

Homocysteine stimulates phosphorylation of NADPH oxidase p47^{phox} and p67^{phox} subunits in monocytes via protein kinase C β activation

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Hyperhomocysteinaemia is an independent risk factor for cardiovascular diseases due to atherosclerosis. The development of atherosclerosis involves reactive oxygen species-induced oxidative stress in vascular cells. Our previous study [Wang and O (2001) *Biochem. J.* **357**, 233–240] demonstrated that Hcy (homocysteine) treatment caused a significant elevation of intracellular superoxide anion, leading to increased expression of chemokine receptor in monocytes. NADPH oxidase is primarily responsible for superoxide anion production in monocytes. In the present study, we investigated the molecular mechanism of Hcy-induced superoxide anion production in monocytes. Hcy treatment (20–100 μ M) caused an activation of NADPH oxidase and an increase in the superoxide anion level in monocytes (THP-1, a human monocytic cell line). Transfection of cells with p47^{phox} siRNA (small interfering RNA) abolished Hcy-induced superoxide anion production, indicating the involvement of NADPH oxidase. Hcy treatment resulted in phosphorylation and subsequently membrane translocation of p47^{phox} and p67^{phox} subunits leading to NADPH oxidase activation. Pretreatment of cells with PKC (pro-

tein kinase C) inhibitors Ro-32-0432 (bisindolylmaleimide XI hydrochloride) (selective for PKC α , PKC β and PKC γ) abolished Hcy-induced phosphorylation of p47^{phox} and p67^{phox} subunits in monocytes. Transfection of cells with antisense PKC β oligonucleotide, but not antisense PKC α oligonucleotide, completely blocked Hcy-induced phosphorylation of p47^{phox} and p67^{phox} subunits as well as superoxide anion production. Pretreatment of cells with LY333531, a PKC β inhibitor, abolished Hcy-induced superoxide anion production. Taken together, these results indicate that Hcy-stimulated superoxide anion production in monocytes is regulated through PKC-dependent phosphorylation of p47^{phox} and p67^{phox} subunits of NADPH oxidase. Increased superoxide anion production via NADPH oxidase may play an important role in Hcy-induced inflammatory response during atherogenesis.

Key words: atherosclerosis, homocysteine, monocyte, NADPH oxidase, reactive oxygen species, superoxide anion.

INTRODUCTION

Hyperhomocysteinaemia, a condition of elevated blood Hcy (homocysteine) levels, is a common and independent risk factor for cardiovascular diseases [1–4]. Abnormal elevation in Hcy levels up to 100–250 μ M in the blood has been reported in patients with hyperhomocysteinaemia [5,6]. Hcy is a thiol-containing amino acid formed during the metabolism of methionine. A significant proportion of patients (up to 30–40%) with coronary artery disease had elevated blood Hcy levels [7]. Although the precise mechanisms of Hcy-associated atherosclerosis remain unclear, inflammatory reaction in vascular cells due to oxidative stress is considered to be one of the important mechanisms contributing to vessel injury [8,9].

Many risk factors for atherosclerosis share a common feature of generating intracellular ROS (reactive oxygen species) causing oxidative stress [10–12]. Dysregulation of superoxide anion production can lead to cell injury. NADPH oxidase is primarily responsible for superoxide anion generation in phagocytes such as neutrophils and monocytes [13,14]. In phagocytes, this oxidase is an enzyme complex consisting of membrane-bound components, termed flavocytochrome *b*₅₅₈ (p22^{phox} and gp91^{phox} subunits) and

cytosolic components (p47^{phox}, p67^{phox} and p40^{phox}). The membrane-associated subunits p22^{phox} and gp91^{phox} (the catalytic subunit of the oxidase) are present as a heterodimer-containing NADPH-binding site, whereas the subunits p47^{phox}, p67^{phox} and p40^{phox} reside in the cytosol as a complex [15–17]. Activation of NADPH oxidase requires the assembly of cytosolic subunits with p22^{phox} and gp91^{phox} subunits in the membrane. It is generally believed that phosphorylation and subsequent translocation of cytosolic subunits to the membrane are essential steps for the activation of NADPH oxidase [15–17]. Upon stimulation, p47^{phox} is rapidly phosphorylated at several serine residues and undergoes a conformational change. Phosphorylated p47^{phox} then interacts with p22^{phox} and gp91^{phox} to promote the oxidase assembly and subsequent activation [16,18]. Phosphorylation of p67^{phox} is also involved in the activation of NADPH oxidase [18]. NADPH oxidase activity can also be regulated at the gene level of oxidase subunits. It has been shown that NADPH oxidase activity is significantly elevated in atherosclerotic lesions, leading to increased superoxide anion production [19,20]. The activation of PKC (protein kinase C) is associated with increased production of superoxide anions in phagocytes as well as in other vascular cells [21–24].

Abbreviations used: CCR2, chemoattractant protein-1 receptor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hcy, homocysteine; L-NAME, N^G-nitro-L-arginine methyl ester; MCP-1, monocyte chemoattractant protein-1; NBT, Nitro Blue Tetrazolium; NF- κ B, nuclear factor κ B; PKC, protein kinase C; cPKC, conventional PKC; ROS, reactive oxygen species; RT, reverse transcriptase; siRNA, small interfering RNA; SOD, superoxide dismutase; PEG-SOD, poly(ethylene glycol)-bound SOD.

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In our previous study, Hcy treatment resulted in an elevation of superoxide anion levels in THP-1 monocytes [25] and in endothelial cells [26]. We further demonstrated that increased intracellular level of superoxide anions was responsible for enhanced mRNA expression of MCP-1 (monocyte chemoattractant protein-1) [25] and its receptor CCR2 (chemoattractant protein-1 receptor 2) in monocytes [27]. Our results suggest that Hcy-induced MCP-1 and CCR2 expression due to increased oxidative stress may facilitate the recruitment of monocytes into the arterial wall [25,27]. Hcy was able to activate NF- κ B (nuclear factor κ B), an oxidative stress-responsive transcription factor, in endothelial cells via oxidative stress [26]. We also observed that the PKC signalling pathway and oxidative stress played a role in Hcy-induced NF- κ B activation in vascular smooth muscle cells [28]. Monocytes/macrophages serve as a major source of superoxide anions contributing to the progression of atherosclerosis [29]. However, the mechanism through which Hcy stimulates superoxide anion production in monocytes is not well understood. In the present study, we investigated the mechanism of Hcy-induced superoxide anion production via NADPH oxidase in human monocytic cells. Our results clearly demonstrated that PKC β played an important role in Hcy-induced NADPH oxidase activation, leading to dysregulation of superoxide anion production in monocytes.

EXPERIMENTAL

Reagents

Goat polyclonal antibodies against NADPH oxidase subunits (p47^{phox}, p67^{phox}, p22^{phox} and gp91^{phox}), rabbit polyclonal antibodies against PKC isoenzymes (PKC α , PKC β and PKC γ), goat polyclonal antibodies against actin and p47^{phox} siRNA (small interfering RNA) duplex were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Mouse anti-phosphoserine monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The corresponding secondary antibodies were purchased from Zymed (Carlsbad, CA, U.S.A.). Phosphorothioate-modified oligonucleotides that were purified by HPLC were purchased from Sigma-Genosys (St. Louis, MO, U.S.A.). All other reagents were from Sigma-Aldrich, except where specified. L-Hcy was prepared from L-Hcy thiolactone as described previously [30,31]. In brief, L-Hcy thiolactone was dissolved and hydrolysed in potassium hydroxide (5 M) to remove the thiolactone group, followed by neutralization with potassium phosphate. The concentration of L-Hcy in the preparation was quantified with the IMx Hcy assay (Abbott Laboratories, Abbott Park, IL, U.S.A.). Freshly prepared L-Hcy was used in all of the experiments.

Cell culture

A human monocytic cell line THP-1 was purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were cultured in RPMI 1640 medium containing 10% (v/v) low-endotoxin fetal bovine serum as described previously [27]. For experiments, cells were cultured in the RPMI 1640 medium and the cell density was maintained at less than 5×10^5 cells/ml to prevent cell differentiation [27].

Determination of superoxide anion levels

After cells were incubated with Hcy and other defined compounds for various time periods, the intracellular superoxide anion level was measured by the NBT (Nitro Blue Tetrazolium) reduction assay described previously [25–27]. In brief, cells were incubated

in Krebs–Henseleit buffer (120 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.2 mM MgSO₄ · 7H₂O, 1.2 mM NaH₂PO₄, 1.27 mM CaCl₂ · 2H₂O and 5.5 mM D-glucose, pH 7.4) in the presence of NBT (1.0 mg/ml) for 60 min. The formazan was generated by the reduction of NBT in the presence of superoxide anions, which was proportional to the amount of superoxide anion generated intracellularly. At the end of the incubation period, the culture medium was removed and cells were washed with warm (37 °C) Krebs–Henseleit buffer. Cells were lysed with a phosphate buffer (6.8 mM NaH₂PO₄ and 73.2 mM Na₂HPO₄, pH 7.8) containing 5% (w/v) SDS and 0.45% gelatin. The cell lysate was centrifuged for 5 min at 13 000 g. The supernatant was used for the measurement of the absorbance at 540 nm (formazan) and 450 nm. The relative concentration of superoxide anion was calculated based on the amount of formazan formed. In control experiments, Hcy (100 μ M) was added to the cell lysis buffer and the absorbance values at 540 nm (formazan) and 450 nm were measured. In addition, experiments were performed using SOD (superoxide dismutase)-inhibitable cytochrome *c* reduction assay [32,33]. After cells were incubated with Hcy or other compounds for a certain period of time, cytochrome *c* (80 μ M) with or without the superoxide anion scavenger SOD (100 units/ml) was added to the collected culture medium and the absorbance at 550 nm was measured using a spectrophotometer. The amount of superoxide anions secreted by cells was calculated. Both NBT reduction assay and cytochrome *c* reduction assay were also performed in the culture medium containing Hcy (100 μ M) in the absence of monocytes. Results were used as a control for the autoxidation of Hcy to generate superoxide. Effects of Hcy on superoxide anion levels were calculated by subtracting the data obtained from Hcy-treated cells from the control data for the autoxidation of Hcy.

Determination of NADPH oxidase activity

NADPH oxidase activity was measured using lucigenin chemiluminescence assay [34]. After cells were incubated with Hcy or other compounds, lucigenin (5 μ M) was added and equilibrated for 2 min. After equilibration, 100 μ M NADPH was added and the chemiluminescent signal was measured every 15 s for 3 min using a luminometer (Lumet LB9507; Berthold Technologies, Bad Wildbad, Germany). The chemiluminescent signal yielded in the samples was corrected for background in each measurement. A standard curve was prepared with xanthine (100 μ M) and xanthine oxidase (Sigma-Aldrich) at various concentrations [32]. NADPH oxidase activity was calculated based on the amount of superoxide anion produced in the reaction mixture [34].

Western-blot analysis

Cell lysate was prepared by homogenizing cells in a buffer containing 10 mM Tris/HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 0.2 mM PMSF, 20 μ M leupeptin, 0.5 mM dithiothreitol and 0.15 μ M pepstatin A. Western-blot analysis was performed to determine cellular protein levels of NADPH oxidase subunits p47^{phox}, p67^{phox}, p22^{phox} and gp91^{phox} as well as PKC isoenzymes. The membrane fraction of cell lysate was prepared by ultracentrifugation. In addition, the protein levels of p47^{phox} and p67^{phox} in the membrane fraction were also determined by Western-blot analysis. In brief, proteins in the cell lysate or in the membrane fraction were separated by SDS/10% PAGE. The proteins on the gel were transferred on to a nitrocellulose membrane. The primary and secondary antibodies were used at the dilution of 1:1000 and 1:2000 respectively. Bands corresponding to individual NADPH oxidase subunits or PKC isoforms were visualized with ECL[®] (enhanced chemiluminescence) reagent (Amersham Biosciences,

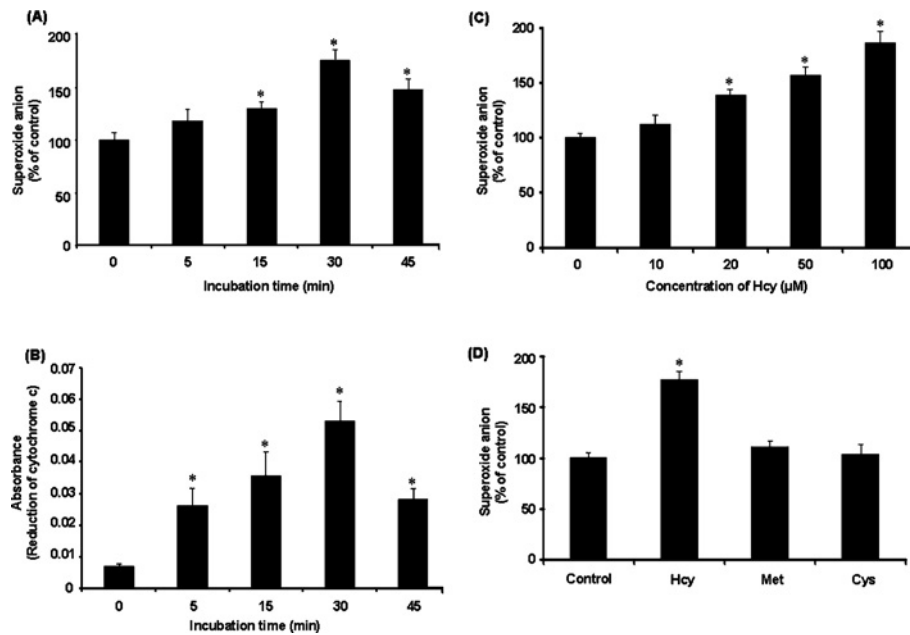


Figure 1 Superoxide anion levels in monocytes

Cells were incubated with or without Hcy (100 μM) for various time periods. At the end of the incubation, the intracellular superoxide anion was measured by NBT reduction assay (A) and the extracellular superoxide anion was measured using cytochrome *c* reduction assay (B). (C) Cells were incubated with Hcy (10–100 μM) or without Hcy for 30 min. (D) Cells were incubated for 30 min in the absence (Control) or presence of Hcy (100 μM), cysteine (Cys; 100 μM) or methionine (Met; 100 μM). In (C and D), the levels of superoxide anions in these cells were determined by NBT reduction assay. The level of superoxide anions in control cells was expressed as 100%. Results were expressed as means ± S.E.M. for five separate experiments each performed in duplicate. *Significantly different from the control value obtained from cells incubated in the absence of Hcy ($P < 0.05$).

Little Chalfont, Bucks., U.K.) and exposed to Kodak X-Omat Blue XB-1 films. The results were analysed with Quantity One image analysis software (version 4.2.1; Bio-Rad, Hercules, CA, U.S.A.).

Measurement of NADPH oxidase mRNA

Total RNAs were isolated from cultured cells with TRIzol® reagent (Invitrogen, Carlsbad, CA, U.S.A.). The mRNA expression of p22^{phox}, gp91^{phox}, p47^{phox} and p67^{phox} were examined by semi-quantitative RT (reverse transcriptase)–PCR analysis. The primers used for p22^{phox} in the PCR reactions were: (i) 5'-GTTTGTG-TGCCTGCTGGAGT-3' and (ii) 5'-TGGGCGGCTGCTTGATG-GT-3'. The primers used for gp91^{phox} were: (i) 5'-GCTGTTCA-ATGCTTGTGGCT-3' and (ii) 5'-TCTCCTCATCATGGTGCA-CA-3'. The primers used for p47^{phox} were: (i) 5'-ACCCAGCC-AGCACTATGTGT-3' and (ii) 5'-AGTAGCCTGTGACGTCGT-CT-3'. The primers used for p67^{phox} were: (i) 5'-CGAGGAA-CCAGCTGATAGA-3' and (ii) 5'-CATGGGAACACTGAGCTT-CA-3'. All primers were synthesized by Invitrogen. In the reverse transcription reaction, 2 μg of total RNA was converted into cDNA. The reaction mixture of PCR contained 0.2 mM dNTP, 0.4 μM 5'- and 3'-primers, 2 units of Taq DNA polymerase, 1.5 mM MgCl₂ and 2 μg of cDNA product from the reverse transcription reaction. The numbers of cycles of PCR amplification were 30 cycles for all the subunits (95 °C for 55 s, 60 °C for 55 s and 72 °C for 90 s). An additional 7 min extension was carried out at 72 °C. The PCR products were separated by agarose gel electrophoresis (1.5% agarose containing ethidium bromide) and visualized under UV light with a gel documentation system (Bio-Rad Gel Doc 1000). Human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was used as an internal standard to verify equal PCR product loading for each experiment.

The values were expressed as a ratio of p47^{phox}, p67^{phox}, p22^{phox} or gp91^{phox} to GAPDH.

Transfection of cells with p47^{phox} siRNA

Cells were transfected with p47^{phox} siRNA duplex (Santa Cruz Biotechnology). In brief, THP-1 cells were seeded in a six-well plate (2 × 10⁵ cells/well) and transfected with p47^{phox} siRNA duplex (sc-29422) according to the manufacturer's instructions. For a negative control, cells were transfected with a control siRNA duplex (sc-36869) consisting of a scrambled sequence that did not knock down cellular RNA (Santa Cruz Biotechnology). At 48 h after transfection, cells were washed three times with RPMI 1640 medium. Cells were incubated in the absence or presence of Hcy for 30 min. The intracellular superoxide anion level was measured by NBT reduction assay as described in the 'Determination of superoxide anion levels' subsection. The mRNA and protein levels of p47^{phox} in transfected cells were determined by RT–PCR and Western-blot analysis respectively.

Immunoprecipitation and immunoblotting analysis

Immunoprecipitation followed by immunoblotting analysis was performed to detect the phosphorylation status of p47^{phox} and p67^{phox} subunits. In brief, cells were lysed in a buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10 mM EGTA, 10 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1% (v/v) Triton X-100, 20 μM leupeptin, 1 mM PMSF and 0.15 μM pepstatin. Immunoprecipitation was performed by incubating cell lysate with goat polyclonal anti-p47^{phox} or anti-p67^{phox} antibodies for 2 h, followed by precipitation with agarose-immobilized Protein A. The immunoprecipitated proteins were then subjected to Western-blot

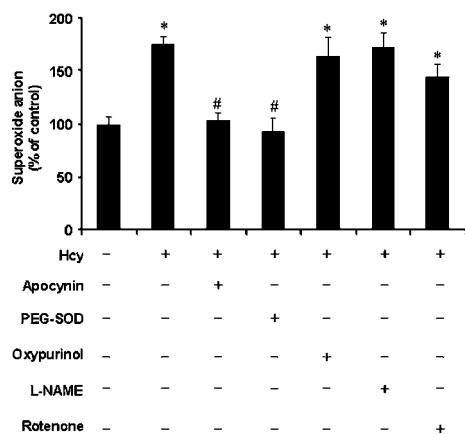


Figure 2 Effect of inhibitors on intracellular superoxide anion levels

Cells were pre-incubated with apocynin (300 μ M), PEG-SOD (300 units/ml), oxyprurinol (10 μ M), L-NAME (100 μ M) or rotenone (1 μ M) for 30 min, followed by incubation with Hcy (100 μ M) for another 30 min. At the end of incubation, levels of intracellular superoxide anions were determined by the NBT reduction assay. Results are expressed as means \pm S.E.M. for five separate experiments each performed in duplicate. The level of superoxide anions in control cells (cultured in the absence of inhibitors and Hcy) was expressed as 100%. *Significantly different from the control value ($P < 0.05$). #Significantly different from the value obtained with Hcy-treated cells ($P < 0.05$).

analysis using a mouse monoclonal anti-phosphoserine antibody. The protein levels of p47^{phox} and p67^{phox} in the immunoprecipitates were also determined by Western-blot analysis using goat polyclonal anti-p47^{phox} and anti-p67^{phox} antibodies. Phosphorylated p47^{phox} and p67^{phox} protein levels were normalized to the total p47^{phox} and p67^{phox} proteins detected in the immunoprecipitates.

Transfection of cells with sense and antisense oligonucleotides

Phosphorothioate-modified oligonucleotides were used in the present study to minimize degradation. The antisense and sense oligonucleotides were derived from published results [22]. The sequence for PKC α isoenzyme-specific antisense oligonucleotide was 5'-CGCCGTGGAGTCGTTGCCCG-3' and its control sense sequence was 5'-CGGGCAACGACTCCACCGCG-3'. The sequence for PKC β isoenzyme-specific antisense oligonucleotide was 5'-AGCGCACGGTGCTCTCCTCG-3' and its control sense sequence was 5'-CGAGGAGAGCACCGTGCGCT-3'. For experiments, individual phosphorothioate-modified oligonucleotides (4–8 μ M) were delivered into cells with Oligofectamine reagent according to the manufacturer's instructions (Invitrogen). The transfection efficiency was determined 48 h later by measuring protein levels of individual PKC isoenzymes in transfected cells using Western-blot analysis.

Assay for PKC activity

PKC activity was measured with an assay kit purchased from Upstate Biotechnology (Charlottesville, VA, U.S.A.). The assay was based on the phosphorylation of a specific substrate peptide (QKRPSQRSKYL) catalysed by PKC. In brief, after treatment with Hcy and other defined compounds, cells were harvested in lysis buffer containing 50 mM Hepes (pH 7.5), 0.2% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 85 μ M leupeptin, 2 mM benzamide and 0.4 mM PMSF. The cell lysate was centrifuged at 100 000 g for 20 min to obtain a detergent-soluble fraction (supernatant) [35]. An aliquot of the supernatant (10 μ l containing 40 μ g of proteins) was incubated with a reaction mixture containing 100 μ M [γ -³²P]ATP (PerkinElmer Life Sciences, Boston, MA, U.S.A.) for 10 min at 30 $^{\circ}$ C. At the

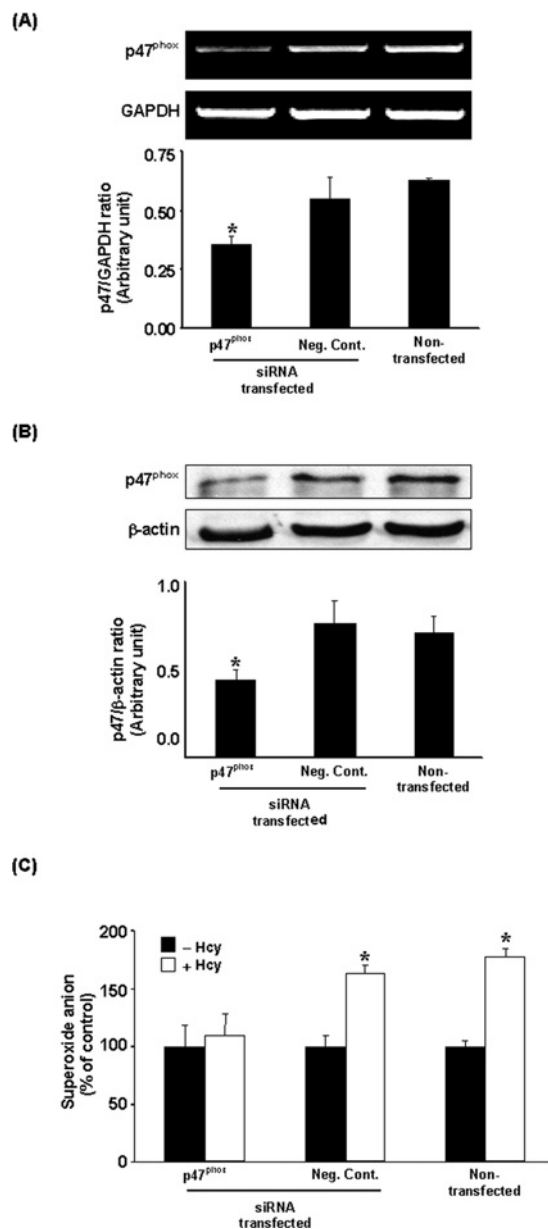


Figure 3 Effect of siRNA transfection on p47^{phox} expression and superoxide anion production

At 48 h after transfection of cells with p47^{phox} siRNA or a negative control siRNA (Neg. Cont.), the mRNA levels of p47^{phox} were determined by RT-PCR analysis (A). GAPDH mRNA was used as an internal standard to verify equal PCR product loading for each experiment. (B) The protein levels of p47^{phox} were determined by Western-blot analysis. β -Actin was used to confirm an equal amount of protein loading for each sample. Representative blots were obtained from four separate experiments. *Significantly different from the value obtained from cells transfected with negative control siRNA ($P < 0.05$). (C) In different sets of experiments, transfected and non-transfected cells were incubated with Hcy (100 μ M; +Hcy) or without Hcy (–Hcy) for 30 min. The intracellular superoxide anion was measured by the NBT reduction assay. Results are expressed as means \pm S.E.M. for five separate experiments each performed in duplicate. *Significantly different from the value obtained from cells incubated in the absence of Hcy (–Hcy) ($P < 0.05$).

end of the incubation, the reaction mixture was transferred to P81 ion-exchange chromatography paper, followed by four washes with 0.75% (v/v) phosphoric acid and one wash with acetone, according to the manufacturer's instructions. The radioactivity incorporation into the peptide substrate was determined by a liquid-scintillation counter.

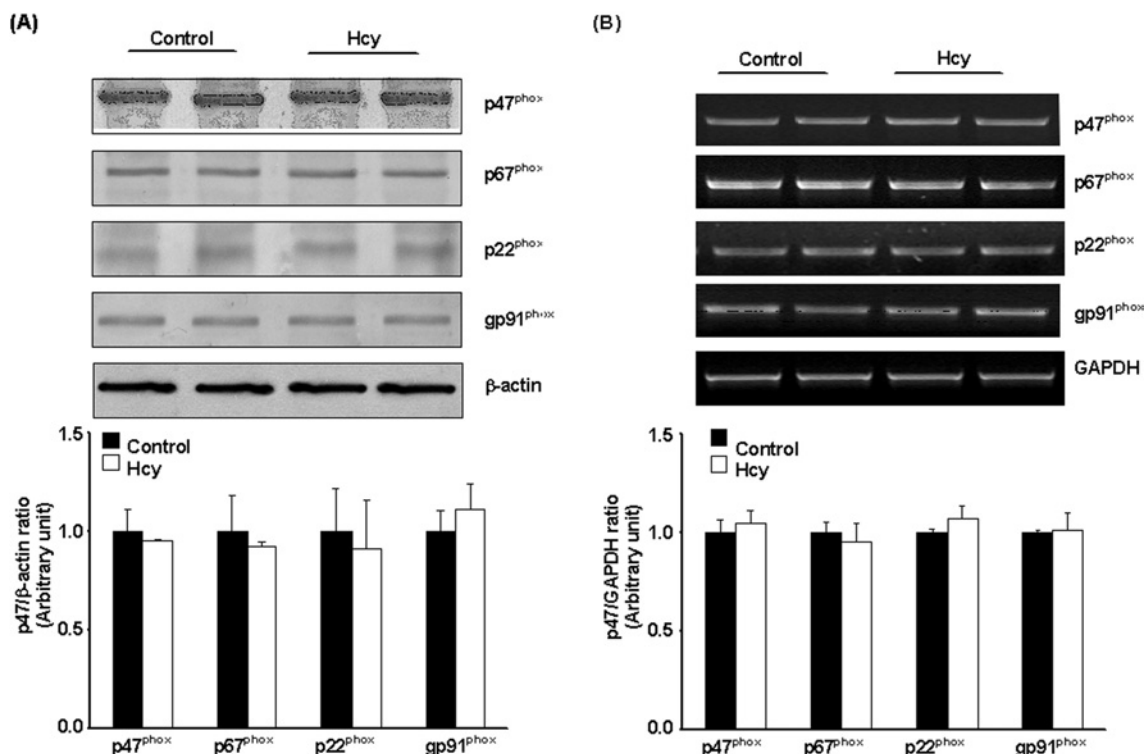


Figure 4 NADPH oxidase subunit protein and mRNA levels in monocytes

Cells were incubated for 30 min in the absence (Control) or presence of Hcy (100 μM). (A) Cellular extracts were prepared and the protein levels of p47^{phox}, p67^{phox}, p22^{phox} and gp91^{phox} were determined by Western-blot analysis. β -Actin was used to confirm equal amount of protein loading for each sample. (B) Total mRNA was isolated and the mRNA levels of individual NADPH oxidase subunits were analysed by RT-PCR. GAPDH mRNA was used as an internal standard to verify equal PCR product loading for each experiment. Representative photos were obtained from three separate experiments. Results are expressed as means \pm S.E.M.

Statistics

Results were analysed using ANOVA followed by Dunnett's post-hoc test. Results are means \pm S.E.M. $P < 0.05$ was considered significant.

RESULTS

Effect of Hcy on intracellular levels of superoxide anions

Hcy elicited a time-dependent elevation in superoxide anion level in monocytes, which reached a maximum at 30 min of incubation (Figure 1A). The production of superoxide anion by monocytes was also determined using cytochrome *c* reduction assay (Figure 1B). A significant elevation in superoxide anion level was observed in cells treated with Hcy in a concentration-dependent manner (Figure 1C). To determine whether such a stimulatory effect was not a generalized effect of thiol-containing amino acids but related specifically to Hcy, the effect of other thiol-containing amino acids, namely cysteine and methionine, on superoxide anion production in monocytes was also examined. There was no significant change in the level of superoxide anions in cells incubated with either cysteine (100 μM) or methionine (100 μM) (Figure 1D). These results indicated that the stimulatory effect on superoxide anion production was related specifically to Hcy. Next, to examine the possible source of increased superoxide anion levels in Hcy-treated cells, several inhibitors of superoxide-producing enzymes were added to the culture medium. Pre-

treatment of these cells with a specific inhibitor (apocynin) for the NADPH oxidase abolished Hcy-induced elevation in intracellular superoxide anion levels, indicating that the NADPH oxidase system might be activated upon Hcy treatment (Figure 2). There was a significant increase in NADPH oxidase activity in cells incubated with Hcy for 30 min [$203.43 \pm 37.50 \mu\text{mol} \cdot \text{min}^{-1} \cdot (10^6 \text{ cells})^{-1}$ versus $3.02 \pm 0.50 \mu\text{mol} \cdot \text{min}^{-1} \cdot (10^6 \text{ cells})^{-1}$ in the control group]. Addition of apocynin to cultured cells completely blocked Hcy-induced activation of NADPH oxidase [$2.75 \pm 0.45 \mu\text{mol} \cdot \text{min}^{-1} \cdot (10^6 \text{ cells})^{-1}$ versus $203.43 \pm 37.50 \mu\text{mol} \cdot \text{min}^{-1} \cdot (10^6 \text{ cells})^{-1}$ in the Hcy-treated group]. In addition, cells were pretreated with inhibitors specific for other candidate enzymes followed by incubation with Hcy. Pretreatment of cells with oxypurinol (an inhibitor for xanthine oxidase), L-NAME (*N*^G-nitro-L-arginine methyl ester; an inhibitor of nitric oxide synthase) or rotenone (an inhibitor of mitochondrial respiratory chain complex I) did not affect intracellular superoxide anion levels upon Hcy treatment (Figure 2). In contrast, pretreatment of monocytes with cell-permeant PEG-SOD [poly(ethylene glycol)-bound SOD], a well-known superoxide scavenger, significantly reduced intracellular superoxide anion (Figure 2). To further confirm the involvement of NADPH oxidase in Hcy-induced superoxide anion production in monocytes, cells were transfected with p47^{phox} siRNA. mRNA and protein levels of p47^{phox} were markedly reduced in cells transfected with p47^{phox} siRNA compared with cells transfected with control siRNA (Figures 3A and 3B). Inhibition of p47^{phox} expression abolished Hcy-induced superoxide anion production (Figure 3C).

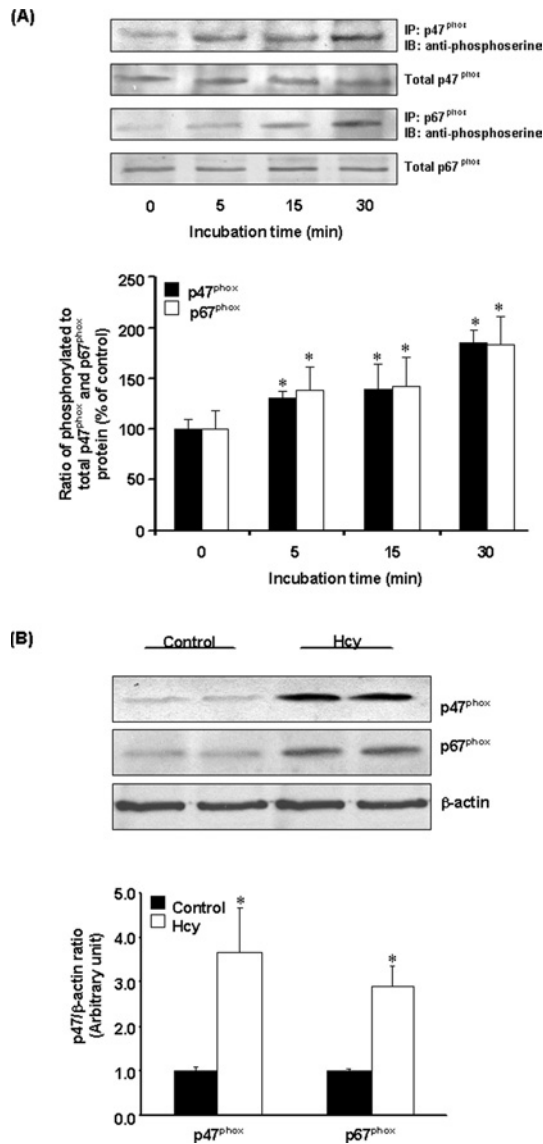


Figure 5 Phosphorylation of p47^{phox} and p67^{phox} subunits

(A) Cells were incubated with Hcy (100 μ M) or without Hcy for various time periods. p47^{phox} and p67^{phox} proteins were immunoprecipitated (IP) using goat anti-p47^{phox} or p67^{phox} antibodies followed by Western-blot analysis (IB) using anti-phosphoserine antibodies to detect serine-phosphorylated proteins. The immunoblots were analysed by densitometry. Results were normalized to total p47^{phox} and p67^{phox} proteins in the immunoprecipitates and are means \pm S.E.M. for five separate experiments. The relative densitometric units for the ratio of phosphorylated to total p47^{phox} or p67^{phox} proteins in the control cells (incubated in the absence of Hcy) were expressed as 100%. (B) Cells were incubated for 30 min in the absence (Control) or presence of Hcy (100 μ M). The membrane fraction was prepared for detection of p47^{phox} or p67^{phox} proteins by Western-blot analysis. β -Actin was used to confirm equal amount of protein loading for each sample. Representative blots were obtained from five separate experiments. *Significantly different from the control value obtained from cells incubated in the absence of Hcy ($P < 0.05$).

Mechanism of Hcy-induced NADPH oxidase activation in monocytes

To determine whether increased NADPH oxidase-mediated superoxide anion production was due to an increase in the protein levels of NADPH oxidase, Western-blot analysis was performed. There was no change in the protein levels of p47^{phox}, p67^{phox}, p22^{phox} and gp91^{phox} in Hcy-treated cells (Figure 4A). Further analysis revealed that there was no change in the mRNA levels of

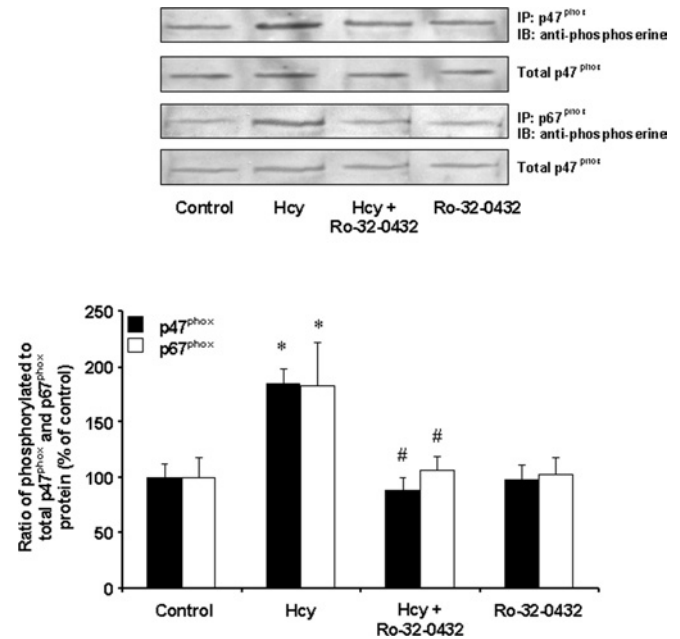


Figure 6 Effect of PKC inhibition on the phosphorylation of p47^{phox} and p67^{phox} subunits

Cells were incubated for 30 min in the absence (Control) or presence of Hcy (100 μ M). In some experiments, cells were pre-incubated with a PKC inhibitor (Ro-32-0432; 100 nM) for 30 min, followed by incubation with Hcy (100 μ M) or without Hcy for another 30 min. The cell lysate was prepared for immunoprecipitation by antibodies against p47^{phox} or p67^{phox} protein. Serine-phosphorylated p47^{phox} and p67^{phox} proteins in the immunoprecipitates were determined by Western-blot analysis using anti-phosphoserine antibodies. Results were normalized to total p47^{phox} and p67^{phox} proteins in the immunoprecipitates and are means \pm S.E.M. for five separate experiments. The relative densitometric units for the control levels were expressed as 100%. *Significantly different from the control values ($P < 0.05$). #Significantly different from the values obtained from Hcy-treated cells ($P < 0.05$).

individual oxidase subunits (Figure 4B). These results suggested that activation of NADPH oxidase in Hcy-treated cells was not due to an increase in the enzyme protein levels. To further investigate the mechanism by which Hcy treatment activated NADPH oxidase-dependent superoxide anion generation in monocytes, the phosphorylation status of the oxidase subunits was examined. Immunoprecipitation and immunoblotting analyses were carried out to detect the phosphorylation status of p47^{phox} and p67^{phox} subunits. There was a significant increase in serine phosphorylation of p47^{phox} and p67^{phox} subunits in Hcy-treated cells (Figure 5A). The levels of p47^{phox} and p67^{phox} proteins were also found to be increased in the membrane fraction of Hcy-treated cells as compared with control cells (Figure 5B). Hcy-induced phosphorylation of p47^{phox} and p67^{phox} subunits was completely abolished by a selective PKC inhibitor, Ro-32-0432 (bisindolylmaleimide XI hydrochloride) (Figure 6). These results indicated that Hcy-induced NADPH oxidase activation in monocytes was due to increased phosphorylation and subsequent membrane translocation of p47^{phox} and p67^{phox}.

Role of PKC in Hcy-stimulated NADPH oxidase activation

To investigate the involvement of PKC in Hcy-induced NADPH oxidase activation, PKC activity was measured in monocytes. PKC activity was significantly increased in cells treated with Hcy in a time-dependent manner (Figure 7A). Such a stimulatory effect was completely blocked by the PKC inhibitor Ro-32-0432 (Figure 7B). In accordance with these results, pretreatment of

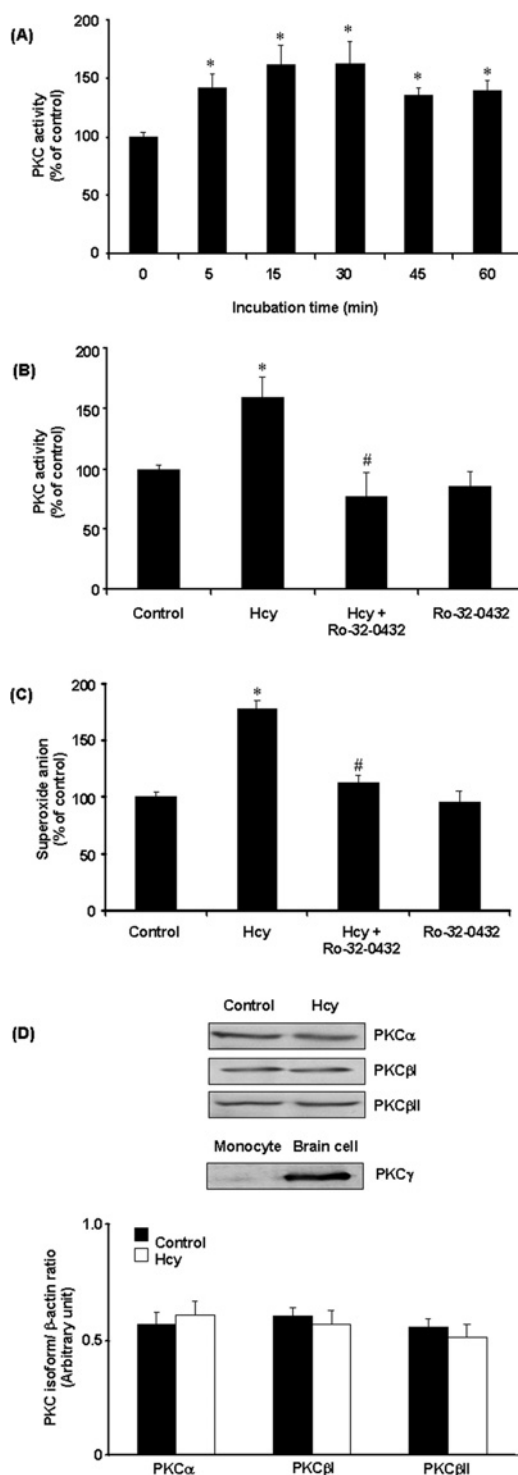


Figure 7 Effect of Hcy on PKC activity in monocytes

(A) Cells were incubated in the presence of Hcy (100 μ M) for various time periods. (B) Cells were incubated for 30 min in the absence (Control) or presence of Hcy (100 μ M). In some experiments, cells were pre-incubated with Ro-32-0432 (100 nM) for 30 min, followed by incubation with Hcy (100 μ M) or without Hcy for another 30 min. PKC activity in control cells was expressed as 100% (151.9 ± 20.06 pmol \cdot min $^{-1}$ \cdot mg of protein $^{-1}$). (C) Intracellular superoxide anion levels were measured by the NBT reduction assay. (D) The cellular protein levels of PKC α , PKC β and PKC γ isoenzymes were determined by Western-blot analysis. Proteins prepared from primary cortical cells of rat brain were used as a positive control for PKC γ . The results are means \pm S.E.M. for five experiments each performed in duplicate. *Significantly different from the control value ($P < 0.05$). #Significantly different from the value obtained from Hcy-treated cells ($P < 0.05$).

cells with Ro-32-0432 also abolished Hcy-induced superoxide anion production in these cells (Figure 7C). Together with the fact that inhibition of PKC activation could abolish Hcy-induced phosphorylation of p47^{phox} and p67^{phox} subunits in monocytes, these results suggested that PKC signalling pathway might play an important role in Hcy-induced NADPH oxidase activation. Ro-32-0432 is a selective cell-permeant inhibitor of PKC α , PKC β and PKC γ isoenzymes. Addition of this inhibitor to the culture medium could abolish NADPH oxidase-dependent superoxide anion production, indicating that activation of PKC was a prerequisite for Hcy-induced phosphorylation of p47^{phox} and p67^{phox}. It has been suggested that PKC α and PKC β (PKC β I and PKC β II) but not PKC γ are present in monocytes [29]. Consistent with these findings, only PKC α and PKC β , but not PKC γ , were detected in THP-1 monocytes (Figure 7D). Hcy treatment did not alter the intracellular protein levels of PKC α , PKC β I and PKC β II (Figure 7D). To identify whether PKC α and/or PKC β were responsible for Hcy-induced phosphorylation of NADPH oxidase subunits, cells were transfected with PKC α or PKC β antisense oligonucleotides. In cells transfected with PKC α or PKC β antisense oligonucleotides, Western-blot analysis revealed a 50, 65 and 60% reduction in PKC α , PKC β I and PKC β II protein levels respectively. On the other hand, protein levels of PKC α or PKC β were not affected in cells transfected with PKC α and PKC β sense oligonucleotides. Furthermore, transfection of antisense PKC β oligonucleotides effectively inhibited Hcy-induced phosphorylation of p47^{phox} and p67^{phox} (Figure 8A) as well as preventing Hcy-induced superoxide anion production in monocytes (Figure 8B). However, no significant changes in NADPH oxidase subunit phosphorylation and superoxide anion production were observed in cells transfected with PKC α antisense oligonucleotide or PKC α and PKC β sense oligonucleotides (Figure 8). To further confirm the role of PKC β in Hcy-induced superoxide anion production in monocytes, cells were treated with a specific PKC β inhibitor (LY333531). In the presence of LY333531, the stimulatory effect of Hcy on PKC activity was completely abolished (Figure 9A). Furthermore, LY333531 treatment also blocked Hcy-induced phosphorylation of p47^{phox} and p67^{phox} (Figure 9B). Addition of LY333531 to the culture medium completely abolished Hcy-induced superoxide anion production in monocytes (Figure 9C). These results clearly indicated that PKC β played an essential role in Hcy-induced NADPH oxidase activation in monocytes via phosphorylation of both p47^{phox} and p67^{phox} subunits leading to increased production of superoxide anions. To determine whether Hcy-induced phosphorylation of both p47^{phox} and p67^{phox} subunits via PKC signalling pathway was calcium-dependent, cells were pretreated with verapamil, a calcium channel blocker, prior to incubation with Hcy. Such a treatment completely reversed Hcy-induced phosphorylation of p47^{phox} and p67^{phox} (Figure 10A). Similar results were obtained after cells were treated with another calcium channel blocker, diltiazem (Figure 10A). Inhibition of calcium influx by calcium channel blockers also antagonized Hcy-induced superoxide anion production (Figure 10B). These results suggested that changes in calcium influx affected Hcy-induced phosphorylation of p47^{phox} and p67^{phox} and subsequently increased superoxide anion generation in monocytes.

DISCUSSION

The present study has clearly demonstrated that Hcy activates NADPH oxidase in monocytes. The PKC signalling pathway is responsible for NADPH oxidase activation in monocytes upon Hcy treatment. PKC β appears to play an important role

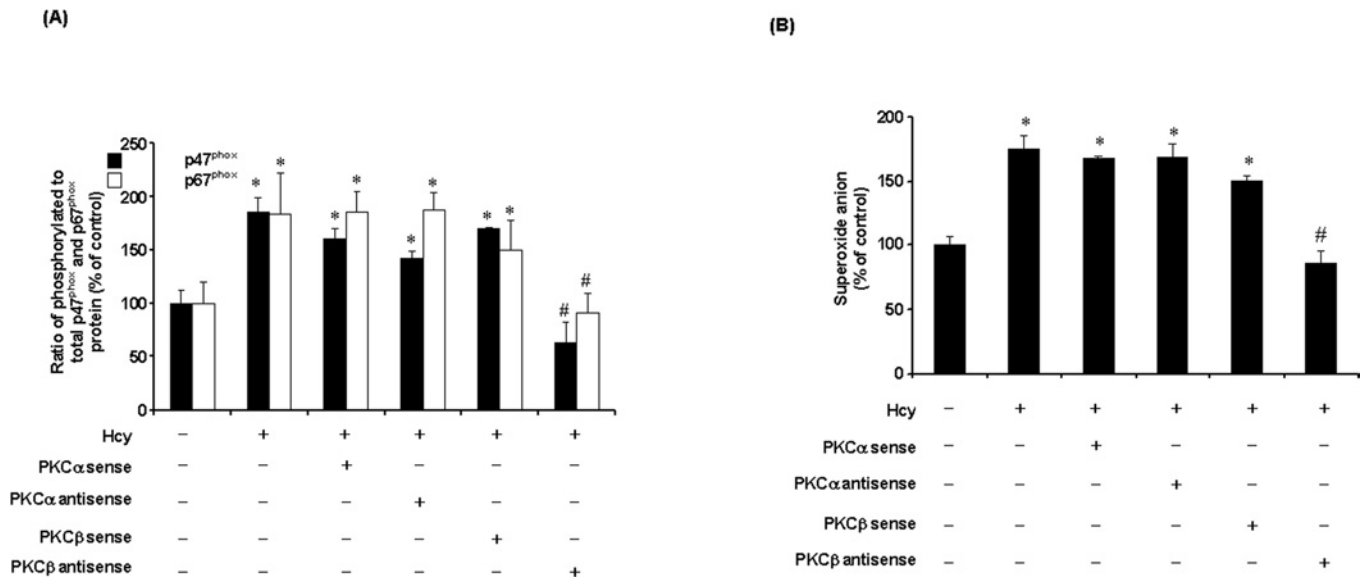


Figure 8 Effect of sense and antisense PKC isoenzyme oligonucleotide transfections on NADPH oxidase subunit phosphorylation

(A) After 48 h of transfection, cells were incubated with Hcy (100 μ M) for 30 min and the levels of serine-phosphorylated p47^{phox} and p67^{phox} proteins were determined by immunoprecipitation and Western-blot analysis. Results were normalized to total p47^{phox} and p67^{phox} proteins in the immunoprecipitates. The relative densitometric units for the control levels were expressed as 100%. (B) Intracellular superoxide anion levels were measured by the NBT reduction assay after cells were transfected with sense or antisense PKC isoenzyme oligonucleotide for 48 h. The level of superoxide anions in control cells was expressed as 100%. Results are means \pm S.E.M. for three experiments each performed in duplicate. *Significantly different from the control value ($P < 0.05$). #Significantly different from the value obtained from Hcy-treated cells ($P < 0.05$).

in stimulating phosphorylation of p47^{phox} and p67^{phox} subunits leading to the activation of NADPH oxidase in Hcy-treated cells.

Previous studies have suggested that oxidative stress is involved in Hcy-mediated cardiovascular risk [9,11,12]. The Hcy-induced ROS production has been indicated as one of the mechanisms causing cell injury in the vasculature [26–28]. One of the early inflammatory responses in monocytes is the production of superoxide anions. NADPH oxidase is considered to be the major source of superoxide anions in monocytes. In the present study, several lines of evidence suggest that NADPH oxidase is responsible for Hcy-induced elevation of superoxide anion levels in monocytes. First, there was a significant increase in NADPH oxidase activity in cells treated with Hcy. Addition of a specific NADPH oxidase inhibitor (apocynin) to cultured cells completely blocked Hcy-induced activation of NADPH oxidase and reduced superoxide anion levels to that of the control. Secondly, phosphorylation of p47^{phox} and p67^{phox} is one of the important mechanisms for NADPH oxidase activation. Indeed, we observed a significant increase in the levels of phosphorylated p47^{phox} and p67^{phox} proteins in Hcy-treated cells. Thirdly, phosphorylation of p47^{phox} and p67^{phox} by PKC is an important mechanism for NADPH oxidase activation in monocytes. Inhibition of PKC activity by either PKC inhibitors or PKC β antisense oligonucleotides not only attenuated Hcy-induced phosphorylation of p47^{phox} and p67^{phox}, but also blocked Hcy-induced superoxide anion production in these cells. Addition of inhibitors specific for other superoxide anion-producing enzymes to cultured cells failed to inhibit Hcy-induced superoxide anion production, indicating that enzymes other than NADPH oxidase were not directly involved in Hcy-stimulated superoxide anion production in monocytes. Furthermore, inhibition of p47^{phox} expression by siRNA abolished Hcy-induced superoxide anion production. Taken together, these results suggest that NADPH

oxidase is the major source of superoxide anion production in monocytes upon Hcy treatment.

We also investigated the possible mechanism by which Hcy activated NADPH oxidase in monocytes. Phosphorylation of p47^{phox} and p67^{phox} subunits is thought to be a key step that precedes membrane translocation of the cytosolic subunits. In the present study, there was a significant increase in phosphorylation of p47^{phox} and p67^{phox} subunits in monocytes upon Hcy treatment. We further investigated the molecular mechanism of increased phosphorylation of these two subunits via PKC signalling pathway. Several PKC isoforms, such as PKC α , PKC β I, PKC β II, PKC ϵ , PKC δ and PKC ζ , have been found in monocytes [29,36]. The first three isoforms (PKC α , PKC β I and PKC β II) belong to the classic or cPKC (conventional PKC) subfamily and their activation is calcium-dependent. The cPKCs expressed in monocytes are PKC α , PKC β I and PKC β II isoforms. In the present study, several lines of evidence suggested that PKC β was involved in Hcy-induced phosphorylation of p47^{phox} and p67^{phox} in monocytes. First, when cells were pretreated with an inhibitor (Ro-32-0432) that specifically inhibited PKC α , PKC β and PKC γ activities, Hcy-induced phosphorylation of p47^{phox} and p67^{phox} was completely abolished (Figure 6). Secondly, Western-blot analysis revealed that PKC α , PKC β I and PKC β II were present in monocytes, whereas little PKC γ was detected (Figure 7D). These results suggested that PKC α and PKC β might be the potential candidates playing an important role in Hcy-induced phosphorylation of p47^{phox} and p67^{phox} subunits. Thirdly, to differentiate whether PKC α and/or PKC β were involved in Hcy-induced phosphorylation of p47^{phox} and p67^{phox}, an antisense strategy was used to down-regulate PKC α and PKC β expression prior to Hcy treatment. Suppression of PKC β , but not PKC α , expression by antisense oligonucleotides completely abolished Hcy-induced serine phosphorylation of both p47^{phox} and p67^{phox} subunits in monocytes (Figure 8A). As a result, Hcy-induced

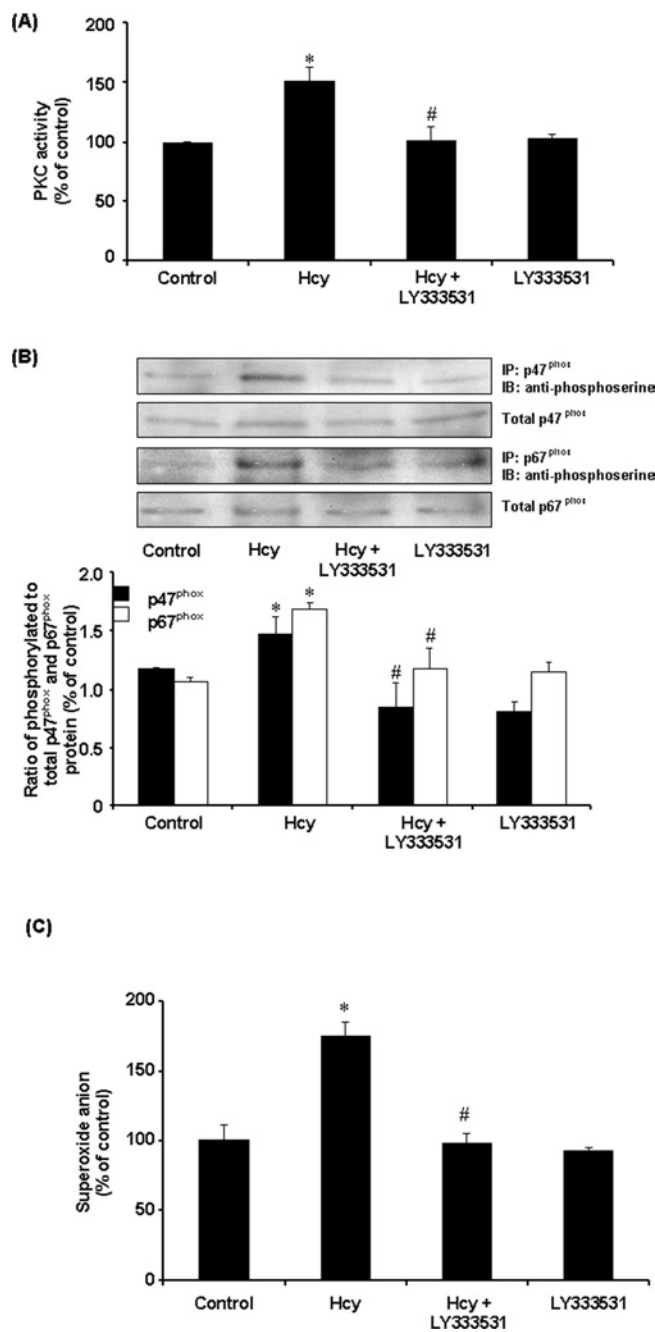


Figure 9 Effect of LY333531 on PKC activity, NADPH oxidase subunit phosphorylation and superoxide anion levels in monocytes

(A) Cells were incubated in the absence (Control) or presence of Hcy (100 μ M) for 30 min. In some experiments, cells were pre-incubated with a PKC inhibitor (LY333531; 5 nM) for 30 min, followed by incubation with Hcy (100 μ M) or without Hcy for another 30 min. PKC activity was measured. (B) Serine-phosphorylated p47^{phox} and p67^{phox} protein subunits were determined by immunoprecipitation and Western-blot analysis. Results are expressed as means \pm S.E.M. for three separate experiments. (C) Intracellular superoxide anion levels were measured by the NBT reduction assay. The level of superoxide anions in control cells was expressed as 100%. Results are expressed as means \pm S.E.M. for four separate experiments each performed in duplicate. The values obtained from control cells were expressed as an arbitrary unit of 100%. *Significantly different from the control value ($P < 0.05$). #Significantly different from the value obtained from Hcy-treated cells ($P < 0.05$).

superoxide anion production was prevented (Figure 8B). Finally, to further confirm the role of PKC β in Hcy-induced NADPH oxidase activation, a specific PKC β inhibitor (LY333531) was

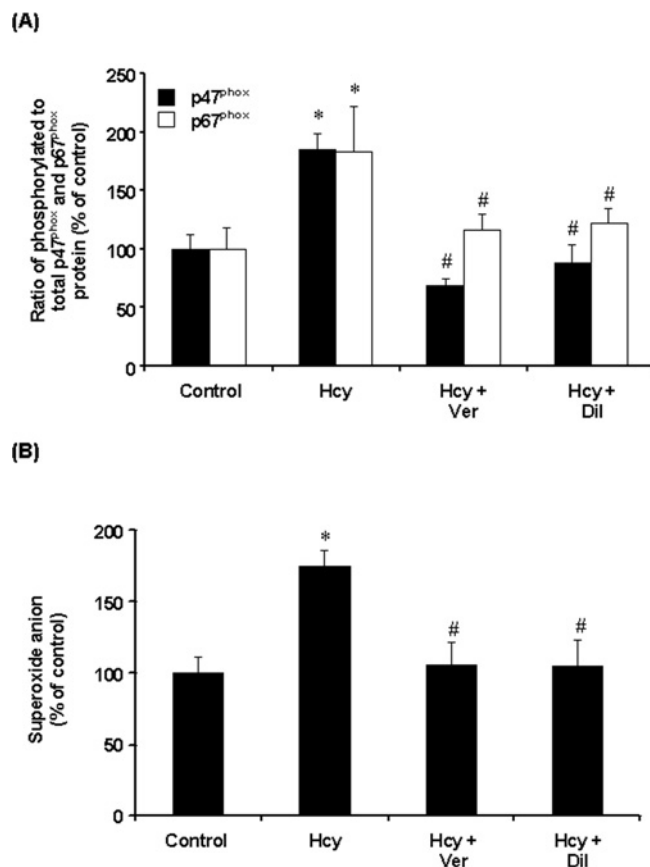


Figure 10 Effect of calcium channel blockers

Cells were incubated in the absence (Control) or presence of Hcy (100 μ M) for 30 min. In some experiments, cells were pre-incubated with verapamil (Ver; 10 nM) or diltiazem (Dil; 100 nM) for 30 min, followed by incubation with Hcy (100 μ M) or without Hcy for another 30 min. (A) Serine-phosphorylated p47^{phox} and p67^{phox} proteins were determined by immunoprecipitation and immunoblotting analysis. Results were normalized to total p47^{phox} and p67^{phox} proteins in the immunoprecipitates. (B) Intracellular superoxide anion levels were measured by the NBT reduction assay. The level of superoxide anions in control cells was expressed as 100%. Results are expressed as means \pm S.E.M. for four separate experiments each performed in duplicate. *Significantly different from the control value ($P < 0.05$). #Significantly different from the value obtained from Hcy-treated cells ($P < 0.05$).

used. Inhibition of PKC β not only abolished Hcy-induced PKC activation (Figure 9A), but also prevented Hcy-induced phosphorylation of p47^{phox} and p67^{phox} in monocytes (Figure 8B). Taken together, these results indicated that PKC activation was a prerequisite for Hcy-induced NADPH oxidase activation via phosphorylation of p47^{phox} and p67^{phox} subunits. Based on the results obtained from the present study, we propose the following mechanism by which Hcy induces superoxide anion production in monocytes: (i) Hcy activates PKC β in monocytes and, once activated, PKC β is responsible for serine phosphorylation of p47^{phox} and p67^{phox} subunits; and (ii) phosphorylation followed by membrane translocation of cytosolic subunits results in the activation of NADPH oxidase and superoxide anion production in these cells. Although our results suggest that Hcy-induced phosphorylation of p47^{phox} and p67^{phox} in monocytes appears to be mediated via PKC β , it does not exclude the possibility that other PKC isoforms can also phosphorylate p47^{phox} and p67^{phox} in monocytes activated by other stimuli. The involvement of these isoforms in Hcy-induced oxidative stress remains to be investigated in future studies.

In summary, the present study has clearly demonstrated that Hcy stimulates superoxide anion production in monocytes via activation of NADPH oxidase. PKC β plays an important role in phosphorylation of p47^{phox} and p67^{phox} leading to NADPH oxidase activation in monocytes. Dysregulation of superoxide anion levels in monocytes via NADPH oxidase may represent one of the mechanisms that contribute to Hcy-associated athero sclerosis.

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