIONS ON [^{3}H] THYMIDINE INCORPORATION IN VASCULAR SMOOTH MUSCLE CELLS

Catalase concentrations	*cpm/mg protein*
10% FCS (control)	17,533.5 ± 2,509.5
10% FCS + catalase 26 U/ml	15,554.0 ± 3,440.8
10% FCS + catalase 260 U/ml	14,529.5 ± 4,384.8
10% FCS + catalase 2600 U/ml	5,662.0 ± 749.5[a]

Compared to control [a]$p < 0.05$.
The values represented as mean ± SD of 5 separate experiments (n = 5).

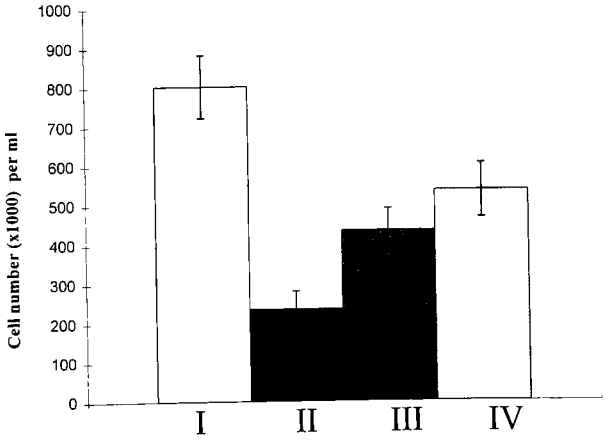

FIG. 1. Effect of homocysteine on vascular smooth muscle cell proliferation. Serum-deprived quiescent cells were stimulated to grow by FCS in the presence of 2,600 U/ml catalase and different homocysteine concentrations. After 48 hr, the cell number was measured as described in Materials and Methods. Viability was >95%. Results are expressed as the mean ±SD of 10 independent experiments. The bars represent data from 10% FCS (bar I), 10% FCS + catalase (bar II), 10% FCS + catalase + 0.25 m*M* homocysteine (bar III), 10% FCS + catalase + 1.0 m*M* homocysteine (bar IV).

FCS and homocysteine in the presence of 2,600 U/ml catalase. As shown in Fig. 1, homocysteine increased proliferation of the cells in the presence of catalase in a dose-dependent manner. At 1.0 m*M* homocysteine concentration, the response was maximum both in terms of increased cell proliferation as well as [^{3}H]thymidine incorporation (Fig 2.).

DISCUSSION

The mechanism by which homocysteine induces coronary atherosclerotic lesions is unknown. The development of a complicated atherosclerotic plaque in humans involves proliferation of smooth muscle cells. It is unclear, however, whether the effects on DNA synthesis and cell proliferation, are produced by homocysteine itself or by some of its metabolites. How these effects are mediated remains obscure. Among others, the role of free radicals and H_2O_2 produced through the oxidation of homocysteine to the thiolactone have been suggested (Starkebaum and Harlan, 1986). Furthermore, it has been demonstrated that signal transduction pathways induced by the growth factor, platelet-derived growth factor, requires the generation of H_2O_2, which may act as signaling molecule for smooth muscle cell growth (Sundearesan *et al.*, 1995).

Studies have shown that the action of homocysteine on smooth muscle cells is associated with the induction of genes important for cell proliferation, *e.g.*, c-*fos* and c-*myc* (Dalton *et al.*, 1997). Tsai *et al.* (1996) have shown that mRNA levels of cyclins D_1 and A are elevated by homocysteine. It was recently demonstrated that homocysteine activates the ERK2 signal transduction pathway and originates growth-promoting signals in smooth muscle cells (Brown *et al.*, 1998). To test whether H_2O_2 was an early signal for proliferation in vascular smooth muscle cells, we used the peroxide-scavenging enzyme catalase. This enzyme rapidly degrades H_2O_2 to water and molecular oxygen. Under the conditions of this study, an increase in the amount of added catalase prevented the stimulation of cell proliferation and DNA synthesis. These results are similar to data reported by Sundaresan *et al.* (1995). In this study, we have shown that homocysteine produces a two-fold stimulation of cell growth and 1.5-fold increase of DNA synthesis in catalase-treated vascular smooth muscle cells. This line of evidence suggests that the effect of homocysteine was mediated via a H_2O_2-independent pathway.

In conclusion, the data shown in this study and similar ones present in the literature (Tsai *et al.*, 1996; Dalton *et al.*, 1997; Brown *et al.*, 1998) strongly suggest that the mitogenic signals of homocysteine in vascular smooth muscle cells are alternative to those produced by H_2O_2. By promoting the proliferation of vascular smooth muscle cells via a pathway alternative to H_2O_2, the effects of homocysteine may sum together with the known initiating events produced by an oxidative stress and accelerate the progression of atherosclerosis.

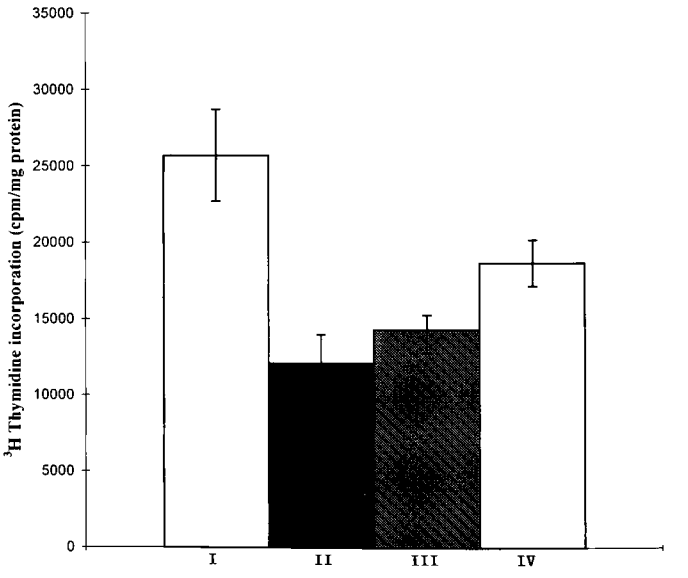

FIG. 2. Effect of homocysteine on DNA synthesis in vascular smooth muscle cells. Quiescent cells were stimulated to grow by FCS in the presence of 2,600 U/ml catalase and different homocysteine concentrations for 20 hr. The cells were pulsed with [^{3}H]thymidine during the last 2 hr of incubation. [^{3}H]Thymidine incorporation was determined as described in Materials and Methods. Results are expressed as the mean ±SD of 10 independent experiments. The bars represent data from 10% FCS (bar I), 10% FCS + catalase (bar II), 10% FCS + catalase + 0.25 m*M* homocysteine (bar III), 10% FCS + catalase + 1.0 m*M* homocysteine (bar IV).

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ABBREVIATIONS

FCS, Fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; H_2O_2, hydrogen peroxide; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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