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Homocysteine as a modulator of platelet-derived growth factor action in vascular smooth muscle cells: a possible role for hydrogen peroxide

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1 Homocysteine is an independent risk factor for cardiovascular disease. The mechanisms by which elevated plasma concentrations of homocysteine are related to the pathogenesis of atherosclerosis are not fully understood. Therefore, we examined the effect of homocysteine on cell replication of rat cultured vascular smooth muscle cells (VSMCs) at concentrations similar to those observed in clinical studies.

2 The incorporation of [^{3}H]-thymidine was used as a marker of mitosis. Homocysteine (250–500 μM) was a weak mitogen as compared to platelet-derived growth factor-BB (PDGF-BB, 1 nM) and serum (10%), but it potentiated the mitogenic effect of PDGF-BB four fold at 500 μM. This enhancement of mitogenesis was blunted by the addition of the scavenging enzyme catalase or the antioxidant N-acetyl-L-cysteine.

3 Furthermore, stimulation of VSMC with homocysteine (25–500 μM) decreased the glutathione peroxidase activity of the cells to 50% of control at 500 μM. Inversely, homocysteine enhanced the superoxide dismutase (SOD) activity to 137% of control at 500 μM, but it had no effect on the catalase activity.

4 Homocysteine decreased the activity of bovine purified liver cytosolic glutathione peroxidase in a time- and dose-dependent manner. The maximum decrease was 50%.

4 In summary, homocysteine has a weak mitogenic effect on VSMC, but it dramatically enhances the mitogenic response of PDGF-BB, presumably by disturbing the activity of antioxidant enzymes.

Keywords: Homocysteine; platelet derived growth factor-BB; superoxide dismutase; catalase; glutathione peroxidase; vascular smooth muscle cell; mitosis; atherosclerosis

Introduction

The conventional risk factors for coronary artery disease include smoking, hyperlipidaemia, hypertension, diabetes mellitus and a positive family history. However, many patients have precocious atherosclerosis without having any of these standard risk factors (Stampfer & Malinow, 1995). An elevated level of homocysteine, an intermediate metabolite of methionine, has been identified as an important and independent risk factor for atherosclerosis (Harker *et al*., 1974; Clarke *et al*., 1991). Normally the plasma level is about 10 μM. Moderate hyperhomocysteinaemia was found in 20–30% of patients with coronary and peripheral vascular diseases (Malinow *et al*., 1993). Plasma concentrations up to 500 μM have been found in patients who suffer from homocystinuria. Homocystinuria is an inborn error of metabolism mostly due to deficiency of cystathione β-synthase, but deficiencies in 5, 10-methylenetetrahydrofolate reductase and methionine synthase have been found as well (Ueland & Refsum, 1989). Patients with severe homocystinuria may develop atherosclerosis (Malinow *et al*., 1990), vascular occlusion (Malinow, 1990) and thromboembolic events (McDonald *et al*., 1964) at an early age.

Proliferation of vascular smooth muscle cells (VSMCs) is one of the key events in the development of atherosclerotic lesions (Fuster *et al*., 1992). Recently, Tsai *et al*. (1996) have examined the direct effect of homocysteine on VSMC proliferation and demonstrated a growth-promoting effect of homocysteine on VSMC, but the precise mechanism is unknown.

Recent evidence suggests that reactive oxygen species (ROS) may function as second messengers in cyutokine (interleukin-1 and tumour necrosis factor-α)-, and some growth factor-mediated intracellular signal transduction pathways (Chen *et al*., 1995). In particular, reactive oxygen species have been shown to stimulate VSMC growth and protooncogne expression (Gadiparthi & Bradford, 1992). Maitrayee *et al*. (1995) demonstrated that signal transduction induced by platelet-derived growth factor (PDGF)-BB requires the generation of hydrogen peroxide (H_2O_2). Therefore, there may be relationships between homocysteine stimulation, H_2O_2 and VSMC proliferation. To test this hypothesis, we studied the effects of homocysteine on VSMC growth and on the activity of superoxide dismutase (SOD), catalase and glutathione peroxidase activity in the cells. In this study, we show that homocysteine increases VSMC DNA synthesis at concentrations within the patho-physiological plasma range and enhances the mitogenic response to PDGF-BB, probably by dysregulation of antioxidant defence systems in the VSMCs.

Methods

Cell culture

Aortic VSMCs were obtained from the thoracic aorta of the rat as described previously (Nishio *et al*., 1996). The cells (1×10^5) were seeded into 35-mm diameter dishes and maintained in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were used between the third and fifth passage. Cells were grown to confluence, at which time they were rendered quiescent by serum deprivation and maintained in serum-free medium for 36 h before the experiment.

[^{3}H]-thymidine incorporation

VSMCs were seeded at a density of 5×10^4 cells/dish and synchronized at the G_0/G_1 phase of the cell cycle by incubation for 3 days in DMEM containing 0.5% (v/v) foetal calf serum.

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The medium was then removed and the cells were stimulated to proliferate in DMEM containing D, L-homocysteine with or without PDGF-BB, and [^{3}H]-thymidine was added (5 μCi ml^{-1}). After 24 h incubation, the incorportion of [^{3}H]-thymidine into acid-insoluble materials was measured (Janat & Gene, 1992).

Enzymatic assays

For determination of enzymatic activities, monolayers of VSMC on 100 mm-diameter cell culture plates were washed twice with ice-cold Krebs-Ringer solution (10 ml), resuspended in Krebs-Ringer solution (concentration, mM: HEPES 20, glucose 10, NaCl 127, KCl 5.5, $CaCl_2$ 1 and $MgSO_4$ 2, pH = 7.4, 1 ml) by scraping with sterile disposable cell lifter, and spun at 120 g for 10 min in Eppendorf tubes. VSMCs were resuspended in 400 μl of phosphate buffer (50 mM sodium phosphate and 0.5% TritonX-100, pH7.5) and sonicated for two 15 s bursts. Sonicates were spun for 10 min at 15,000 *g* and the supernatants were used immediately for enzymatic assays and protein determinations (Bradford, 1976). Catalase activity of the extracts (20 μl, 5–10 mg ml^{-1}) was measured by monitoring the disappearance of hydrogen peroxide at 240 nm (Hildebrandt *et al*., 1978). Glutathione peroxidase activity of crude extracts (20 μl, 5-10 mg ml^{-1}) was determined by use of a couple assay in which the rate of t-butyl hydroperoxide-dependent NADPH oxidation at 340 nm was monitored (Gunzler & Flohe, 1985). SOD activity of the extracts was measured by monitoring the rate of NADPH oxidation in the presence and absence of the sample at 340 nm (Teixeira *et al*., 1996). Highly purified SOD (from bovine erythrocytes) was used as a standard for this assay. Measurement of activity was expressed as a percentage of the change of the mean absorbance in the absence of the enzymes. One unit of SOD activity is equivalent to 50% inhibition.

Detection of intracellular H_2O_2

Intracellular levels of H_2O_2 were analysed by fluorescence-activated cell sorting (FACS) (Becton Dickinson, Mountain View, CA) with 2′-7′-dichlorofluorecin diacetate (DCFH-DA) as a probe (Lo & Cruz, 1995). Experiments were performed under dim light. Confluent, serum-deprived VSMCs were incubated in DMEM containing 5 mM DCFH-DA for 24 h with additional homocysteine and PDGF then chilled on ice and washed with cold PBS. Washed cells were detached from culture plates by trypsin digestion. The activity of trypsin was quenched with 0.05% BSA in PBS. The fluorescent intensities of DCFH-DA for samples of 10,000 cells were analysed by flow cytometry by a FACScan flow cytometer equipped with an air-cooled argon laser.

Detection of superoxide production

Superoxide anion production was measured as the superoxide dismutase inhibitable reduction of acetylated ferricytochrome c (Heinecke *et al*., 1987). VSMCs were preincubated in DMEM for 15 min, washed once with DMEM, and incubated with 1 ml of medium containing cytochrome c (1 mg ml^{-1}) with or without superoxide dismutase (20 μg ml^{-1}) in humidified air at 37°C on a shaking table. At 100 min, the medium was removed from the cells and the absorbance at 550 nm was read immediately. Superoxide anion-specific reduction of cytochrome c was expressed as the difference in absorbance between cells incubated with or without superoxide dismutase by use of an extinction coefficient of 21.1 mM^{-1} cm^{-1}.

Materials

Aminotriazole (ATZ), D, L-homocysteine and nistroarginine were obtained from Sigma. 2′-7′-Dichlorofluorecin diacetate (DCFH-DA) from Molecular Probes, and [^{3}H]-thymidine from Amersham. N-acetyl-L-cysteine (NAC) was from Aldrich. Catalase was from Boehringer Mannheim. PDGF-BB and ferricytochrome C was from Cosmo Bio Co., Ltd. All cell culture materials were from Life Technologies. Purified bovine glutathione peroxidase was purchased from Toyobo Co., Ltd and was used without further purification.

Statistical analysis

Unless indicated otherwise, all experiments were carried out three or four times on different days and each was made in duplicate. The results are expressed as mean ± s.e.mean. Statistical analysis was performed by the use of one-way analysis of variance followed by a Bonferroni *t* test for multiple comparisons with a P value <0.05 considered statistically significant.

Results

Homocysteine as a mitogen for rat VSMC

Figure 1 shows that homocysteine alone significantly increased the incorporation of [^{3}H]-thymidine into DNA at a concentration of 250–500 μM. PDGF-BB (1 nM) or serum (10%), the positive controls, were more potent mitogens as compared to homocysteine (500 μM) alone (Figure 2). The increase with homocysteine (500 μM) alone was approximately 2 fold above that observed for the control. When homocysteine (500 μM) and PDGF (1 nM) were added together, the increase in [^{3}H]-thymidine incorporation into DNA was approximately 4 fold above that observed for PDGF (1 nM) alone and was approximately 6 fold above that observed for homocysteine (500 μM) alone.

Antioxidants inhibit [^{3}H]-thymidine incorporation

To determine the involvement of ROS in the enhancement of [^{3}H]-thymidine incorporation into DNA by the combination of homocysteine and PDGF-BB, we cultured VSMCs with homocysteine, PDGF-BB and antioxidants. Figure 2 shows that catalase, an enzyme that catalyses the decomposition of hydrogen peroxide to H_2O and O_2, and N-acetyl-L-cysteine (NAC), a glutathione precursor and radical sca-

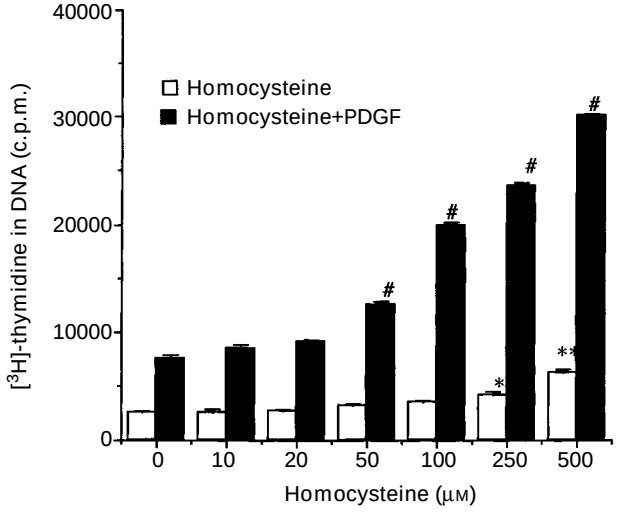

Figure 1 Determination of the mitogenic response of confluent cultured VSMC to different concentrations of homocysteine or homocysteine plus PDGF (1 nM). The data represent [^{3}H]-thymidine incorporated into DNA and are the mean of four independent experiments in duplicate. *$P<0.05$, **$P<0.01$ analysis of variance and Bonferroni *t* test, homocysteine-treated cells different from controls. $^{\#}P<0.01$ homocysteine plus PDGF-treated different from PDGF alone.

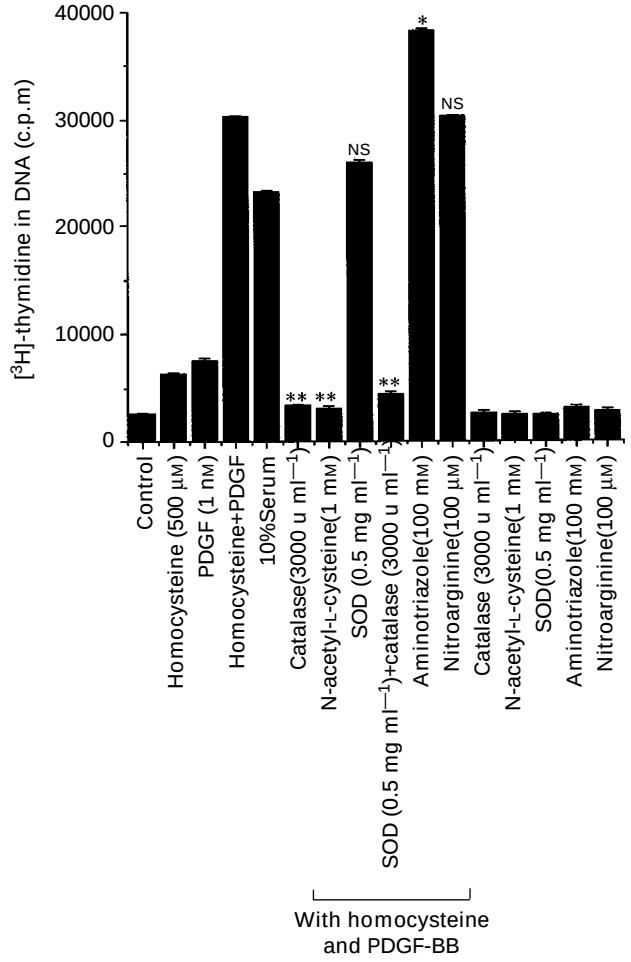

Figure 2 Antioxidants inhibited the increased mitosis evoked by the combination of PDGF-BB and homocysteine in confluent VSMCs. Results represent the mean amount of [^{3}H]-thymidine incorporated into DNA $\pm$ s.d. of four independent experiments in duplicate. $^*P<0.05$, $^{**}P<0.01$, analysis of variance plus Bonferroni t test, different from PDGF (1 nM) and homocysteine (500 μM).

venger, prevented the enhanced [^{3}H]-thymidine incorporation into DNA induced by the combination of homocysteine and PDGF-BB, whereas superoxide dismutase (SOD) was not active. This may be due to hydrogen peroxide formation by superoxide dismutase which catalyzes the dismutation of superoxide anion to hydrogen peroxide. Indeed catalase in combination with superoxide dismutase significantly reduced the enhanced [^{3}H]-thymidine incorporation. Aminotriazole (ATZ), an inhibitor of catalase, increased the enhanced [^{3}H]-thymidine incorporation by the combination of homocysteine and PDGF-BB even further. The nitric oxide synthase inhibitor nitroarginine (0.1 – 1 mM) did not significantly affect the enhanced DNA synthesis by the combination of homocysteine and PDGF-BB (Figure 2, and data not shown). Catalase, N-acetyl-L-cysteine, superoxide dismutase, aminotriazole or nitroarginine alone were without effects on [^{3}H]-thymidine incorporation into DNA as compared to the control.

H_2O_2 content of VSMCs treated with homocysteine and PDGF-BB

To test whether the combination of PDGF-BB and homocysteine induced more reactive oxygen species as compared homocysteine or PDGF-BB alone, we measured the relative concentrations of H_2O_2 in VSMC by use of DCFH-DA and fluorescence-activated cell sorting (FACS). DCFH-DA is oxidized to membrane-impermeable, fluorescent DCFH-DA derivatives in the presence of H_2O_2. As shown in Figure 3, fluorescence intensity induced by the combination of homocysteine and PDGF was augmented as the homocysteine concentrations (0 – 500 μM) increased and a significant augmentation was observed at the homocysteine concentration of 100 μM or more. An increase in fluorescence intensity was also induced by homocysteine alone as compared to the control, and this increase was significant at the concentration of 500 μM. Thus, the addition of homocysteine increased the generation of PDGF-induced reactive oxygen species in VSMCs in a concentration-dependent manner.

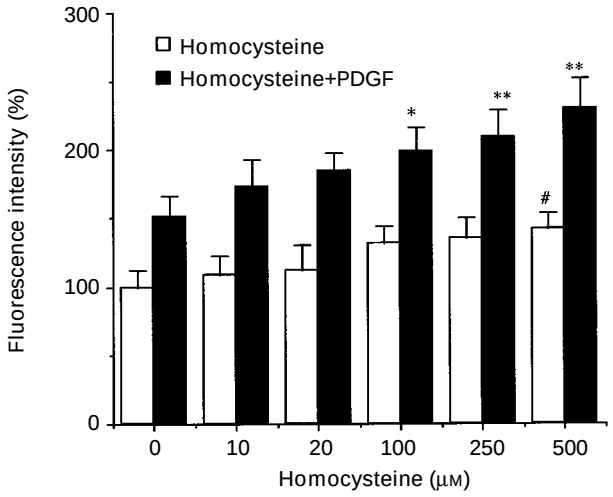

Figure 3 The effect of 24 h exposure to homocysteine with or without PDGF (1 nM) on the content of H_2O_2 in VSMC estimated by FACS analysis as described in Methods. Data are expressed as a percentage change relative to the control cells without homocysteine or PDGF arbitrarily set at 100% and presented as mean $\pm$ s.d. of three independent experiments in duplicate. $^*P<0.05$, $^{**}P<0.01$, analysis of variance plus Bonferroni t test, different from PDGF alone. $^{\#}P<0.05$, analysis of variance plus Bonferroni t test, different from no addition.

The effect of homocysteine on activites of SOD, catalase and glutathione peroxidase

Since the H_2O_2 content was increased by homocysteine and PDGF in VSMCs, we measured the activities of two enzymes which can catabolize H_2O_2, catalase and glutathione peroxidase after incubation with homocysteine for 24 h. Figure 4 shows that homocysteine decreased the activity of glutathione peroxidase in a dose-dependent manner (100 – 500 μM). In contrast, homocysteine was without effect on the activity of catalase within the range of physiological concentrations (25 – 500 μM). To test the possibility that homocysteine stimulated H_2O_2 producing systems, we measured the activity of SOD. Homocysteine significantly increased the activity of SOD in a concentration-dependent manner (100 – 500 μM).

Superoxide anion content of VSMCs treated by homocysteine and PDGF-BB

As inactivation of catalase and glutathione peroxidase by superoxide anion has been demonstrated (Fridorich, 1985), we measured the content of superoxide anion in VSMC treated with the combination of PDGF-BB (1 nM) and homocysteine (10 – 500 μM. Homocysteine caused a concentration-dependent increase in the content of superoxide anion in the presence or absence of PDGF-BB (1 nM). Significant increases were observed at 100 μM or more for the combination with PDGF, or 20 μM or more for homocysteine alone (Figure 5).

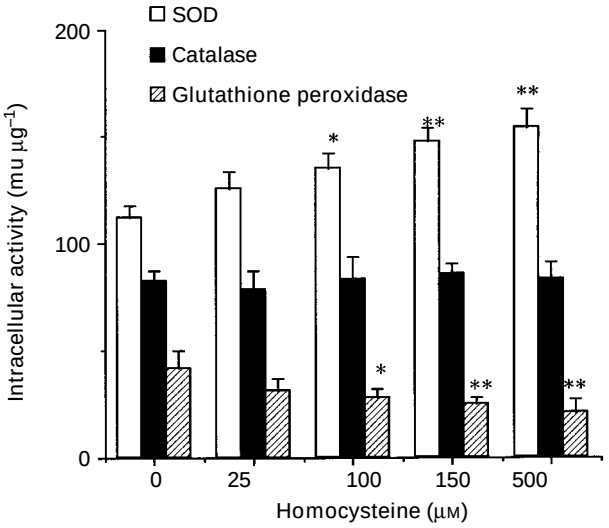

Figure 4 The activities of catalase, glutathione peroxidase and SOD in extracts of confluent VSMCs exposed to homocysteine for 24 h. Measurements of three experiments in duplicate are expressed as mean ± s.d. Units are defined as follows: SOD, amount of SOD inhibiting by 50% autooxidation of NADPH; catalase, micromoles of H_2O_2 consumed per minute; glutathione peroxidase, nanomol of NADPH oxidized per minute. $^*P<0.05$, $^{**}P<0.01$, analysis of variance followed by a Bonferroni t test, homocysteine-treated cells different from control cells.

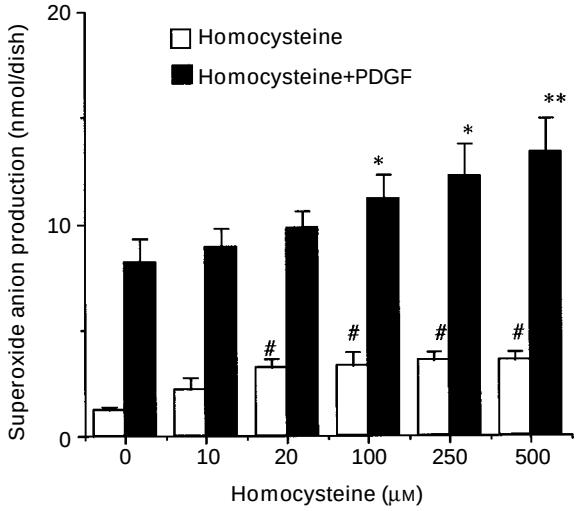

Figure 5 Superoxide anion production at various concentrations of homocysteine in the presence and absence of PDGF (1 nM) was assayed. Cells were preincubated for 15 min in DMEM, washed once and incubated with 1.0 ml of DMEM containing acetylated ferricytochrome c (1 mg ml^{-1}) and the indicated reagents ± superoxide dismutase (20 μg ml^{-1}). Superoxide-specific reduction of cytochrome c was determined after 100 min of incubation as described under Methods. Measurements of three experiments in duplicate, and are expressed as mean ± s.d. $^*P<0.05$, $^{**}P<0.01$, analysis of variance followed by a Bonferroni t test, PDGF (1 nM) and homocysteine-treated cells different from PDGF (1 nM) only treated cells. $^{\#}P<0.05$, homocysteine-treated cells different from control.

Inactivation of purified bovine cytosolic glutathione peroxidase

As homocysteine decreased the activity of glutathione peroxidase in VSMCs, it was of interest to determine whether homocysteine could also directly affect the activity of glutathione peroxidase. Purified bovine liver cytosolic glutathione peroxidase was initially treated with homocysteine and then subjected to glutathione peroxidase assay with hydrogen peroxide as a substrate. The enzyme used was >95% pure on SDS-polyacrylamide gel electrophoresis (data not shown). Figure 6 shows that glutathione peroxidase activity decreased after incubation with homocysteine in a concentration and time-dependent manner.

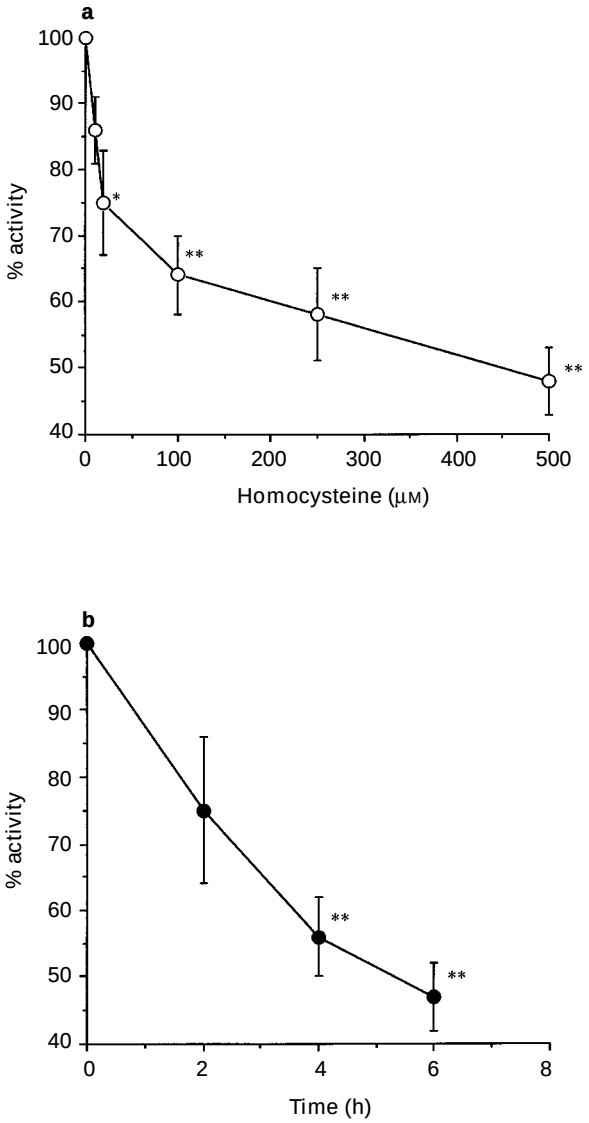

Figure 6 Dose-dependence (a) and the time course (b) of inactivation of bovine glutathione peroxidase. Purified bovine glutathione peroxidase activity at a concentration of 1 mg ml^{-1} was preincubated with various concentrations of homocysteine for 6 h (a) or with 500 μM homocysteine for various times (b) at 37°C. Activities are given as the percentage of the control value. $^*P<0.05$, $^{**}P<0.01$, analysis of variance plus Bonferroni t test, different from control.

The effect of PDGF-BB on activities of SOD, catalase and glutathione peroxidase

As shown in Figure 3, PDGF-BB induced more H_2O_2 content than homocysteine. Therefore, we measured the effects of PDGF (1 nM) alone and in combination with homocysteine (500 μM) on the activity of antioxidant enzymes. Figure 7 shows that PDGF alone increased the activity of superoxide dismutase and catalase as compared to the control. PDGF (1 nM) alone had no effect on the activity of glutathione peroxidase. The combination of PDGF (1 nM and homocysteine (500 μM) increased superoxide dismutase activity, but de-

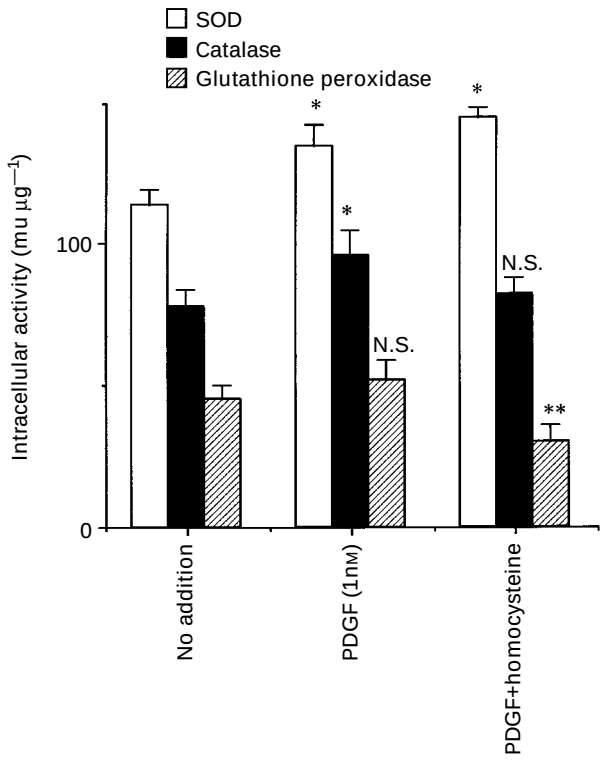

Figure 7 The activities of catalase, glutathione peroxidase and SOD in extracts of confluent SMCs exposed to PDGF-BB (1 nM) or in combination with homocysteine (500 μM) for 24 h. Measurements of three experiments in duplicate, and are expressed as mean $\pm$ s.d. Units are defined as follows: SOD, amount of SOD inhibiting by 50% autooxidation of NADPH; catalase, micromol of H_2O_2 consumed per minute; glutathione peroxidase, nanomol of NADPH oxidized per minute. $^*P<0.05$, $^{**}P<0.01$, analysis of variance followed by a Bonferroni t test, PDGF or homocysteine-treated cells different from control cells.

creased the activity of glutathione peroxidase as compared to the control.

Discussion

The molecular mechanisms by which homocysteine stimulates the development of atherosclerosis are not fully understood, though injury of vascular endothelial cells has been implicated (Wall *et al*., 1980). The injured endothelium produces growth factors, which act on neighbouring VSMCs to promote their proliferation. Thus, previous studies on homocysteine-induced atherosclerosis or thrombosis have focused on the effects of homocysteine on endothelial cells (Wang *et al*., 1993). Recently, it has been shown that homocysteine increases DNA synthesis in VSMC and induces the Cyclin A gene (Tsai *et al*., 1996).

In the present study, we showed that homocysteine within its physiological plasma range weakly increases thymidine uptake in rat VSMCs, but dramatically enhances their mitogenic response to PDGF-BB. Antioxidants, such as N-acetyl-L-cysteine or catalase, inhibited the enhancement of [^{3}H]-thymidine incorporation into DNA by the combination of homocysteine and PDGF-BB. Inversely, the catalase inhibitors, aminotriazole enhanced [^{3}H]-thymidine incorporation into DNA by the combination of homocysteine and PDGF-BB. These results suggest that H_2O_2 or reactive oxygen species derived from H_2O_2 are involved in the enhanced [^{3}H]-thymidine incorporation into DNA induced by the combination of homocysteine and PDGF-BB. Furthermore, homocysteine changed the balance of H_2O_2 metabolism through increasing the activity of superoxide dismutase and reducing the activity of glutathione peroxidase.

Superoxide dismutase, catalase and glutathione peroxidase scavenge active oxygen species, and thus protect cells from free radical-mediated disturbance (Grisham & McCord, 1986). Superoxide dismutase catalyses the dismutation of superoxide anion radicals, catalase catalyses the reduction of hydrogen peroxide and glutathione peroxidase detoxifies both hydrogen peroxide and lipid hydroperoxides. The increase in activity of superoxide dismutase may be a response to enhanced levels of superoxide anion (Figure 5). The decreased glutathione peroxidase activity may be explained by the fact that antioxidant enzymes are inhibited by specific oxygen reactive species (Vessey & Lee, 1993), which is formed from homocysteine (Heinecke *et al*., 1987), an inhibitor of both catalase (Fridorich, 1985) and glutathione peroxidase (Blum & Fridorich, 1985). Therefore, it may be possible that the increased SOD activity was not sufficient to remove completely the homocysteine-formed superoxide anion radicals in this experimental condition (Figure 5). The remaining radicals might inhibit glutathione peroxidase activity and prevent the increase of catalase activity. Furthermore, we demonstrated that homocysteine directly decreased the activity of bovine purified cytosolic glutathione peroxidase in a time- and dose-dependent manner. This suggests that homocysteine decreased the activity of glutathione peroxidase partly independent of homocysteine-induced superoxide anion radicals. However, further studies are needed to clarify the opposing effects of homocysteine on SOD, catalase and glutathione peroxidase.

Figure 3 shows that the H_2O_2 content of VSMC, treated by homocysteine and PDGF (1 nM) together, was significantly higher than in cells treated with PDGF (1 nM) alone at the homocysteine concentration of 100 μM or more, in spite of unchanged catalase activity. The content of catalase is lower than the content of glutathione peroxidase in most cells, except for hepatocytes and erythrocytes, and the K_m value of catalase for hydrogen peroxide is higher than that of glutathione peroxidase (Asahi *et al*., 1995). Therefore these results may imply that glutathione peroxidase is primarily important in VSMC. But as the catalase inhibitor aminotriazole significantly enhanced the mitogenic effect of homocysteine plus PDGF (Figure 2), the role of catalase cannot be excluded.

Lastly, we investigated the effects of PDGF alone and in combination with homocysteine on antioxidant enzyme activities (Figure 7). The results suggested that the increase of H_2O_2 content in cells treated with PDGF-BB is probably due to enhanced biosynthesis rather than decreased degradation by catalase and/or glutathione peroxidase.

In conclusion, our results demonstrate that homocysteine alone slightly stimulates cell proliferation, but dramatically enhances the proliferative action of PDGF-BB in rat VSMCs. Therefore it appears that homocysteine may modify the action of other growth factors or cytokines present in atherosclerotic lesions in a synergistic manner. Furthermore, this phenomenon may be at least partly the result of impairment of the catabolism of reactive oxygen species, caused by a decrease in glutathione peroxidase activity.

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