Journal of Cellular Biochemistry 100:1255–1265 (2007)

A Role for PLCγ2 in Platelet Activation by Homocysteine

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Abstract The aim of this study was to examine the homocysteine effect on phospholipase Cγ2 (PLCγ2) activation and to investigate the signaling pathway involved. We found that homocysteine stimulated the tyrosine phosphorylation and activation of platelet PLCγ2. The tyrosine kinases p60src and p72syk appeared to be involved upstream. Reactive oxygen species were increased in homocysteine treated platelets. Likely oxidative stress could prime the non receptor-mediated tyrosine kinase p60src, inducing phosphorylation and activation of p72syk. The antioxidant *N*-acetyl-L-cysteine prevented the activation of these kinases. The phosphorylation and activation of PLCγ2 were greatly reduced by the inhibition of p72syk through piceatannol. Moreover indomethacin diminished the homocysteine effect on p60src, p72syk and PLCγ2, suggesting that thromboxane A_2 could be involved. In addition the treatment of platelets with homocysteine caused intracellular calcium rise and protein kinase C activation. Finally homocysteine induced platelet aggregation, that was partially reduced by indomethacin and by *N*-acetyl-L-cysteine of 35% or 50% respectively, while the PLCγ2 specific inhibitor U73122 diminished platelet response to homocysteine of 70%. Altogether the data indicate that PLCγ2 plays an important role in platelet activation by homocysteine and that the stimulation of this pathway requires signals through oxygen free radicals and thromboxane A_2. J. Cell. Biochem. 100: 1255–1265, 2007.

Key words: Homocysteine; p60src; p72syk; PLCγ2; platelet activation

Protein tyrosine kinases play a crucial role in many cellular responses, including cellular activation, proliferation, and differentiation. The binding of growth factors and cytokines to their receptors stimulates specific tyrosine kinase activities associated with receptors or non-receptors protein tyrosine kinases. Both types of kinases are downstream signaling events [Ullrich and Schlessinger, 1990; Taniguchi, 1995]. It is known that extracellular stress, ionizing radiation, UV irradiation, hydroperoxide or genotoxic agents can activate the non receptor p72syk and/or src family protein tyrosine kinases [Schieven et al., 1993a; Hardwick and Sefton, 1995; Brumell et al., 1996]. The exact roles of these non-receptors protein tyrosine kinases in stress-activated signaling pathway have to be clarified. The protein-tyrosine phosphorylation is suggested to be involved in the molecular mechanisms of platelet activation, because the tyrosine phosphorylation of many cellular proteins increases in platelets activated by agonists [Golden and Brugge, 1989; Nakamura and Yamamura, 1989; Bachelot et al., 1992; Razdan et al., 1994]. In particular the activation of proteins of the src family was described [Razdan et al., 1994]. Reactive oxygen species (ROS) have been indicated as intracellular messengers required for the activation of a large number of signal transduction mechanisms, especially those mediated by tyrosine kinases [Salmeen et al., 2003; Yada et al., 2003]. ROS stimulate tyrosine phosphorylation by the activation of several kinases, such as proteins of the mitogen-activated protein kinase pathway, JNK, proteins of the src family [Schieven et al., 1993a; Yoshizumi et al., 2000] and p72syk [Schieven et al., 1993b]. Src protein kinases

Abbreviations used: $cPLA_2$, cytosolic phospholipase; A_2, NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; PLC, phospholipase C; PKC, protein kinase C; ROS, reactive oxygen species.

Grant sponsor: Ministero della Salute, Roma, Italy; Grant number: 020306006029.

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Received 25 May 2006; Accepted 28 July 2006

DOI 10.1002/jcb.21123

(52–62 kDa) are highly expressed in human platelets. Among these the most abundant is p60src [Golden and Brugge, 1989]. This protein can be activated by hydrogen peroxide [Rosado et al., 2004], Moreover oxidative stress induces the activation of the tyrosine kinase p72syk in T- and B-lymphocytes [Schieven et al., 1994; Qin et al., 1997] and THP-1 cells [Rezaul et al., 1998] and promotes the association of the p72syk with phospholipase C (PLC) γ1 in porcine blood lymphocytes [Qin et al., 1995]. Additionally, oxidants produce inhibition of protein-tyrosine phosphatase in lymphocytes and macrophages [Hecht and Zick, 1992; Zor et al., 1993]. The non-receptor tyrosine kinase p72syk is expressed in cell lines of hematopoietic origin, including lymphocytes, myeloid cells, and platelets. Several studies have established a critical role for this kinase in hemostasis, as p72syk deficient mice suffer from severe hemorrhagic complications in uterus and die within the perinatal period [Turner et al., 1995]. p72syk has been identified in platelets and is rapidly activated following agonist stimulation [Taniguchi et al., 1993; Clark et al., 1994; Torti et al., 1999; Canobbio et al., 2001]. In addition the role of p72syk activation in platelets stimulated by collagen has been well established [Poole et al., 1997]. When p72syk becomes activated it may promote the tyrosine phosphorylation of downstream substrates including PLCγ2 [Clark et al., 1994].

Platelet hyperactivity seems to be directly involved in thrombotic events. A positive correlation of plasma homocysteine levels and cardiovascular diseases was shown, as increased homocysteine level in patients with stroke or atherosclerosis was measured [Domagala et al., 1998; Cattaneo, 1999]. Endothelial injury is one of the pathological changes in the development of the vascular disease involving platelet activation. Oxidative stress and/or lipid peroxidation has been reported to be implicated in the development of atherosclerosis or thrombosis [Domagala et al., 1998; de Jong et al., 1998; Cattaneo, 1999]. Some evidence indicate that homocysteine generates oxidative damage. Moreover homocysteine impairs nitric oxide (NO) release from endothelial cells [Upchurch et al., 1997] and platelets [Mutus et al., 2001; Leoncini et al., 2003], enhances arachidonic acid release in platelets [Signorello et al., 2002], and stimulates smooth muscle cell proliferation [Jeremy et al., 1999]. The endothelial cell injury in vitro seems to be associated to the progressive loss of NO-mediated inhibition of platelet aggregation [Stamler et al., 1993]. In addition homocysteine increases the adhesiveness of endothelial cells to platelets [Dardik et al., 2000]. Taken together all these effects participate in the platelet hyperactivity described in hyperhomocysteinemia. Increased platelet activation may be one of the mechanisms involved in the elevated risk of thrombosis and atherosclerosis. In the present report we have studied the homocysteine effects on PLCγ2 pathway. We have found that the tyrosine kinases p60src and p72syk are phosphorylated in platelets treated with homocysteine, providing evidence that p72syk may be implicated in the phosphorylation of PLCγ2. Moreover homocysteine induces protein kinase C (PKC) activation and intracellular calcium rise. Likely homocysteine activates PLCγ2. All these mechanisms can contribute to platelet hyperactivity shown in hyperhomocysteinemia.

MATERIALS AND METHODS

Blood Collection and Preparative Procedure

Freshly drawn venous blood from healthy volunteers was collected into 130 mM aqueous trisodium citrate anticoagulant solution (9:1). The donors claimed to have not taken drugs known to interfere with platelet function during 2 weeks prior to blood collection and gave their informed consent. Platelet-rich plasma and washed platelets were prepared as described elsewhere [Leoncini et al., 1990]. Briefly platelet-rich plasma, obtained by centrifugation of the whole blood at 100*g* for 25 min and added to 1 μg/ml apyrase and 1 μM PGE_1, was centrifuged at 1,000*g* for 15 min. Pellet was washed once with pH 4.8 ACD solution (75 mM trisodium citrate, 42 mM citric acid and 136 mM glucose) and centrifuged at 1,000*g* for 15 min, then was resuspended in Ca^{2+}-free pH 7.4 HEPES buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Glucose, 10 mM HEPES). The final pH of platelet suspension was 7.4. All chemicals, if not otherwise indicated, were from Sigma.

ROS Determination

DCFH-DA is a ROS-sensitive probe that can be used to detect ROS production in living cells. DCFH oxidation yields the fluorescent adduct DCF that is trapped inside the cells. DCFH-DA

was prepared from DCF (Fluka) according to Brandt and Keston [1965]. Washed platelets (5.0×10^7 platelets/ml) were incubated for 15 min at 37°C with 10 μM DCFH-DA then centrifuged for 15 min at 1,100*g*. The obtained pellet was resuspended at 5.0×10^7 platelets/ml in pH 7.4 HEPES buffer. Samples, prewarmed for 15 min at 37°C with saline or additions, were incubated with homocysteine as indicated, under unstirring condition. Incubation, prolonged for further 15 min, was stopped by sonicating samples twice for 10 s on ice. After a brief centrifugation, supernatants were appropriately diluted and the fluorescence of suitable aliquots was measured using a Perkin–Elmer fluorescence spectrometer model LS50B with 504 nm excitation and 526 nm emission. The concentration of intracellular DCF was calculated by a standard curve of the commercial compound.

Total Protein Tyrosine Phosphorylation Assay

Platelet suspensions (1.0×10^9 platelets/ml), prewarmed with saline or genistein at 37°C for 15 min, were treated with homocysteine as indicated. Incubation was stopped by addition of 2× Laemmli-SDS reducing sample buffer. Samples heated for 5 min at 100°C, were separated by 5–10% SDS–PAGE and transferred to nitrocellulose membranes (Bio-Rad). Running was performed in the presence of phosphotyrosine molecular weight standard markers. Blots were blocked in 5% fat-free dry milk dissolved in TBST (Tris buffer saline, pH 7.6 10 mM Tris, 150 mM NaCl, containing 0.1% Tween 20) for 30 min at 37°C and then incubated overnight at 4°C with antiphosphotyrosine 4G10 clone (1/1,000 dilution) (Upstate). Membranes were extensively washed and incubated for 60 min at room temperature with horseradish peroxidase-conjugated secondary antibody (Santa Cruz). After further washings, blots were developed using the Amersham ECL system, revealed by Bio-Rad Chemi-Doc and the optical density, reported as arbitrary unit, quantified with the related software package.

Tyrosine Phosphatase Assay

Washed platelets (2.0×10^9 platelets/ml) were resuspended in pH 7.4 HEPES buffer containing 1.0 mM PMSF and 10 μg/ml leupeptin and lysed by sonication. The cell-free extract was centrifuged at 700*g* for 20 min to remove intact cells and large particles, and then incubated with homocysteine or Na_3VO_4, as indicated. Tyrosine phosphatase activity was assayed spectrophotometrically using 10 mM orthophospho-L-tyrosine as substrate in 100 mM pH 4.0 sodium acetate buffer. The rate of dephosphorylation was determined by measuring inorganic phosphate released by the Fiske-Subbarow's method [Leloir and Cardini, 1957]. Proteins were quantified according to the Lowry method [Lowry et al., 1951].

Immunoblotting

Washed platelets (1.0×10^9 platelets/ml), preincubated at 37°C for 15 min with saline or various agents, were treated with homocysteine, as indicated. Incubation was stopped by addition of 2×Laemmli-SDS reducing sample buffer and samples were treated as above described. Blots were incubated with anti-phospho-p60src (tyr^{416}) (Cell Signaling) antibody. At last, nitrocellulose membranes were stripped by incubation with 62.5 mM pH 6.7 Tris/HCl, 2% SDS, 100 mM β-mercaptoethanol for 30 min at 50°C, reprobed with antibody against p60src (Upstate) and protein bands revealed as detailed above.

Immunoprecipitation

Washed platelets (1.0×10^9 platelets/ml), prewarmed for 15 min at 37°C with saline or agents, were incubated with homocysteine as indicated. Incubation was stopped by adding an equal volume of lysis mixture (0.5% SDS, 1% Triton X-100, 0.75% sodium deoxycholate, 10 mM EDTA, 1 mM PMSF, 50 mM NaF, 200 μM Na_3VO_4, 100 μM leupeptin, 100 μg/ml aprotinin, 10 μM staurosporine). Lysates, after a brief centrifugation, were treated with 1.0 μg of anti-p72syk (Upstate), or anti-PLCγ2 (Santa Cruz) antibodies for 2 h at 4°C. The immunocomplexes were precipitated with 100 μl of protein A-sepharose CL-4B (Amersham). After 60 min on ice, immunoprecipitates were washed with 1.0 ml of IP-wash 1 (10 mM pH 7.4 Tris/HCl, 150 mM NaCl, 0.5% Triton X-100), followed by IP-wash 2 (10 mM pH 7.Tris/HCl, 750 mM NaCl, 0.5% Triton X-100) and finally again with IP-wash 1. Immunoprecipitates were extracted with 100 μl of 2×Laemmli-SDS reducing sample buffer, heated at 60°C for 20 min and resolved on 5–10% SDS–PAGE. Gels were transferred to nitrocellulose membranes and blots were treated as detailed above. Blots were stripped, reprobed with antibodies

against p72syk or PLCγ2, and protein bands revealed as described above.

Measurement of p47 kDa Phosphorylation

Washed platelets (2.5×10^9 platelets/ml), resuspended in pH 7.4 HEPES buffer containing 1 mM EGTA and 5% platelet-poor plasma, were incubated at 37°C with [^{32}P] phosphoric acid (1 mCi/ml) (Perkin Elmer), under gentle shaking. After 60 min, platelet suspension was centrifuged and the pellet, washed once, was finally resuspended to 2.0×10^8 platelets/ml in the same buffer. Samples were preincubated for 15 min at 37°C with saline or additions, then homocysteine or agonists were added when required. Incubation was stopped by the addition of suitable aliquots of 2×Laemmli-SDS reducing sample buffer. The samples were heated for 5 min at 100°C and proteins separated by 5–10% SDS–PAGE with molecular weight standard markers. Gels were dried and [^{32}P] phosphorylated bands, revealed by the Packard Cyclone Storage Phosphor System, were quantified by the related software package.

Measurement of Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} concentration was measured as previously described [Rotondo et al., 1997] with slight modifications. Washed platelets (3.0×10^8 platelets/ml) were incubated with 1 μg/ml FURA 2/AM (Calbiochem) for 60 min at 37°C. Two μM PGE_1 and 1 mM EGTA were added before centrifuging loaded platelets for 15 min at 1,100*g*. The pellet, resuspended at 3.0×10^8 platelets/ml in the same buffer, was preincubated at 37°C for 10 min with additions, then 50 μM homocysteine was added. FURA 2-loaded platelet fluorescence was monitored under unstirring conditions at 37°C for 15 min in a Perkin-Elmer fluorescence spectrometer model LS50B with excitation at 340 nm and 380 nm and emission at 509 nm. The fluorescence of fully saturated FURA 2 (F_{max}) was obtained by lysing the cells with 50 μM digitonin in the presence of 2 mM Ca^{2+}, while F_{min} was determined by exposing the lysed platelets to 1 M EGTA. The fluorescence was fully quenched with 5 mM Mn^{2+} to give the autofluorescence value. A software combined with the fluorescence spectrometer converted data into cytosolic Ca^{2+} concentration. The K_d value for FURA 2 and Ca^{2+} was 135 nM.

Platelet Aggregation

Platelet aggregation was monitored according to Born's method [Born, 1962] in a Menarini Aggrecoder PA-3210 aggregometer, and quantified by the light transmission reached within 10 min. Washed platelets (3.0×10^8 platelets/ml) were preincubated with saline or the agents for 10 min at 37°C before the homocysteine addition.

Data Analysis

Data are the mean ± SD of at least three independent determinations. Statistical analysis was performed using the unpaired Student's *t*-test and considering significant the difference between control and each treatment at least at 5% level ($P < 0.05$).

RESULTS

Homocysteine Effect on ROS Formation

Resting platelets generate a continuous basal flux of ROS, including superoxide anion and hydrogen peroxide. Moreover the treatment of platelets with agonists increases the levels of these oxygen radicals [Leoncini et al., 1991]. The data of Figure 1A show that homocysteine, in a dose-dependent manner, is able to increase ROS basal level, reaching the maximal effect at 100 μM. ROS elevation induced by homocysteine is significantly ($P < 0.01$) reduced by the NADPH oxidase inhibitor DPI and by indomethacin, and abolished by $AACOCF_3$ (Calbiochem) and ETYA, which are inhibitors of cytosolic phospholipase A_2 ($cPLA_2$) activation and of arachidonic acid metabolism. In addition, the antioxidant reagent *N*-acetyl-L-cysteine (NAC) exerts a powerful effect, abolishing ROS generated by homocysteine and greatly reducing ROS basal intracellular level (Fig. 1B).

Effect of Homocysteine on Protein Tyrosine Phosphorylation

In Figure 2A a representative phosphotyrosine immunoblotting experiment, in which the whole platelets have been treated with varying homocysteine concentrations, demonstrates that homocysteine increases total tyrosine phosphorylation. In particular several proteins seem to be involved. The phosphoproteins p52–62, p72 and p145 kDa are dominant, being the kinetics of appearance different. As expected the preincubation of platelets with the protein

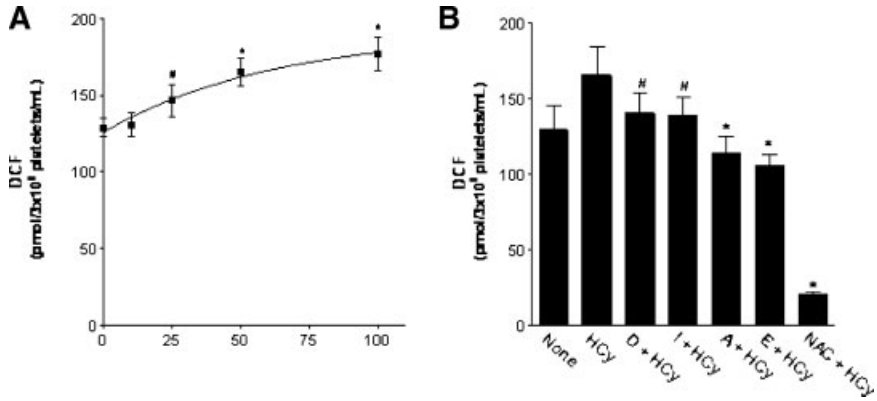

Fig. 1. Effect of homocysteine on ROS formation. **A**: Washed platelets (5.0×10^7 platelets/ml), loaded with 10 μM DCFH-DA, were preincubated for 15 min at 37°C with saline and then incubated in the presence of increasing concentrations of homocysteine for further 15 min. Values are mean ± SD of four separated experiments carried out in triplicate. $^{\#}P < 0.0125$; $^{*}P < 0.0005$. **B**: Washed platelets (5.0×10^7 platelets/ml), loaded with 10 μM DCFH-DA and preincubated for 15 min at 37°C with saline, 50 μM DPI (D), 50 μM indomethacin (I), 50 μM $AACOCF_3$ (A), 50 μM ETYA (E) or 5 mM NAC (N) were incubated with 50 μM homocysteine (HCy) for further 15 min. Results represent the mean ± SD of four experiments carried out in triplicate. $^{\#}P < 0.01$; $^{*}P < 0.0005$ versus homocysteine.

tyrosine kinase inhibitor genistein [Akiyama et al., 1987] abolishes the tyrosine phosphorylation of these proteins. Data (mean ± SD) of the tyrosine phosphorylation patterns obtained from three similar experiments in Figure 2B are shown. The increase in protein tyrosine phosphorylation induced by homocysteine can be dependent on tyrosine kinase activation and/or on tyrosine phosphatases inhibition. Since homocysteine does not affect tyrosine

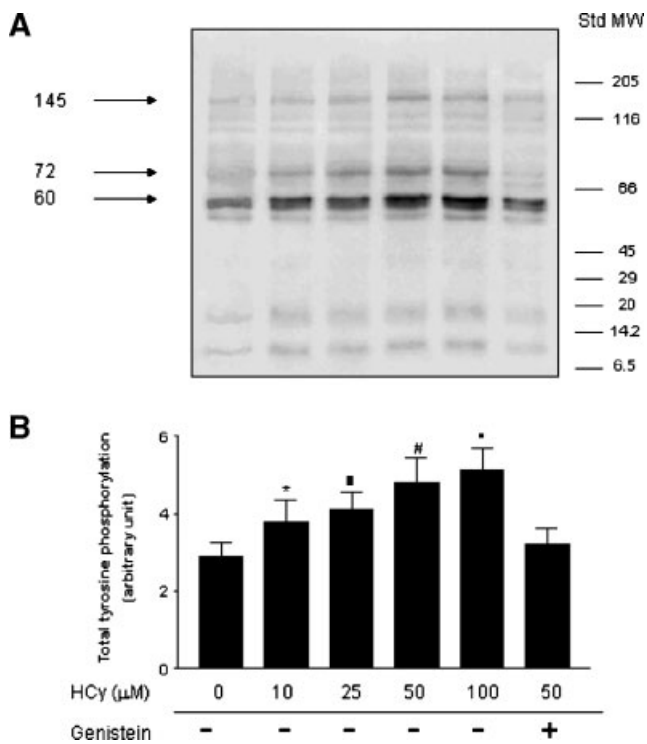

Fig. 2. Effect of homocysteine on protein tyrosine phosphorylation. Washed platelets (1.0×10^9 platelets/ml), prewarmed for 15 min at 37°C with saline or 100 μM genistein, were incubated for 15 min with homocysteine (HCy) as indicated. **A**: Blot is representative of three separated experiments. **B**: Densitometric scanning ± SD of the total tyrosine phosphorylation of three experiments is shown. $^{*}P < 0.05$; $^{\S}P < 0.01$; $^{\#}P < 0.005$; $^{\bullet}P < 0.0025$ versus none.

TABLE I. Effect of Homocysteine on Tyrosine Phosphatase Activity

	Total tyrosine phosphatase activity (nmol/mg/min)
None	42.1 ± 0.1
10 μM HCy	41.6 ± 0.2
25 μM HCy	44.5 ± 0.2
50 μM HCy	45.6 ± 0.1
100 μM HCy	43.2 ± 0.3
1 mM Na_3VO_4	9.2 ± 0.5*

The cell-free extracts were incubated with homocysteine or Na_3VO_4 as indicated. Tyrosine phosphatase activity was determined by measuring released inorganic phosphate by the Fiske–Subbarow's method. Values are the mean ± SD of three experiments carried out in triplicate.
*$P < 0.0005$ versus none.

phosphatase activities (Table I), likely homocysteine stimulates protein tyrosine kinases. Since p60src, p72syk and PLCγ2 (145 kDa) seem to specifically involved, the homocysteine effect on these proteins was deeply examined.

Homocysteine Effect on p60src Phosphorylation

Homocysteine is able to increase the ROS basal level [Leoncini et al., 2006]. ROS activate a large number of signal transduction steps, especially those mediated by tyrosine kinases [Salmeen et al., 2003; Yada et al., 2003]. In particular the tyrosine kinase p60src is considered a target of oxidant species [Schieven et al., 1993; Yoshizumi et al., 2000]. Thus we wanted to investigate whether p60src could be phosphorylated in platelets treated with homocysteine. Results of Figure 3A demonstrate that homocysteine induces the phosphorylation of p60src in a dose-dependent manner, giving a relevant effect at 50 μM. The homocysteine effect is abolished by NAC and is partially reduced by indomethacin, suggesting that oxidative mechanisms and thromboxane A_2 could be involved in p60src phosphorylation (Fig. 3B).

Homocysteine Effect on p72syk and PLCγ2 Phosphorylation

Previous studies have shown that p72syk is rapidly activated in human platelets stimulated by agonists [Taniguchi et al., 1993; Clark et al., 1994; Torti et al., 1999; Canobbio et al., 2001] and in response to oxidative stress [Schieven et al., 1994; Qin et al., 1997]. Since homocysteine elevates ROS concentration (Fig. 1A), we aimed to measure the homocysteine effect on p72syk phosphorylation. A significant increase in tyrosine phosphorylation of this kinase in homocysteine treated platelets was shown. The dose-dependent effect is relevant at 25 μM and peakes at 100 μM homocysteine (Fig. 4A). The p60src kinase specific inhibitor PP1 (Calbiochem) and the antioxidant agent NAC abolish

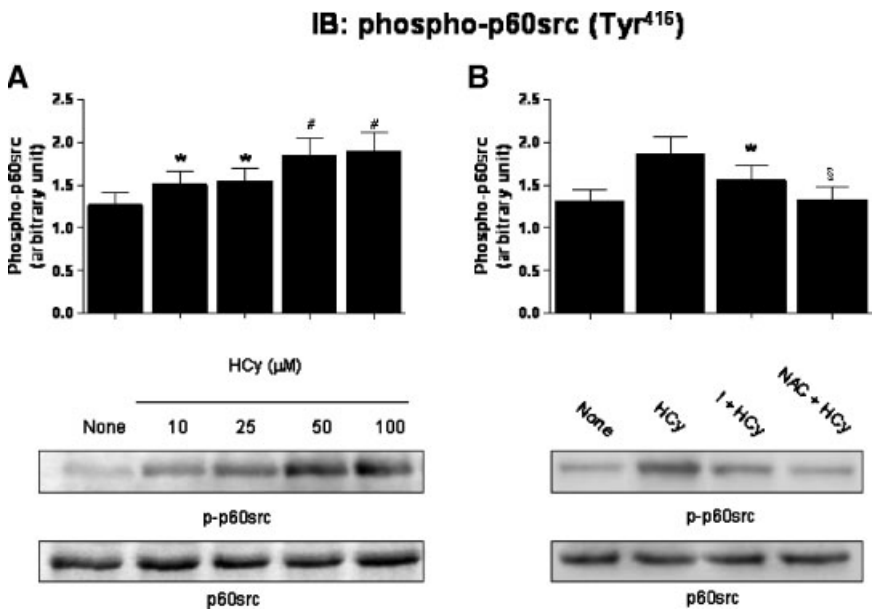

Fig. 3. Homocysteine induced tyrosine phosphorylation of p60src. Washed platelets (1.0×10^9 platelets/ml), prewarmed for 15 min at 37°C with saline, were incubated for 15 min at 37°C with homocysteine (HCy) as indicated (**A**). **B**: Washed platelets, pretreated for 15 min at 37°C with saline, 50 μM indomethacin (I) or 5 mM NAC were incubated for 15 min at 37°C with 50 μM homocysteine (HCy). Phospho-p60src(tyr^{416}) was immunoblotted with the specific antibody. Blots are representative of three independent but similar experiments. In the **upper panels** the densitometric scanning ± SD of the p60src phosphorylation of three experiments is shown. (A) *$P < 0.05$; #$P < 0.005$ versus none. (B) *$P < 0.05$; §$P < 0.01$ versus HCy.

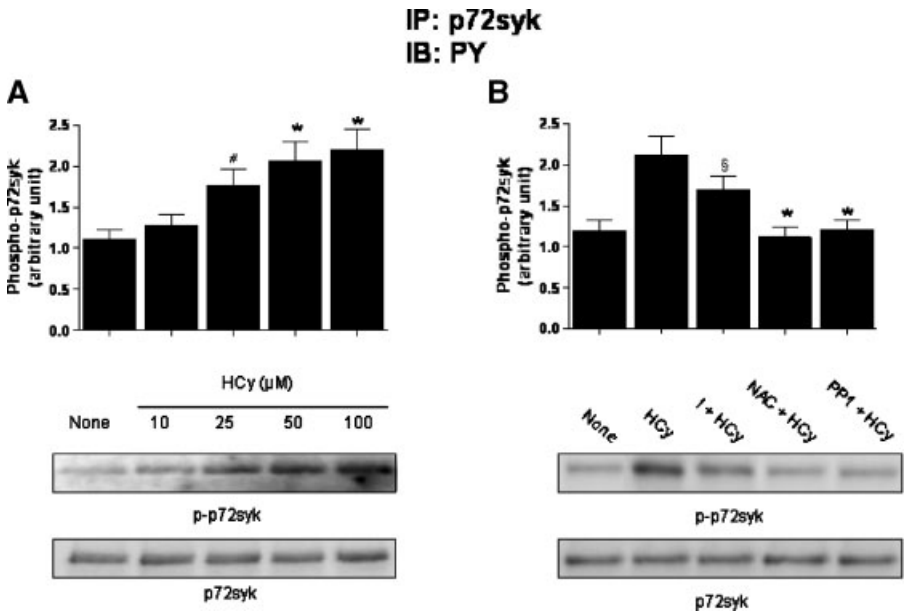

Fig. 4. Homocysteine-induced tyrosine phosphorylation of p72syk. Washed platelets (1.0×10^9 platelets/ml), prewarmed for 15 min at 37°C with saline, were incubated for 15 min at 37°C with homocysteine (HCy) as indicated (**A**). **B**: Washed platelets, pretreated for 15 min at 37°C with saline, 50 μM indomethacin (I), 5 mM NAC or 20 μM PP1 were incubated for 15 min at 37°C with 50 μM homocysteine (HCy). p72syk was immunoprecipitated with the specific antibody and immunoblotted with antiphosphotyrosine antibody. Blots are representative of three independent and similar experiments. In the upper panels the densitometric scanning ± SD of the p72syk phosphorylation of three experiments is shown. (A) $^{\#}P < 0.005$; $^{*}P < 0.0025$ versus none. (B) $^{\S}P < 0.05$; $^{*}P < 0.0025$ versus HCy.

the p72syk phosphorylation induced by homocysteine. Moreover indomethacin greatly reduces the p72syk tyrosine phosphorylation, indicating that thromboxane A_2 is involved (Fig. 4B). It is noteworthy that p60src and p72syk phosphorylation are strictly correlated ($y = 1.735 \times -1.130$; $r^2 = 0.8975$).

We further investigated whether p72syk could lead to tyrosine phosphorylation of PLCγ2. Results indicate that homocysteine dose-dependently induces PLCγ2 phosphorylation (Fig. 5A). The homocysteine effect is largely reduced by the p72syk inhibitor piceatannol, suggesting that PLCγ2 lies downstream of activated p72syk. A strict correlation between p72syk and PLCγ2 phosphorylation was shown ($y = 0.6548 \times +0.511$; $r^2 = 0.8273$). Moreover PLCγ2 phosphorylation is diminished by the preincubation of platelets with indomethacin. Likely the phosphorylation of PLCγ2 in homocysteine treated platelets is downstream $cPLA_2$ and requires the production of thromboxane A_2 (Fig. 5B).

Effect of Homocysteine on PKC Activation

Activation of PLC leads to the formation of diacylglycerol, which in platelets activates PKC. Calcium is also required for PLC activation. The main platelet substrate for PKC is the p47 protein (pleckstrin), that represents a reliable marker for PLC activation. Thus to evaluate the homocysteine effect on PLCγ2 activation we have measured the p47 protein phosphorylation. The phosphorylation of pleckstrin induced by homocysteine in [^{32}P]-labeled platelets is a dose-dependent mechanism (data not shown). Moreover comparing homocysteine with known platelet agonists able to induce activation of PLCγ2, we have shown that 50 μM homocysteine produces a similar response to collagen (Mascia Brunelli), but smaller than U46619 (Calbiochem) and PMA (Fig. 6A). The homocysteine effect on PLCγ2 activation is abolished by the PKC inhibitor RO-31-8220 (Calbiochem), by NAC, genistein and PP1. In addition piceatannol and indomethacin strongly decrease p47 phosphorylation induced by homocysteine (Fig. 6B).

Effect of Various Agents on Calcium Elevation

Homocysteine induces calcium elevation: in the presence of 50 μM homocysteine the calcium basal level is increased from 95.7 ± 4.3 to 180.2 ± 6.1 nM. Since PLCγ2 induces

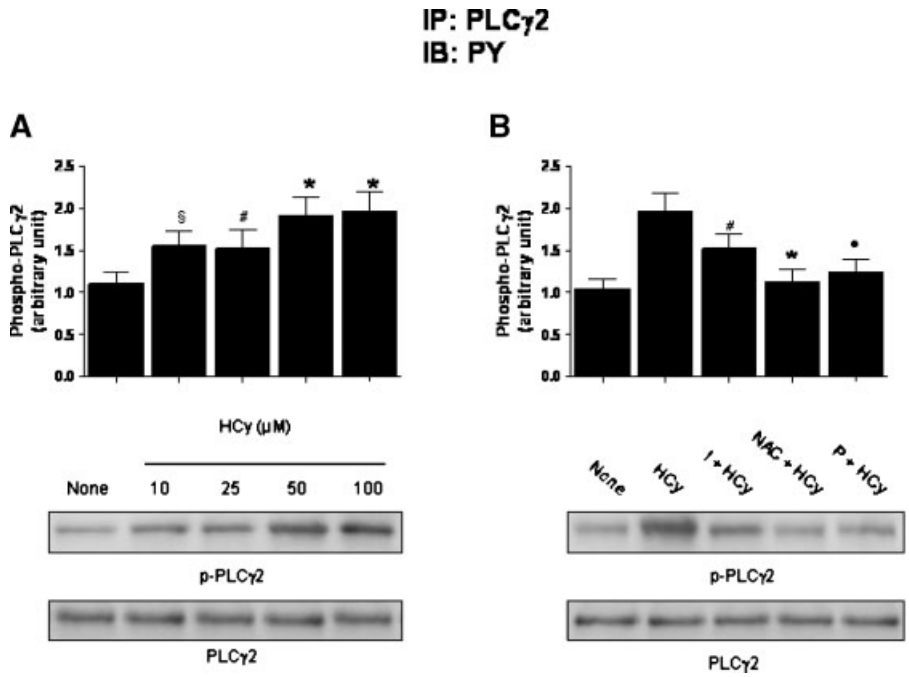

Fig. 5. Homocysteine induced tyrosine phosphorylation of PLCγ2. Washed platelets (1.0×10^9 platelets/ml), prewarmed for 15 min at 37°C with saline, were incubated for 15 min at 37°C with homocysteine (HCy) as indicated (**A**). **B**: Washed platelets, prewarmed 15 min at 37°C with saline, 50 μM indomethacin (I), 5 mM NAC or 30 μM piceatannol (P), were incubated for 15 min at 37°C with 50 μM homocysteine (HCy). PLCγ2 was immunoprecipitated with the specific antibody and immunoblotted with antiphosphotyrosine antibody. Blots are representative of three independent and similar experiments. In the **upper panels** the densitometric scanning ± SD of the PLCγ2 phosphorylation of three experiments is shown. (A) $^{\S}P < 0.0125$; $^{\#}P < 0.025$; $^{*}P < 0.0025$ versus none. (B) $^{\#}P < 0.025$; $^{*}P < 0.0025$; $^{\bullet}P < 0.005$ versus HCy.

intracellular calcium rise through the inositol 3,4,5 triphosphate release, we measured the effect of known inhibitors of specific steps of the p60src/PLCγ2 signaling cascade. Data of Figure 7 show that calcium rise promoted by homocysteine is partially (about 30%) reduced by PP1 and ETYA. Piceatannol and indomethacin produce an inhibition of 50% or 58%,

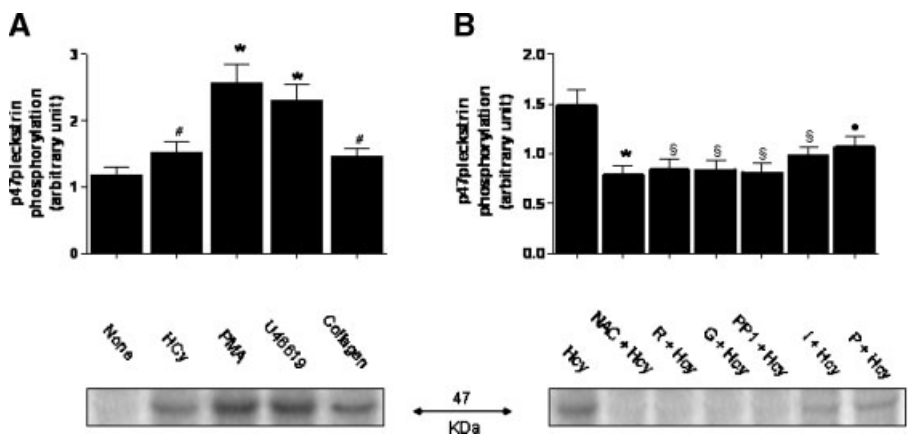

Fig. 6. Effect of homocysteine on p47pleckstrin phosphorylation. [^{32}P]-Labeled washed platelets (2.0×10^8 platelets/ml), preincubated for 15 min at 37°C with saline, were stimulated with 50 μM homocysteine (HCy), 1 μM PMA, 10 μM U46619 or 5 μg/ml collagen (**panel A**). In panel B [^{32}P]-labeled washed platelets were pretreated with 5 mM NAC, 10 μM Ro-31-8220 (R), 100 μM genistein (G), 20 μM PP1, 50 μM indomethacin (I) or 30 μM piceatannol (P) for 15 min at 37°C and then incubated with 50 μM homocysteine (HCy). After 15 min at 37°C, incubation was stopped by the addition of suitable aliquots of 2×Laemmli buffer. Upon separation of platelet proteins by SDS–PAGE, phosphorylated p47pleckstrin was revealed in a Phosphor Imager System. Data are representative of three independent experiments yielding similar results. In the **upper panels** the densitometric scanning ± SD of p47pleckstrin phosphorylation of three experiments is shown. (**A**) $^{\#}P < 0.025$; $^{*}P < 0.0005$ versus none. (**B**) $^{\S}P < 0.0025$; $^{\bullet}P < 0.005$; $^{*}P < 0.0005$ versus HCy.

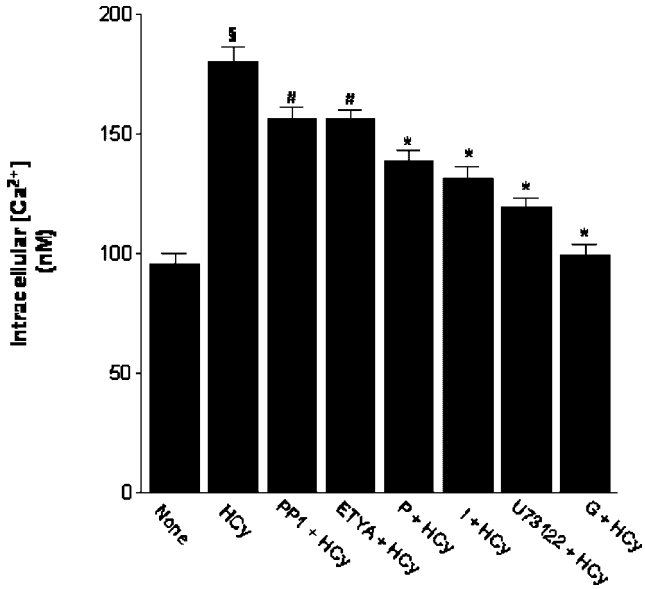

Fig. 7. Effect of inhibitors on intracellular $[Ca^{2+}]$ elevation induced by homocysteine. FURA 2-loaded platelets (3.0×10^8 platelets/ml) were preincubated for 10 min at 37°C with saline, 20 μM PP1, 50 μM ETYA, 30 μM piceatannol (P), 50 μM indomethacin (I), 10 μM U73122, 100 μM genistein (G) and then treated with 50 μM homocysteine (HCy) for another 15 min. Data are the mean ± SD of three experiments carried out in duplicate. $^{\S}P < 0.0005$ versus none; $^{\#}P < 0.0025$, $^{*}P < 0.0005$ versus homocysteine.

respectively. Moreover in the presence of the PLCγ2 inhibitor U73122 the calcium rise is reduced of about 70%, while genistein is more effective.

Platelet Aggregation

Previously we have shown that homocysteine induces platelet aggregation. In particular the platelet treatment with 100 μM homocysteine produced a 35–40% increase of basal response [Leoncini et al., 2006]. Thus we wanted to measure the effect of various agents which interfere with calcium elevation, arachidonic acid release and/or metabolism on homocysteine induced aggregation. We found that indomethacin, ETYA, $AACOCF_3$ and the p38MAPK inhibitor SB203580 (Calbiochem) inhibited aggregation of 35%, 30%, 45%, and 32%, respectively. The effects of PP1 (about 35%) and ETYA on platelet aggregation were quite similar to that exerted on calcium rise. Moreover NAC reduced the aggregation of 45–50% and U73122 was the most potent showing an inhibition of 70%.

DISCUSSION

The present study shows that homocysteine increases total protein tyrosine phosphorylation, an event indicative of platelet activation [Golden and Brugge, 1989; Nakamura and Yamamura, 1989; Bachelot et al., 1992; Razdan et al., 1994]. In particular the tyrosine kinases p60src, p72syk and PLCγ2 seem to be the main targets of the homocysteine effect. Early signals generated by homocysteine can activate p60src and subsequently p72syk. Previous studies [Signorello et al., 2002] have demonstrated that homocysteine promotes arachidonic acid release and thromboxane A_2 formation by the involvement of $cPLA_2$. The $cPLA_2$ activation occurs through the phosphorylation and by the intracellular calcium elevation, which are both stimulated by homocysteine [Leoncini et al., 2006]. In the present report, we show that ROS are generated in platelets treated with homocysteine. Thus oxidative stress could prime the non receptor-mediated tyrosine kinase p60src, which in turn phosphorylates and activates p72syk. The antioxidant NAC abolishes the activation of both p60src and p72syk, stressing the role of the ROS in the stimulation of this pathway. Moreover indomethacin partially reduces the homocysteine effect on p60src and p72syk, indicating that the activation of these enzymes can require the production of thromboxane A_2. PLCγ2 has been reported to be a substrate for p72syk in stimulated platelets [Poole et al., 1997]. To investigate the probable

role of p72syk in the phosphorylation and activation of PLCγ2, we studied the effect of piceatannol on this enzyme. Data clearly show that p72syk is involved in the PLCγ2 phosphorylation (Fig. 5B) and activation (Fig. 6). In addition PLCγ2 activation induced by homocysteine could be regulated by the lipid products of phosphatydilinositol 3-kinase [Poole et al., 1997; Carpenter and Ji, 1999], such as wortmannin (Calbiochem) which inhibits PLCγ2 activity (data not shown). The tyrosine phosphorylation is not sufficient to allow expression of PLCγ2 activity, which requires its specific interaction with the membrane via phosphatydilinositol (3, 4, 5)-trisphosphate generated by phosphatydilinositol 3-kinase. The results stated in the present report suggest that PLCγ2 lies downstream of p60src and p72syk in platelets treated with homocysteine. The phosphorylation of these enzymes seems to be primed by ROS generated by homocysteine itself and by the homocysteine induced arachidonic acid release [Leoncini et al., 2006]. In addition thromboxane A_2 can have a role in PLCγ2 activation. This activated enzyme produces the second messengers inositol 1, 4, 5 trisphosphate and diacylglycerol both of which are involved in platelet activation. Inositol 1, 4, 5 trisphosphate induces calcium mobilisation. Diacylglycerol stimulates PKC, which allows platelet secretion, conformational changes and aggregation. Homocysteine stimulates calcium rise (Fig. 7), that maximally (70%) derives from intracellular calcium store depletion and for the remaining percent from Ca^{2+} influx as previously shown in oxidative stress conditions [Ermak and Davies, 2002]. Moreover homocysteine reduces NO bioavailability [Upchurch et al., 1997; Mutus et al., 2001; Leoncini et al., 2003], contributing to generate platelet hyperactivity.

In conclusion through the mechanisms reported in this study and others previously described [Leoncini et al., 2006], homocysteine induces platelet activation and sensitizes the platelets for the later stimulation by agonists resulting in increased aggregation and secretion. Thus homocysteine could have a role in the pathogenesis of various vascular diseases [Townend et al., 1998].

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