Altered nitric oxide/cGMP platelet signaling pathway in platelets from patients with acute coronary syndromes

Title: Altered nitric oxide/cGMP platelet signalling pathway in platelets from patients with acute coronary syndromes

Article Type: Original Paper

Corresponding Author: PhD Loredana Bergandi,

Corresponding Author's Institution:

First Author: Loredana Bergandi, PhD

Order of Authors: Loredana Bergandi, PhD; Marco Cordero, MD; Matteo Anselmino , PhD; Gaetana Ferraro, MD; Laura Ravera, MD; Paola Dalmasso, MS; Corrado Moiraghi, MD; Gian Paolo Trevi, MD; Dario Ghigo, MD; Amalia Bosia, MD; Serena Bergerone, MD

Abstract: Aim. To investigate whether the nitric oxide (NO)/cyclic GMP (cGMP) signalling pathway, in basal conditions and stimulated by sodium nitroprusside (SNP), may disclose abnormal patterns in platelets from patients with an acute coronary syndrome.
Design. Platelet activation (sP-selectin), inflammation (TNF-α and erythrosedimentation rate), thrombotic state (fibrinogen) and plaque disruption (HsCRP) markers were assessed in ten patients with unstable angina (UA), 14 with acute myocardial infarction (AMI) and 14 age and sex macthed healthy subjects. Platelets homogenates western blot analysis were performed, in basal conditions and stimulated by SNP, to assess cGMP levels and the expression of the sGC isoforms. Upstream (Akt1 protein kinase α phosphorylation at Ser473 and eNOS phosphorylation) and downstream (vasodilator-stimulated phosphoprotein phosphorylation) signalling of the NO/cGMP pathway was tested in the three study groups.
Results. Platelet activation, inflammation, thrombotic state and plaque disruption markers proved significantly higher in both the UA and AMI patients compared to healthy controls. Basal levels of cGMP (pmol/1010 platelets) were higher in platelets from UA (1097±111, $p<0.0001$) and AMI (1122±77, $p<0.0001$) patients compared to those from healthy controls (497±80). Similarly, serine phosphorylation in several proteins of the NO/cGMP signalling pathway (Akt1 protein kinase, NO synthase and VASP) was more represented in platelets from UA and AMI patients compared to controls. Following SNP stimulation AMI platelets disclosed a lack of cGMP increase and of VASP phosphorilation in comparison with healthy controls.
Conclusion. The present study supports the hypothesis that low concentrations of endogenously synthesized NO and cGMP may promote platelet activation. The increased inflammatory state which often accompanies an acute coronary syndrome may be responsible of the platelet activation via the NO/cGMP pathway. Furthermore, platelets from AMI patients seem more resistant to SNP stimulation, exerted not only at the cGMP level but also at other signalling check-points.

Keywords: nitric oxide; cyclic GMP; platelets; sodium nitroprusside; acute coronary syndromes

Suggested Reviewers:

Altered nitric oxide/cGMP platelet signalling pathway in platelets from patients with acute coronary syndromes

Loredana Bergandi PhD[*], Marco Cordero MD†, Matteo Anselmino MD PhD†, Gaetana Ferraro MD†, Laura Ravera MD†, Paola Dalmasso MS[§], Corrado Moiraghi MD‡, Gian Paolo Trevi† MD, Dario Ghigo MD[*], Amalia Bosia MD[*], Serena Bergerone MD†

* Department of Genetics, Biology and Biochemistry, University of Torino, Via Santena 5/bis, 10126 Torino, Italy;

† Cardiology Division, Department of Medicine, San Giovanni Battista Hospital, University of Torino, C.so Dogliotti 14, 10126 Torino, Italy

‡ Department of Emergency, San Giovanni Battista Hospital, C.so Dogliotti 14, 10126 Torino, Italy

§ Department of Public Health and Microbiology, University of Torino, Via Santena 5/bis,

10126 Torino, Italy

Bergandi and Cordero contributed equally to this work.

Running title: altered cGMP platelet signalling in ACS

Corresponding author: Loredana Bergandi, Dipartimento di Genetica, Biologia e Biochimica (Sezione di Biochimica), Via Santena 5/bis, 10126 Torino, Italy. Phone: +39-011-6705851. Fax: +39-011-6705845. E-mail: loredana.bergandi@unito.it

Introduction

Nitric oxide (NO) is an important modulator of both vasomotor tone and platelet function, leading to vasodilation and inhibition of platelet aggregation and adhesion [27]. NO induces these effects by stimulating soluble guanilate cyclase (sGC): the subsequent increase of cyclic guanosine 3',5'-monophosphate (cGMP) synthesis modulates several effectors, such as cGMP-dependent protein kinase (PKG), cGMP-dependent ion channels and cGMP-regulated phosphodiesterases [24]. Chronic as well as acute symptomatic coronary heart disease has been associated with increased platelet aggregability and NO resistance, detected as decreased platelet responsiveness to the antiaggregatory effects of NO donors, such as nitroglycerin and sodium nitroprusside (SNP) [4]. Previous work from our group has suggested that unstable angina (UA) and acute myocardial infarction (AMI) are conditions associated with increased platelet aggregability and NO resistance, measured as decreased platelet responsiveness to the inhibitory effect of the NO donor SNP [21]. The molecular mechanism remains to be elucidated. Although NO, by elevating intracellular cGMP, is known to inhibit platelet activation [18], it has been recently demonstrated that the major NO synthase (NOS) isoform expressed in platelets, the endothelial NOS (eNOS), may play a stimulatory role in low dose agonist-induced platelet activation and promote an *in vivo* thrombotic response in an injury-induced arterial thrombosis model [15,17]. Such stimulatory role of eNOS would be dependent on NO-mediated sGC activation and elevation of cGMP, which in turn would promote the aggregation-dependent platelet secretion of granules contents [15,17]. Additional evidence indicates that low concentrations of NO promote a discrete platelet degranulation [23]. Given these latest findings, the current concept of NO signalling needs to be revised highlighting the plausible biphasic role of NO in platelet activation; at the low concentrations, produced by platelet eNOS, NO would promote platelet secretion and aggregation, while at higher concentrations NO would inhibit platelet activation [15,17].

The present study aimes to investigate whether the NO/cGMP signalling pathway - upstream (Akt and eNOS phosphorylation) and downstream (VASP phosphorylation) - may disclose abnormal patterns, in basal conditions and stimulated by SNP, in platelets from patients with an acute coronary syndrome (unstable angina and acute myocardial infarction).

Materials and methods

Study population

The study population included 24 unrelated patients consecutively admitted at the Emergency Department with a diagnosis of acute coronary syndrome as a first manifestation of coronary artery disease. For inclusion the chest pain onset had to be within 8 hours from the admission. The endorsed exclusion criteria were known history of coronary heart disease; previous coronary revascularizations; malignant hypertension; aortic stenosis; hypertrophic cardiomyopathy; anemia; hypovolemia; chronic therapy with salicilates (or similars), statins or other antiplatelet and anticoagulant drugs; altered values of circulating platelets and coagulation factors; chronic inflammatory diseases; chronic steroid treatment (in the last 15 days from admission); surgical procedures or major trauma within the last month.

Ten patients presented with unstable angina and 14 with an acute myocardial infarction (defined on the Joint European Society of Cardiology/American Heart Association definitions) [26]. Eventually the control group included in the study constituted of 14 healthy subjects, macthed for age and sex with the previous groups and not presenting clinical evidence of coronary heart disease. All subjects gave written informed consent for participation in the study, which was approved by the institutional ethic committee and was performed according to the principles of the latest update of the Helsinki Declaration.

Laboratory testing

Platelets count, eritrosedimentation rate, fibrinogen and high sensitivity C-reacting protein were performed on blood samples collected with free-fall technique to avoid aggregation by local stasis, in one-tenth volume of 3.8% trisodium citrate and centrifuged for 10 minutes at 3000 rpm. The venipuncture was performed in the Emergency Department before any drug administration (aspirin, nitrates or anticoagulants). Measurements were performed by conventional clinical chemistry methods.

Measurement of serum soluble selectin-P and tumor necrosis factor-α

Levels of serum soluble selectin-P (sP-selectin) and tumor necrosis factor-α (TNF-α) were measured, respectively, with the human sP-selectin ELISA Kit (R&D Systems, Minneapolis, MN, USA) and the human TNFα ELISA Kit (Endogen, Pierce Biotechnology, Rockford, USA).

Materials

Plasticware was produced by Falcon (Becton Dickinson, Franklin Lakes, NJ, USA). Tris buffer saline (TBS) 10X was composed as follows: 25 mM Tris base, 0.2 M glycine, 20% metanol, pH 8.5. The electrophoresis reagents were produced by Bio-Rad Laboratories (Richmond, CA, USA). The protein contents of platelet-rich plasma (PRP) was assessed with the BCA kit from Pierce (Rockford, IL, USA). Unless otherwise specified, reagents were purchased from Sigma Aldrich (Milano, Italy).

Measurement of platelet cGMP

The measurement of platelet cGMP was performed as previously described [4] in the absence or presence of SNP (12.5 μM), 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) (50

μM), 8-bromo guanosine-3',5'-cyclic monophosphate (8-Br-cGMP) (100 μM), 8-bromo guanosine-3',5'-cyclic monophosphorothioate, and Rp-isomer (Rp-cGMPS; Calbiochem-Novabiochem Corporation, San Diego, CA, USA) (100 μM).

Western blot analysis

Aliquots of PRP from the three population groups were pre-treated for 15 minutes at 37°C with the NO donor SNP (12.5 μM), the sGC inhibitor ODQ (50 μM), a membrane-permeable analog of cGMP 8 Br-cGMP (100 μM), and the PKG inhibitor Rp-cGMPs (100 μM). Platelets were then sedimented by centrifugation at 2000×*g* for 5 minutes and solubilized directly in lysis buffer (1% SDS, 0.1% Triton X-100, 10 mM Tris–HCl, 10% β-mercaptoethanol, 0.002% bromophenol blue, pH 7.4), supplemented with a protease inhibitor cocktail set III (100 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, 80 mM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, 1 mM pepstatin; Calbiochem-Novabiochem Corporation, San Diego, CA, USA). After centrifugation at 13,000×*g* for 15 minutes, aliquots containing 30 μg of proteins were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (12% polyacrylamide), transferred to polyvinylidene difluoride filter membrane (Immobilon P,

Millipore, Bedford, MA, USA) and probed with the followings: a rabbit polyclonal antibody (diluted 1:100 in TBS 1X- Non Fat Dry Milk 3% with 0.05% Tween-20) specific for a chicken polyclonal antibody anti-sGC $\alpha_1\beta_1$ (1:500); a sheep polyclonal antibody anti-Akt1/PKBα (0.5 μg/ml) (Upstate, D.B.A.; catalog no. 06-558) and a sheep polyclonal antibody anti-phospho-Akt1/PKBα(Ser^{473}) (0.5 μg/ml) (Upstate, D.B.A.; catalog no. 06-801); a mouse polyclonal antibody anti-human eNOS (0.25 μg/ml) (Transduction Laboratories, Lexington, KY; catalog no. 30030) and a rabbit polyclonal anti-phospho-eNOS(Ser_{1177}) antibody (0.5 μg/ml) (Cell Signaling, Celbio, Beverly, MA; catalog no. 9571), a vasodilator-stimulated phosphoprotein (anti-VASP) (0.2 μg/ml) (Cell Signaling, Celbio, Beverly, MA;

catalog no. 3112); a mouse monoclonal antibody, clone 16C2, anti-VASP phosphorylated at serine 239 [anti-phospho-VASP(Ser^{239}) (0.2 μg/ml) (Upstate, D.B.A., Lake Placid, New York; catalog no. 05-611)].

After an overnight incubation, the membrane was washed with PBS-Tween 0.1% and subjected for 1 h to the following: peroxidase-conjugated antibody (diluted 1:1000 in PBS-Tween with Blocker Non Fat Dry Milk 5%, Biorad Laboratories); anti-chicken γ-globulin (Calbiochem-Novabiochem Corporation; catalog no. 345877); anti-sheep IgG (Upstate, D.B.A.; catalog no. 12-342); anti-mouse IgG or anti-rabbit (donkey; Amersham International; catalog no. NA931V and NA934V). The membrane was washed again with PBS-Tween, and proteins were visualized by the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Molecular weight standards were used in all gels**.**

Statistical analysis

Continuous variables, presented as means and standard deviations (SD), were compared by Student's *t* test for equality of means after a normalized distribution was assured and by repeated-measures one-way ANOVA followed by Bonferroni multiple comparison test. Categorical variables, presented as counts and percentages, were compared in cross tabulations tables by means of the Pearson chi-square test and likelihood ratio. All statistical analysis was performed with the program package STATA® v 8.0. The level of significance was taken as two-tailed p=0.05.

Results

Clinical and biochemical features of healthy subjects, UA and AMI patients are reported in Table 1. The platelets count (x $10^3/mm^3$) in patients with UA (278±48) resulted significantly

lower compared to both patients with AMI (372±62, p<0.02) and healthy subjects (342±128, p<0.02).

Serum markers of thrombophilic status and inflammation

Serum markers of platelet activation (sP-selectin), inflammation (TNF-α and erythrosedimentation rate), thrombotic state (fibrinogen) and plaque disruption (HsCRP) proved higher in both UA and AMI patients compared to healthy controls (Table 1).

Basal platelet cGMP (pmol/10^{10} platelets) was higher in both UA (1097±111, p<0.0001) and AMI patients (1122±77, p<0.0001) compared to healthy subjects (497±80) (Figure 1A).

In SNP-treated platelets the absolute values of cGMP did not differ among the three study groups (Figure 1A), but when comparing the difference between SNP-stimulated and basal cGMP levels in each group, the SNP stimulation in UA patients proved more effective (Δ 3300±364 pmol/10^{10} platelets) than in healthy subjects (Δ 1961±343 pmol/10^{10} platelets, p<0.01) and AMI patients (Δ 1296±153 pmol/10^{10} platelets, p<0.02) (Figure 1B). The SNP stimulation effect was instead not significantly dissimilar within AMI patients and healthy controls (Figure 1B).

Basal expression of the sGC subunits α1 and of subunit β1 by western blot analysis of platelets homogenates disclosed comparable patterns within the three study groups (Figure 2).

Akt and eNOS phosphorylation

Compared with platelets from healthy subjects, UA and AMI platelets showed a significant increase in the activation of the signalling pathway modulated by NO, involving the NOS activation by a cascade of protein kinases: the Akt1/PKBα phosphorylation at Ser^{473} (Figure 3) and the eNOS phosphorylation at Ser^{1177} (Figure 4).

VASP phosphorylation at serine 239

While Phospho-VASP(Ser^{239}), a marker of PKG activation, was not detectable in platelets from healthy subjects, VASP phosphorylation was clearly present in UA and even more in AMI platelets (Figure 5). SNP and 8-bromo-cGMP incubation induced VASP phosphorylation in control and UA platelets to levels moreless superimposable to those basally observed in AMI platelets. Concerning VASP phosphorylation, AMI platelets revealed unsensible to SNP and 8-bromo-cGMP incubation (Figure 5). In healthy and UA platelets ODQ reverted the SNP-induced effect, and Rp-8-Br-cGMPS reverted the 8-bromo-cGMP-induced effect, Suggesting, respectively, a sGC and PKG mediation. The two inhibitors, instead, did not decrease phospho-VASP levels in AMI platelets, already maximally expressed under basal conditions (Figure 5).

Discussion

The main finding of the present work is that platelets from patients with acute coronary syndromes not on therapy with salicilates (or derivatives), statins, or any other antiplatelet and anticoagulant drug, exhibit abnormal function. Furthermore patelets from patients presenting with UA or AMI disclosed different characteristics, both basal than SNP-induced.

In comparison with healthy subjects, UA and AMI patients exhibited higher levels of serum sP-selectin (a sensitive marker of in vivo platelet activation [2]) and basal intraplatelet cGMP. Western blot analysis of the sGC isoforms expression excluded that this finding could have been attributable to a dissimilar isoform costitution compared to platelets from controls; indeed the basal expression of the sGC subunits α1 and of subunit β1 was comparable in the platelets from all the three study groups.

Although the 30 year old dogma that NO and cGMP play inhibitory roles in platelet activation, proven by the inhibited platelet function by high concentrations of NO donors and

cGMP analogs [18], recent evidences suggested that, instead, low concentrations of endogenously synthesized NO and cGMP may promote platelet secretion and aggregation [6,7,8]. The NO/cGMP pathway has therefore lately been entitled of a biphasic role in platelet activation.

sGC activity, in several cell types, is modulated by NO, via a signalling pathway implicating the NOS activation by a cascade of protein kinases, such as PI3K and Akt/PKB [2]. PI3K has been observed to promote Akt activation during platelet stimulation [16,25], and two different isoforms of Akt, Akt1/PKBα and Akt2/PKBβ, have been found to play a role in platelet activation [3]. Once phosphorylated, Akt is known to phosphorylate and activate in its turn other proteins, including eNOS [9]. In fact, several platelet agonists may sequentially activate PI3K, Akt, and eNOS, which synthesizes NO [22] and NO stimulates sGC, causing an increase of intracellular cGMP levels.

In our experience this mechanism seemed apparently working in both UA and AMI platelets, as both the major downstream effector of PI3K (Akt) and the major downstream effector of Akt (eNOS) exhibited a significant increase in basal phosphorylation. The product of NOS activation, NO, may be responsible for the enhanced baseline concentrations of cGMP in both UA and AMI platelets, responsive to SNP in terms of cGMP production.

Unfortunately the evaluation of the levels of NO produced by platelets themselves is struggling, due to the impossibility to maintain these cells in culture for the incubation times (at least 12-24 hours) generally needed to measure detectable amounts of nitrite, the stable derivative of NO. Furthermore, in an *ex vivo* study like the present, to administrate NOS inhibitors *in vivo* to patients and healthy controls checking the effect on basal platelet cGMP is not possible. Given these limitations the finding of higher Akt and eNOS phosphorylation, upstream steps of the NO/cGMP signalling pathway, seems highly relevant.

The eNOS-generated NO, by stimulating sGC, may induce production of cGMP, which until recently has been entitled to inhibit platelet activation by activating PKG. Recent experimental evidences, instead, have suggested that PKG may play an important stimulatory role in platelet activation. Mouse platelets knockout for PKG and human platelets treated with PKG inhibitors showed significantly reduced platelet aggregation in response to low dose agonists, including thrombin, thromboxane B_2, von Willebrand factor and collagen [15]. VASP (46-50 kDa) has been identified as a substrate for both PKG and cAMP-dependent protein kinase (PKA). This phosphoprotein is preferentially phosphorylated at Ser^{239} by PKG and at Ser^{157} by PKA and the levels of phospho-VASP(Ser^{239}) have been described as a reliable marker of PKG activation [14,19]. The presented finding of a significantly higher level of phosphorylated VASP in UA and AMI platelets compared to controls supports the hypothesis of an activated pathway, specifically downstream, in patients with acute coronary syndromes.

Eventually the present work supports the evidence that the cGMP pathway is, in basal conditions, more activated in platelets from acute coronary syndrome patients than in controls. One of the triggers of this phenomeon may be the proinflammatory cytokine TNF-α, as its circulating levels were significantly increased in both UA and AMI patients [8]. Indeed, TNF-α is known to promote platelet aggregation in patients with heart failure [20], and has been recently shown to stimulate eNOS phosphorylation via the PI3K/Akt signal transduction pathway [13,1,7].

Previous literature has reported that acute coronary syndormes relate with NO resistance, as decreased platelet responsiveness to the antiaggregatory effects of NO donors [4]. Interestingly, our investigation showed that the ability of the NO donor SNP to further increase cGMP was fully maintained in platelets from UA patients, while it was strongly impaired in AMI platelets, suggesting dissimilar platelet reactions in these two groups. The

resistance of AMI platelets was exhibited not only at the sGC step, but also at the PKG step: both SNP and 8-Br-cGMP were unable to increase VASP phosphorylation levels. Such finding could be consequence of the already high and stable basal level of VASP phosphorylation in AMI platelets not revertable either by the sGC inhibitor ODQ or the PKG inhibitor Rp-8-Br-cGMPS.

Conclusion

The onset of NO resistance observed in patients with acute coronary syndromes has been mainly attributed to a not yet clearly defined impairment of sGC and/or to the scavenging of NO by the superoxide anion radical [10], however the precise molecular modifications responsible for the desensitisation of the NO/cGMP pathway has hitherto not been identified. The present data suggest that NO resistance in this setting of patients may involve other steps than the sGC alone: in UA and AMI patients the alteration of the cGMP pathway is more complex than the previously hypothesized, showing quantitative differences among the two groups of patients. It is conceivable that the increased phosphorylation of VASP in UA and AMI platelets is a relatively stable modification, caused by a prolonged NO-mediated stimulation of the cGMP pathway and no longer sensitive to sGC and PKG inhibitors. Platelet hyperaggregability combined with hyporesponsiveness to exogenous NO donors in AMI patients may reflect an impaired physiological response to endogenous NO, and could contribute to the increased risk of ischemic events. Moreover, nitrate therapy may be least likely to obtain beneficial results [6]. Indeed Willoughby et al. showed that a reduced platelet response to NO administration in the first hours from the onset of symptoms was associated with a worse prognosis in UA patients, as to relapsed incidence and increased mortality [28]. In fact, in AMI patients, there is lack of evidence supporting nitrovasodilators efficacy in

inhibiting platelet activation, and these drugs have not proved beneficial in terms of mortality reduction [5].

References

1. Barsacchi R, Perrotta C, Bulotta S, Moncada S, Borgese N, Clementi E (2003) Activation of endothelial nitric-oxide synthase by tumor necrosis factor-alpha: a novel pathway involving sequential activation of neutral sphingomyelinase, phosphatidylinositol-3' kinase, and Akt. Mol Pharmacol 63:886-895

2. Brazil DP, Yang ZZ, Hemmings BA (2004) Advances in protein kinase B signalling: AKTion on multiple fronts. Trends Biochem Sci 29:233-242

3. Chen J, De S, Damron D, Chen WS, Hay N, Byzova TV (2004) Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice. Blood 104:1703-1710

4. Chirkov YY, Holmes AS, Willoughby SR, Stewart S, Wuttke RD, Sage PR, Horowitz JD (2001) Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets. J Am Coll Cardiol 37:1851-1857

5. Collaborative Group (1995) ISIS-4 (Fourth International Study of Infarct Survival): a randomised factorial trial assessing early oral captopril, oral mononitrate, and intravenous magnesium sulphate in 58,050 patients with suspected acute myocardial infarction. Lancet 345:669-685

6. Daiber A, Wenzel P, Oelze M, Münzel T (2008) New insights into bioactivation of organic nitrates, nitrate tolerance and cross-tolerance. Clin Res Cardiol 97(1):12-20

7. De Palma C, Meacci E, Perrotta C, Bruni P, Clementi E (2006) Endothelial nitric oxide synthase activation by tumor necrosis factor alpha through neutral sphingomyelinase 2, sphingosine kinase 1, and sphingosine 1 phosphate receptors: a novel pathway relevant to the pathophysiology of endothelium. Arterioscler Thromb Vasc Biol 26:99-105

8. Debrunner M, Schuiki E, Minder E, Straumann E, Naegeli B, Mury R, Bertel O, Frielingsdorf J. (2008) Proinflammatory cytokines in acute myocardial infarction with and without cardiogenic shock. Clin Res Cardiol 97(5):298-305

9. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399:601-605

10. Friebe A, Koesling D (2003) Regulation of nitric oxide-sensitive guanylyl cyclase. Circ Res 93:96-105

11. Ignarro LJ (1993) Nitric oxide-mediated vasorelaxation. Thromb Haemostasis 70:148-151

12. Ikeda H, Takajo Y, Ichiki K, Ueno T, Maki S, Noda T Sugi K, Imaizumi T (1995) Increased soluble form of P-selectin in patients with unstable angina. Circulation 92:1693–1696

13. Kawanaka H, Jones MK, Szabo IL, Baatar D, Pai R, Tsugawa K (2002) Activation of eNOS in rat portal hypertensive gastric mucosa is mediated by TNF-alpha via the PI 3-kinase-Akt signaling pathway. Hepatology 35:393-402

14. Kwiatkowski AV, Gertler FB, Loureiro JJ (2003) Function and regulation of Ena/VASP proteins. Trends Cell Biol 13:386-392.

15. Li Z, Xi X, Gu M, Feil R, Ye RD, Eigenthaler M, Hofmann F, Du X (2003) A stimulatory role for cGMP-dependent protein kinase in platelet activation. Cel 112:77-86

16. Li Z, Zhang G, Le Breton GC, Gao X, Malik AB, Du X (2003) Two waves of platelet secretion induced by thromboxane A2 receptor and a critical role for phosphoinositide 3-kinases. J Biol Chem 278:30725-30731

17. Marjanovic JA, Li Z, Stojanovic A, Du X (2005) Stimulatory roles of nitric-oxide synthase 3 and guanylyl cyclase in platelet activation. J Biol Chem 280:37430-37438

18. Massberg S, Sausbier M, Klatt P, Bauer M, Pfeifer A, Siess W Fässler R, Ruth P, Krombach F, Hofmann F (1999) Increased adhesion and aggregation of platelets lacking cyclic guanosine 3',5'-monophosphate kinase I. J Exp Med 189:1255-1264

19. Oelze M, Mollnau H, Hoffman N, Warnholtz A, Bodenschatz M, Smolenski A Walter U, Skatchkov M, Meinertz T, Münzel T (2000) Vasodilator-stimulated phosphoprotein

serine 239 phosphorylation as a sensitive monitor of defective nitric oxide/cGMP signalling and endothelial dysfunction. Circ Res 87:999-1005

20. Pignatelli P, De Biase L, Lenti L, Tocci G, Brunelli A, Cangemi R Riondino S, Grego S, Volpe M, Violi F (2005) Tumor necrosis factor-alpha as trigger of platelet activation in patients with heart failure. Blood 106:1992-1994

21. Pistono M, Bergerone S, Carrieri, L Paglia I, Stefano D, Capizzi A, Ferri M, Pescarmona G, Bosia A, Trevi G. (2002) Platelet cyclic GMP levels in unstable angina and myocardial infarction. Platelets 13:307-311

22. Randriamboavonjy V, Fleming I (2005) Endothelial nitric oxide synthase (eNOS) in platelets: how is it regulated and what is it doing there? Pharmacol Rep 57 Suppl:59-65

23. Randriamboavonjy V, Schrader J, Busse R, Fleming I (2004) Insulin induces the release of vasodilator compounds from platelets by a nitric oxide-G kinase-VAMP-3-dependent pathway. J Exp Med 199:347-356

24. Schlossman J, Feil R, Hofmann F. (2003) Signaling through NO and cGMP-dependent protein kinases. Ann Med 35:21-27

25. Stojanovic A, Marjanovic JA, Brovkovych VM, Peng X, Hay N, Skidgel RA, Du X. (2006) A phosphoinositide 3-kinase-AKT-nitric oxide-cGMP signaling pathway in stimulating platelet secretion and aggregation. J Biol Chem 281:16333-16339

26. The Joint European Society of Cardiology/American College of Cardiology Committee. (2000) Myocardial Infarction redefined – A consensus document of The Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of Myocardial Infarction. Eur Heart J 21:1502–1513

27. Walford G, Loscalzo J. (2003) Nitric oxide in vascular biology. J Thromb Haemost 1:2112-2118

28. Willoughby S C, Stewart S, Holmes A S, Chirkov Y Y, Horowitz JD (2005) Platelet Nitric Oxide Responsiveness. A Novel Prognostic Marker in Acute Coronary Syndromes. Arterioscler Thromb Vasc Bio 25:2661-2666

Tables

Table 1. Clinical and biochemical features of healthy subjects, unstable angina and acute myocardial infarction patients.

Variable	healthy subjects (n=14)	UA patients (n=10)	AMI patients (n=14)
Age	64.3 ± 0.98	66 ± 1.29	68.5 ± 1.29
Sex, % male	57% (8/14)	40% (4/10)	64% (9/14)
Platelets count (x 10^3/mm^3)	342 ± 128	278 ± 48 * °	372 ± 62
ESR (mm/h)	1.5 ± 0.4	16.9 ± 1.6 **	12.7 ± 1.6 **
Fibrinogen (mg/dl)	210.6 ± 6.5	610.1 ± 32.6 ***	673.3 ± 54.6 ***
HsCRP (mg/dl)	2.6 ± 0.24	15.2 ± 0.71 ***	17.5 ± 1.13 ***
sP-selectin (ng/ml)	82 ± 19	107 ± 21 *	123 ± 22 **
TNF-α (pg/ml)	7.8 ± 1.4	19.2 ± 3.2 *	22.4 ± 3.8 *
Diabetes	4/14 (30%)	6/10 (60%)	11/14 (78%)
Hypertension	8/14 (60%)	8/10 (80%)	13/14 (93%)
Familiarity	2/14 (10%)	2/10 (20%)	2/14 (14%)
Dyslipidemia	5/14 (35%)	6/10 (60%)	12/14 (85%)

UA, unstable angina; AMI, acute myocardial infarction; ESR = erythrosedimentation rate, HsCRP = Hight sensibility C-reactive protein, TNF-α = tumor necrosis factor-α

* $p < 0.02$, ** $p < 0.01$, *** $p < 0.0001$ vs. healthy subjects

° $p < 0.02$ vs. patients with AMI

Figure 1.

A. Basal and SNP-dependent cGMP synthesis in platelets of healthy (CTRL) subjects (n=14), in UA patients (n=10) and in AMI patients (n=14). Platelets were incubated for 15 minutes at 37° C in the absence (basal) or presence of 12.5 μM SNP. cGMP concentration was determined as described under Materials and Methods. Data are presented as mean ± SD. * $p < 0.0001$ vs. respective basal; ♦ $p < 0.0001$ vs. basal of healthy control patients.

B. Elaboration of data presented in panel A, showing the SNP-dependent cGMP increase from basal level in platelets from healthy (CTRL) subjects, UA patients and AMI patients. Data are presented as mean ± SD. * $p < 0.01$ vs. healthy control patients; ♦ $p< 0.02$ vs. SNP-dependent cGMP increase in AMI patients.

Figure 2.

Expression of sGC subunits α_1 (73-82 kDa) and β_1 (70-72 kDa) in healthy subjects (CTRL), in UA patients and AMI patients. Western blot analysis was performed as described under the Materials and Methods section.

Top. The figure is representative of the experiments performed on each healthy subject or patient.

Bottom. The protein bands obtained from 14 healthy subjects (CTRL), 10 UA patients and 14 AMI patients have been quantitated densitometrically. Values, expressed as arbitrary units,

are represented as means ± SD.

Figure 3.

Top. Expression of Akt1/PKBα and phosphorylation of Akt1/PKBα at Ser^{473} in healthy subjects (CTRL), in UA patients (UA) and AMI patients (AMI). Western blot analysis was performed as described under the Materials and Methods section. The figure is representative of the experiments performed on each healthy subject or patient.

Bottom. The protein bands obtained from 14 healthy subjects (CTRL), 10 UA patients and 14 AMI patients have been quantitated densitometrically. Values, expressed as arbitrary units,

are represented as means ± SD; * $p < 0.02$ and ** $p < 0.01$ vs. healthy subjects; ° $p < 0.02$ vs. UA patients.

Figure 4.

Top - Expression of eNOS and phosphorylation of eNOS at Ser^{1177} in healthy subjects (CTRL), in UA patients (UA) and AMI patients (AMI). Western blot analysis was performed as described under the Materials and Methods section. The figure is representative of the experiments performed on each healthy subject or patient.

Bottom - The protein bands obtained from 14 healthy subjects (CTRL), 10 UA patients and 14 AMI patients have been quantitated densitometrically. Values, expressed as arbitrary units, are represented as means ± SD; *$p < 0.001$ and ** $p < 0.0001$ vs. healthy subjects; ° $p < 0.01$ vs. UA patients.

Figure 5.

Top. VASP phosphorylation at Ser^{239} in healthy subjects (panel A), in UA patients (panel B) and AMI patients (panel C). PRP was incubated for 15 minutes at 37° C with 12.5μM SNP (snp) and 500 μM 8-Br-cGMP (br) alone or in presence, respectively, of 50 μM ODQ (snp+odq), a guanylate cyclase inhibitor, and of 500 μM Rp-8-Br-cGMPS (br+rp), a selective inhibitor of PKG. ODQ and Rp-8-Br-cGMPS alone gave results superimposable to ctrl (data not shown). The figure is representative of the experiments performed on each healthy subject or patient. Western blot analysis was performed as described under the Materials and Methods section.

Bottom. The protein bands obtained in three experiments from 14 healthy subjects (CTRL), 10 UA patients and 14 AMI patients have been quantitated densitometrically. VASP Ser^{239} was analyzed by immunoblotting using an anti-phospho-VASP antibody (16C2) against the Ser^{239} of phosphorylated VASP. Values, expressed as arbitrary units, are means ± SEM of

three separate experiments. * $p < 0.01$ and ** $p < 0.001$ vs.ctrl; ° $p < 0.02$ vs. snp and °° $p < 0.002$; ◊ $p < 0.01$ and ◊◊ $p < 0.001$ vs. br

Table

Table 1. Clinical and biochemical features stratified by the three study groups. Values are expressed as means ± SD.

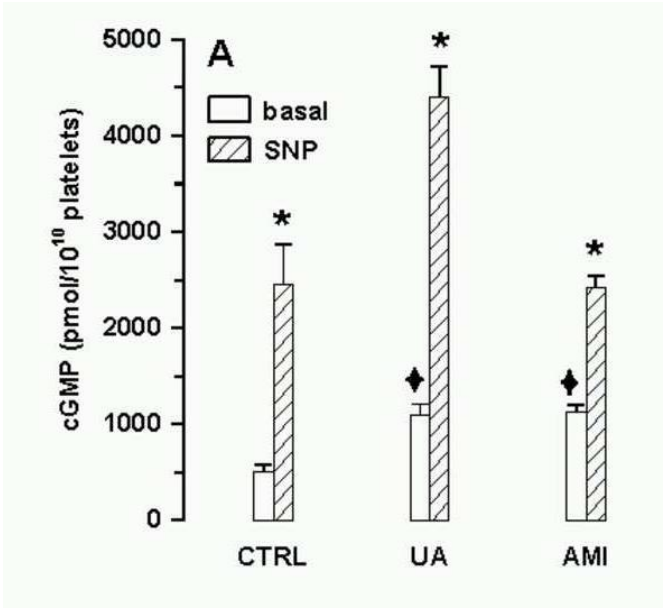
Variable	healthy subjects (n=14)	UA patients (n=10)	AMI patients (n=14)
Age	64.3 ± 10.98	66 ± 11.29	68.5 ± 11.29
Sex, % male	57% (8/14)	40% (4/10)	64% (9/14)
Platelet count (x10³/mm³)	342 ± 128	278 ± 48 *	372 ± 62
ESR (mm/h)	1.5 ± 0.4	16.9 ± 1.6 **	12.7 ± 1.6 **
Fibrinogen (mg/dl)	210.6 ± 6.5	410.1 ± 32.6 ***	475.3 ± 34.6 ***
HsCRP (mg/dl)	2.6 ± 0.24	15.2 ± 0.71 ***	17.5 ± 1.13 ***
sP-selectin (ng/ml)	82 ± 19	107 ± 21 *	123 ± 22 **
TNF-α (pg/ml)	7.8 ± 1.4	19.2 ± 3.2 *	22.4 ± 3.8 *
Diabetes	4/14 (30%)	4/10 (40%)	11/14 (78%)
Hypertension	8/14 (60%)	8/10 (80%)	13/14 (93%)
Familiarity	2/14 (10%)	2/10 (20%)	2/14 (14%)
Dyslipidemia	5/14 (35%)	4/10 (40%)	12/14 (85%)

UA, unstable angina; AMI, acute myocardial infarction; ESR = erythrosedimentation rate, HsCRP = High sensibility C-reactive protein, TNF-α = tumor necrosis factor α.

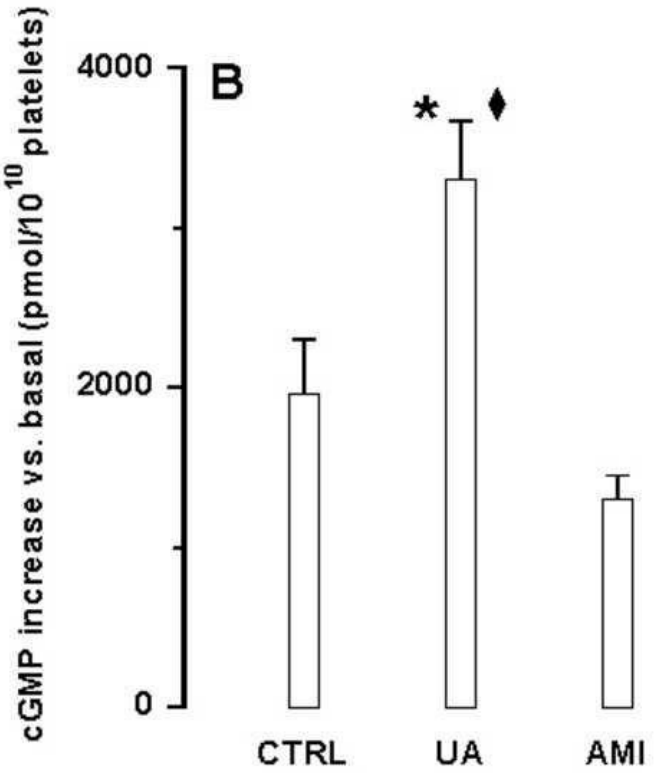
* $p < 0.02$, ** $p < 0.01$, *** $p < 0.0001$ vs. healthy subject

$p < 0.02$ vs. patients with AMI

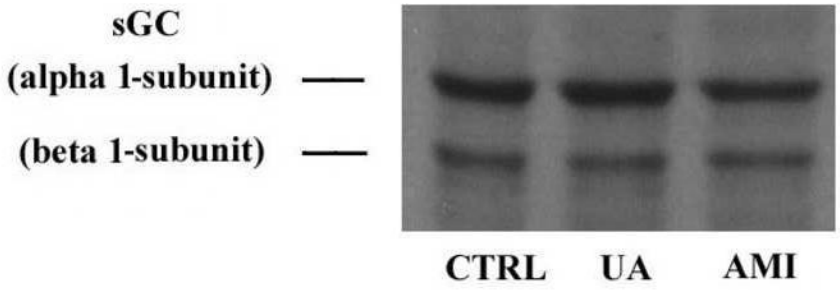
Figure


A
basal
SNP
cGMP (pmol/10^10 platelets)
5000
4000
3000
2000
1000
0
*
*
*
CTRL
UA
AMI

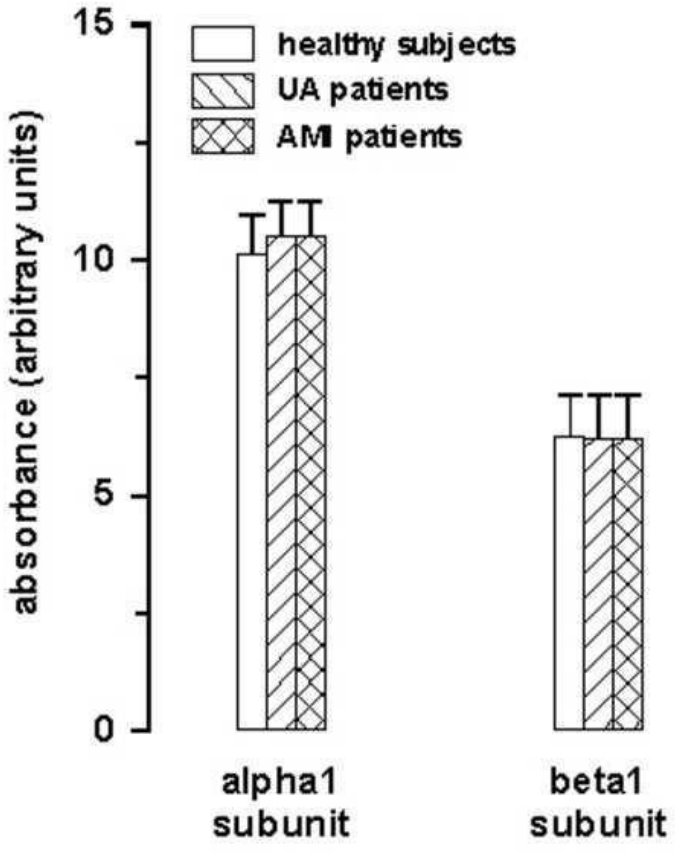
Figure


B
cGMP increase vs. basal (pmol/10^10 platelets)
4000
2000
0
*
♦
CTRL
UA
AMI

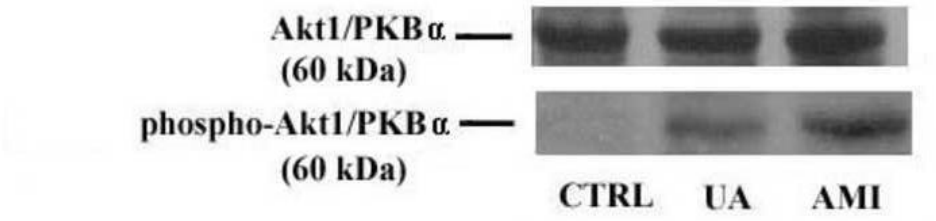
Figure



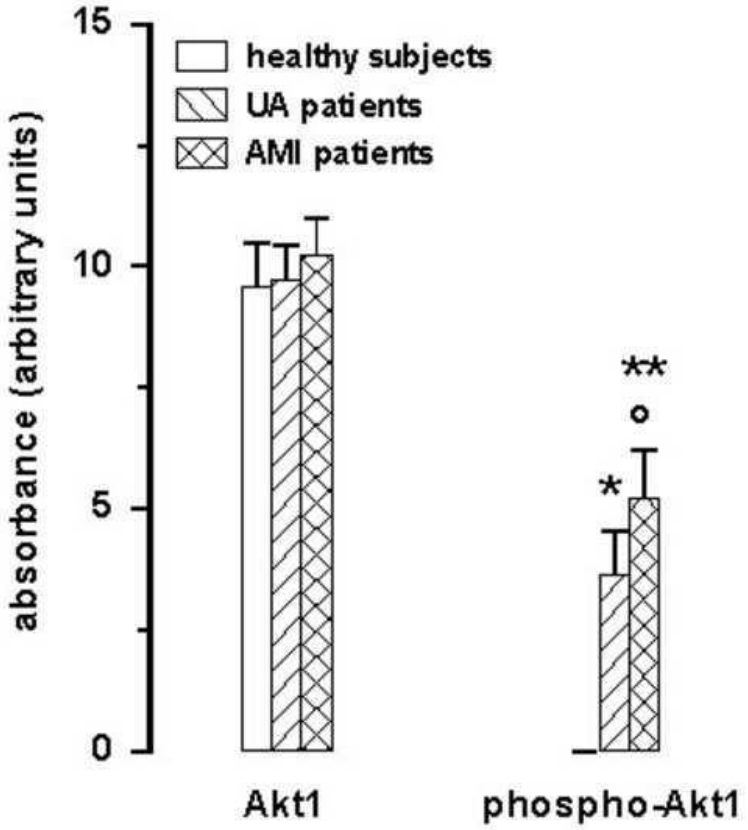
Figure


15
10
5
0
absorbance (arbitrary units)
healthy subjects
UA patients
AMI patients
alpha1 subunit
beta1 subunit

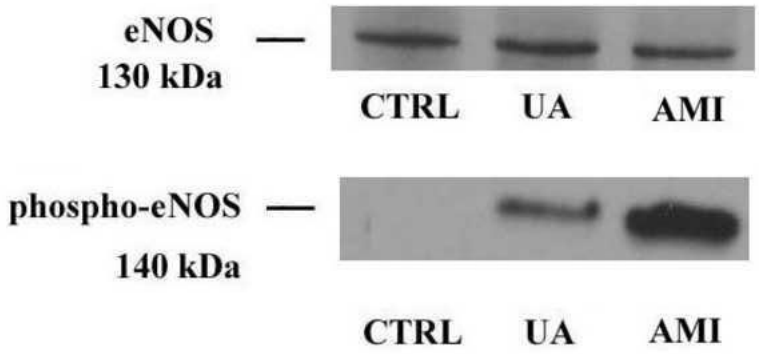
Figure



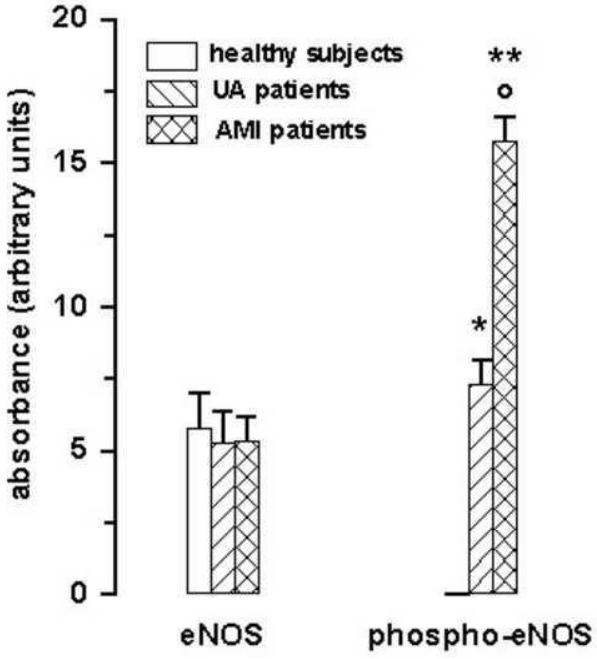
Figure

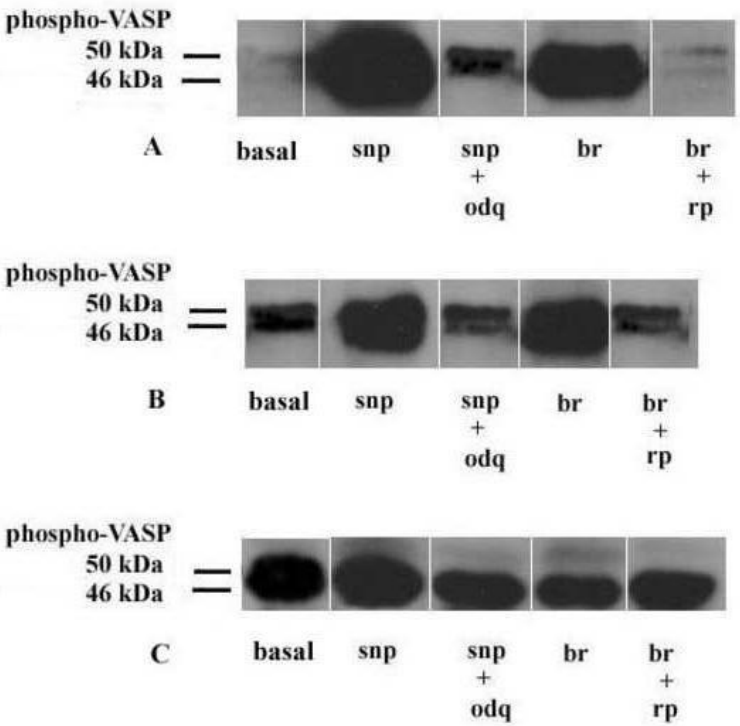

15
10
5
0
absorbance (arbitrary units)
healthy subjects
UA patients
AMI patients
**
°
*
Akt1
phospho-Akt1

Figure



Figure


20
15
10
5
0
absorbance (arbitrary units)
healthy subjects
UA patients
AMI patients
**
o
*
eNOS
phospho-eNOS


phospho-VASP
50 kDa
46 kDa
A
basal
snp
snp + odq
br
br + rp
phospho-VASP
50 kDa
46 kDa
B
basal
snp
snp + odq
br
br + rp
phospho-VASP
50 kDa
46 kDa
C
basal
snp
snp + odq
br
br + rp

Figure

A
absorbance (arbitrary units)
10
8
6
4
2
0
**
**
oo
◇◇
basal
stp
stp+edtj
br
br+stp
B
absorbance (arbitrary units)
10
8
6
4
2
0
*
*
o
◇
basal
stp
stp+edtj
br
br+stp
C
absorbance (arbitrary units)
10
8
6
4
2
0
basal
stp
stp+edtj
br
br+stp