Platelet aggregation and P-selectin levels during exercise treadmill test in patients with ischaemic heart disease

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Abstract

Introduction: Coronary artery disease (CAD) is associated with higher platelet activation sometimes despite aspirin use. There are conflicting data concerning platelet activation course during physical exercise in patients on aspirin with CAD.

Aim: To assess platelet activation pattern during physical exercise in patients with CAD.

Methods: The study included 35 patients (20 men, 15 women) aged 64.7±10 years with CAD (CCS II) on aspirin treatment (75 mg daily) and a control group of 10 healthy subjects adjusted for age and gender. Treadmill testing was performed using the Bruce protocol. Platelet aggregation was measured with optical aggregation with the agonists ADP (10 μM), collagen (2 μg/ml) and arachidonic acid (0.5 mg/ml) before and at peak exercise; P-selectin platelet and soluble expression (basal and after stimulation with thrombin) was assessed with cytofluorometry before, at peak exercise and 1 hour after.

Results: There were no differences in collagen and ADP aggregation between patients and the control group. There was a significant increase of ADP aggregation at peak exercise in the control group ($p < 0.05$). There was a positive correlation between platelet aggregation before exercise and at peak exercise with ADP ($r = +0.86$) and with collagen ($r = +0.61$). There was no difference in soluble P-selectin concentration between patients and the control group. Platelet P-selectin expression without stimulation with thrombin 1 hour after exercise was significantly higher in patients than in the control group ($p < 0.05$).

Conclusions: 1. Physical exercise does not intensify platelet aggregation in patients with CAD on 75 mg aspirin daily. 2. Despite taking aspirin, platelet activation measured with the expression of platelet P-selectin increases and there is further intensification during exercise testing. 3. The concentration of soluble P-selectin in patients with CAD does not reflect the expression of platelet P-selectin.

Key words: platelet aggregation, P-selectin, coronary artery disease

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Introduction

Aspirin (acetylosalicylic acid) inhibiting platelet aggregation has been used for many years in secondary prevention of ischaemic heart disease (IHD), stroke and some types of atrial fibrillation. The effectiveness of this therapy was shown in a metaanalysis published in 2002, performed by the Antithrombotic Trialists' Collaboration. It revealed 34% reduction in fatal myocardial infarction (MI) and 15% reduction of cardiovascular mortality in patients receiving antiplatelet agents for secondary prevention [1]. Patients taking aspirin have the feeling of effective prevention of MI. Pathophysiological studies and clinical findings show, however, that in some patients aspirin inhibits platelet aggregation insufficiently or only partially, which is referred to as "aspirin resistance" [2]. The antiaggregatory effect of aspirin

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may also be inadequate in subjects with primary hypersensitivity to acetylsalicylic acid while under special circumstances, e.g. during physical exercise. Strenuous exercise in healthy individuals may lead to excessive activation of the coagulation system, including platelet activation, and simultaneous inhibition of fibrinolysis [3]. Patients with IHD present disproportionate activation of the coagulation system even in standard conditions [4]. This phenomenon may progress and lead to myocardial ischaemia during exercise [5, 6].

Verification of this hypothesis may be of great importance for antiplatelet therapeutic regimens in patients with IHD. Assessment of coagulation profile at rest but also during exercise and its pharmacological modification may influence the course of the disease, incidence of acute ischaemic events and progression of atherosclerosis of the coronary arteries.

P-selectin is used as an indicator of *in vivo* platelet activation [7]. It acts as an adhesion molecule that facilitates binding of leukocytes to activated platelets and endothelium. Additionally, it is a vital component of both haemostatic and inflammatory processes [8].

The aim of this study was to evaluate platelet aggregation and P-selectin levels in patients with stable angina pectoris (CCS class II) during controlled physical exercise.

Methods

Study group

A total of 35 subjects with CCS class II IHD confirmed on coronary angiography were enrolled in the study, including 15 females and 20 males, at the mean age of 64.7±10 years. Patients were treated with antianginal drugs according to current guidelines of the Polish Cardiac Society, including 75 mg aspirin daily for at least 30 days. Neither agents that might affect platelet aggregation, such as thienopyridine derivates or non-steroid anti-inflammatory drugs, nor nitrates were used during exercise tests. Exclusion criteria were as follows: blood platelet count below 150 k/mm^3 and above 440 k/mm^3 and haemoglobin level below 8 g%. The study population underwent coronary angiography via femoral artery access using standard fluoroscopic views. Coronary artery stenosis of at least 70% was considered significant.

The control group consisted of 10 subjects, including 4 females and 6 males, at the mean age of 64.4±11 years, receiving 75 mg aspirin daily for at least 30 days and taking no other medications that might significantly affect platelet aggregation, such as thienopyridine derivates or non-steroid anti-inflammatory drugs. Ischaemic heart disease was excluded in this group based on negative outcome of clinical evaluation, electrocardiographic exercise test and dobutamine stress echocardiography.

Characteristics of the study and control groups are detailed in Table I.

Measurement of platelet aggregation

Platelet aggregation indices were measured twice: before exercise test and at peak exercise. For these measurements blood was drawn from the antecubital vein without using a tourniquet in both patients and healthy individuals, into 4 tubes injected with 3.8% sodium citrate in the ratio of one unit of anticoagulant and nine units of blood. At the same time blood was drawn into one tube with ethylenediaminetetraacetic acid (EDTA) to determine complete blood count and for evaluation of blood platelet count. Beckton Dickinson (USA) tubes were used in this study. Subsequently, sodium citrate blood was immediately transferred to the laboratory, where it was centrifuged, while EDTA blood was used for determination of complete blood count. Two tubes were centrifuged at 100 g for 15 minutes in order to obtain platelet-rich plasma. Two tubes were centrifuged at 2,400 g for 20 minutes in order to obtain platelet-poor plasma. The platelet-rich plasma tube was used to determine blood platelet count, which ranged from 200,000/mm^3 to 300,000/mm^3. If platelet count in 1 mm^3 exceeded acceptable concentrations, platelet-rich plasma was diluted with platelet-poor plasma. After preparation of platelet-rich and platelet-poor plasma pools, platelet aggregation tests were started.

Table I. Characteristics of patients and control group

Variable	Study group N=35	Control group N=10	p
Arterial hypertension	17	7	NS
Diabetes mellitus	7	2	NS
Smoking	8	5	NS
Platelet count	193,600±40,232	232,000±78,164	NS
Past myocardial infarction	19	0	<0.0001
Past PTCA	15	0	<0.001

Aggregation was assessed using an optical method with a Chrono-Log platelet aggregometer (model 560 Ca, USA), an optical method with ADP (10 µM) and arachidonic acid (0.5 mg/ml).

Platelet aggregation as assessed with the optical method was expressed as a percentage of platelets which aggregated within five minutes of agonist action. Model 560 Ca automatically calculates this value using built-in Chrono-Log AgroLink software.

The following preparations of agonists were added to consecutive platelet-rich plasma cuvettes to verify platelet aggregation: 0.5 mg/ml arachidonic acid, 10 µM adenosine diphosphate and 2 µg/ml collagen.

Arachidonic acid-induced aggregation was performed to confirm that patients were taking aspirin. It was less than 20% in all patients which confirmed the regular use of the drug [9].

ADP, acting as an agonist, initiates platelet shape changes and their reversible primary aggregation. Subsequent secondary aggregation is induced by the ADP-dependent pathway of thromboxane A_2 synthesis. ADP-induced platelet aggregation is decreased in patients taking thienopyridine derivatives or aspirin, and with afibrinogenemia and Glanzman thrombasthenia [10]. Collagen stimulates platelet aggregation following binding to its specific platelet membrane receptor. Similarly to ADP, collagen increases platelet activation and causes their aggregation partially by increase of thromboxane A_2 synthesis [9].

Blood for evaluation of platelet function was drawn three times: before, at peak and one hour after exercise test. Platelet activation was measured at baseline and after administration of thrombin by means of P-selectin (sP-selectin) level assessment using ELISA method (Bender MedSystems GmbH Human sP-selectin BMS 219/3CE, Austria) and flow cytofluorometer (Dako, Netherlands). Reference range for healthy individuals is 111-299 ng/ml at mean concentration of 190 ng/ml ± 50 ng/ml. No interaction with sE-selectin, sL-selectin and other tested proteins was observed. Within- and between-assay variability was 2.4% and 5.2%, respectively.

Measurement of P-selectin expression

Blood samples were also used to determine membrane P-selectin expression prior to and after platelet stimulation with thrombin. Following blood collection one part was fixed with 1% paraformaldehyde in PBS buffer and then incubated for 30 minutes at room temperature with anti-CD61 antibodies labelled with fluoresceine isothiocyanate (FITC). The rest of the blood was incubated for 4 minutes at room temperature with 30 µl of newly prepared thrombin solution, and subsequently fixed with paraformaldehyde solution and labelled with anti-CD61. Furthermore, labelling with nonselective antibodies to IgG1 conjugated with phycoerythrin was performed. A Partec cytofluorometer was used for analysis. Platelet area was determined based on the size and granulation distribution. Results are given as a percentage of CD61postive platelets.

Exercise treadmill test

Exercise test was performed using the Burdick T 600 treadmill and Sicard 460 S software and recorder (Siemens), using the Bruce protocol. Test discontinuation criteria included reaching age-specific METs value and/or age-specific submaximum heart rate, presence of electrocardiographic signs of myocardial ischaemia and/or angina, fatigue and patient's request.

Statistical analysis

Statistical analysis was performed using Statistica 5.5 software (StatSoft Inc.). In all patients mean values and standard deviations and distribution of studied variables were evaluated applying the following normality tests: Kolmogorov-Smirnov test, Lillefors test and Shapiro-Wilks' W test. Significance of differences between the groups was determined with Student's *t*-test for unpaired variables of normal distribution and Mann-Whitney U test for variables not following normal distribution. Correlations between variables were estimated using a correlation matrix, calculating Pearson's correlation coefficient r for normal distribution variables and Spearman R for variables not following normal distribution. Statistical significance of differences between dichotomous variables in both groups was assessed with χ^2 test, χ^2 with Yates correction or exact Fisher test depending on the number of patients. Correlations between dichotomous variables were evaluated with Φ coefficient. For all analyses statistical significance was considered for $p < 0.05$.

Results

Results of tests are detailed in Table II.

In both study and control groups, mean workload reached during ECG exercise treadmill test was 7 METs. Exercise phase duration in the study group was 531±148 s and did not differ significantly from the controls (452±210 s).

Baseline ADP- and collagen-induced aggregation indices did not differ significantly between study and control groups.

No significant change of ADP- and collagen-induced platelet aggregation was observed in the study group during exercise test compared to the resting parameters.

In the control group, there was a significant increase of platelet aggregation induced by ADP observed at peak

Table II. Aggregation parameters and P-selectin measurements in the study and control groups

Parameter	Study group N=35	Control group N=10	p
ADP-induced aggregation before exercise test (%)	84.9±11.7	82.1±16.9	NS
ADP-induced aggregation at peak exercise (%)	83.6±13.6	89.1±10.9*	NS
Collagen-induced aggregation before exercise test (%)	64.5±19.9	65.5±23.9	NS
Collagen-induced aggregation at peak exercise (%)	72.0±14.4	82.1±11.5	NS
P-selectin on platelet membrane before exercise (without stimulation with thrombin) (%)	0.48±0.60	0.15±0.32	NS
P-selectin on platelet membrane at peak exercise (without stimulation with thrombin) (%)	0.40±0.87	0.18±0.46	NS
P-selectin on platelet membrane at 1 hour post exercise (without stimulation with thrombin) (%)	2.3±6.3	0.06±0.10	<0.05
P-selectin on platelet membrane before exercise (after stimulation with thrombin) (%)	74.8±15.4	60.4±19.3	0.06
P-selectin on platelet membrane at peak exercise (after stimulation with thrombin) (%)	79.7±8.8	68.5±14.5	<0.03
P-selectin on platelet membrane at 1 hour post exercise (after stimulation with thrombin) (%)	79.5±10.1	64.6±14.6	<0.01
Soluble P-selectin before exercise (ng/ml)	208.8±91.9	190.0±97.6	NS
Soluble P-selectin at peak exercise (ng/ml)	213.0±100.8	193.1±90.7	NS
Soluble P-selectin at 1 hour post exercise (ng/ml)	198.7±82.8	172.5±80.3	NS

** p <0.05 compared to platelet aggregation before exercise*

exercise in comparison to baseline values (p <0.05). No significant change of platelet aggregation was shown after activation with collagen in this group.

The P-selectin expression on platelet membrane without thrombin stimulation did not differ significantly between groups before exercise test and at peak exercise, becoming considerably more pronounced at 1 hour after exercise in the study group (p <0.05).

The P-selectin secretion on platelet membrane after thrombin stimulation showed significant differences between both groups at peak exercise (p <0.03) and at 1 hour after exercise (p <0.01), whereas the difference was statistically borderline before exercise treadmill test (p=0.06).

In the study group no significant change of soluble P-selectin expression was recorded before, at peak and at 1 hour after exercise. Also, no significant differences were found in soluble P-selectin expression in analogous testing conditions in participating controls.

Correlations

A negative correlation was found between reached METs on exercise treadmill test and participants' age (r=–0.47) and presence of diabetes mellitus (r=–0.39). Also, exercise phase duration negatively correlated with age (r=–0.42).

The ST-segment changes observed during exercise positively correlated with presence of angina (r=+0.76), severity of lesions found on coronary angiography (r=+0.40) and history of MI (r=+0.43).

There was a correlation between baseline and peak exercise ADP-induced platelet aggregation intensity (r=+0.86). In addition, a correlation was found between baseline and peak exercise collagen-induced platelet aggregation intensity (r=+0.61).

Discussion

Our study revealed a heterogeneous protective effect of aspirin on mechanisms of platelet activation in patients with IHD. The antiaggregatory effect of aspirin which prevents potential risk of thrombosis was maintained during exercise. On the other hand, inhibition of P-selectin expression was not observed and thus the substance, considered an important haemostatic and inflammatory factor, may increase the risk of post-exercise coronary events in patients with IHD.

Atherosclerosis is a chronic inflammatory process which is induced and supported by endothelium activation. P-selectin is stored in Weibel-Palade bodies of endothelial cells and is secreted and released by activated endothelium. P-selectin is also present in inactive platelets and is redistributed on their surface after platelet activation. P-selectin is rapidly translocated to the cell surface, but it also quickly returns to baseline position after discontinuation of the stimulating factor [11]. Burger and Wagner evaluated release of soluble P-selectin in mice species, in which P-selectin was secreted only from endothelium or only from platelets. Their study revealed that soluble P-selectin is mainly produced in the endothelium as

well as that its release noticeably increases on a cholesterol-rich diet [12].

Extensive physical exercise induces shear forces at the vascular walls. Shear-induced platelet aggregation (SIPA) is an important mechanism increasing the potential of thrombogenesis. Wang et al. evaluated mechanisms involved in enhanced thrombogenesis, such as platelet aggregation, von Willebrand factor binding to platelets, GP IIb/IIIa activation, platelet membrane expression of P-selectin and soluble P-selectin concentration [13]. The results indicate that physical exercise measured by means of maximum oxygen consumption of 80% resulted in platelet aggregation, increased von Willebrand factor activation and its binding to platelets, increased activation of GP IIb/IIIa and caused greater secretion of P-selectin on platelet membranes. Strenous exercise induces SIPA through an increase of binding capacity of von Willebrand factor to platelets with subsequent activation of GP IIb/IIIa complexes and extensive expression of P-selectin [13].

Indeed, there are reports available showing decreased expression of GP IIb/IIIa receptor and CD 62p after exercise and exposure to thrombin, although they are still in a minority [14]. These studies indicate increased release of endogenous mediators inhibiting platelet function as a potential cause of depleted platelet activation and their increased sensitivity to prostacycline and nitrous oxide.

Enhanced thrombotic state was thought to be associated with increased risk of sudden death during strenous exercise. Although fibrinolysis is activated at the same time, its activation ends with exercise termination, whereas thrombosis activation lasts for about another 30 minutes. On the other hand, Tanasescu et al. documented that more intensive physical exertion reduces the risk of IHD and not exercise duration but its intensity remains a considerable predictor of beneficial prognosis [15].

Interestingly, aspirin does not prevent increased platelet aggregation as a response to physical exercise and does not reduce P-selectin expression on platelet membrane and soluble P-selectin levels. Exercise is always associated with hypercatecholaminemia, which triggers ADP and thrombin that activate platelets. According to Hurlen et al., aspirin shows limited antiplatelet and antithrombotic protection in patients during exercise [16].

Available literature shows that risk of sudden death persists 30 minutes after termination of intense exercise [17]. In papers involving populations of healthy individuals coagulation indices increased after intensive exercise (60–70% $VO_{2\,max}$), whereas observations regarding exercise of moderate intensity (40–50% $VO_{2\,max}$) are not so uniform [18, 19]. Physical exercise can be measured as maximum oxygen consumption or workload expressed as METs [20]. Physical exercise of maximum oxygen consumption of >60% corresponds to 6 METs; in our study both patients and healthy individuals performed comparably with a physical effort of mean 7 METs, which may be found intensive in the light of previously presented definitions.

The finding that baseline expression of P-selectin on platelet membrane without stimulation with thrombin is higher in patients is very interesting, whereas activation with thrombin allows the difference to reach borderline statistical significance. Thrombin activation makes the platelets of patients more similar, to some extent, to those of healthy subjects; however, physical exercise reveals a significantly larger growth trend of P-selectin secretion on platelet membrane in patients with ischaemic heart disease. Moreover, in our study secretion of P-selectin on platelet membrane, particularly one hour after discontinuation of exercise, was significantly higher in patients than in the healthy population, which may indicate more intense and prolonged activation of platelets in subjects with IHD. P-selectin expression on platelet membrane in IHD patients after termination of exercise test was significantly higher, both before and after stimulation with thrombin.

A wide range of P-selectin secretion on platelet membrane in patients before thrombin stimulation indicates very large individual variability of this parameter. Different and delayed increase of P-selectin secretion on platelet membrane in individuals after termination of physical exercise indicates incomplete protection offered by aspirin against platelet activation. The occurrance of significant differences between studied groups not before one hour after discontinuation of exercise confirms findings that coagulation activation persists long after discontinuation of exercise.

The reported changes are shown with respect to secretion of P-selectin on platelet membrane; however, similar significant changes were not observed for soluble P-selectin concentrations. Moreover, no significant correlations were found between soluble and platelet membrane P-selectin. Changes observed in serum concentrations cannot be identified with intracellular ones.

In the control group, a significant increase of ADP-induced platelet aggregation was found at peak exercise along with a tendency to increase collagen--induced aggregation; however, it failed to reach statistical significance. In patients with IHD platelet aggregation in the same conditions did not change significantly.

These detailed findings revealed a heterogeneous protective effect of aspirin on mechanisms of platelet activation in patients with IHD. The antiaggregatory

effect of aspirin which prevents potential risk of thrombosis is maintained during exercise. On the other hand, inhibition of P-selectin expression was not observed and thus the substance, as an important haemostatic and inflammatory factor, may increase the risk of post-exercise coronary events in patients with IHD.

Conclusions

1. Exercise does not exaggerate platelet aggregation in patients with IHD taking aspirin on a regular basis.
2. In patients with IHD, despite taking aspirin, platelet activation measured by means of P-selectin expression on platelet membrane is increased and is further enhanced during exercise treadmill test.
3. Concentration of soluble P-selectin does not reflect expression of P-selectin on platelet membranes in patients with IHD.

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Agregacja płytek krwi i stężenie P-selektyny podczas próby wysiłkowej u osób z chorobą niedokrwienną serca

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Streszczenie

Wstęp: Aspiryna, lek hamujący agregację płytek krwi, od wielu lat jest stosowana w prewencji wtórnej choroby niedokrwiennej serca, udarów mózgu i w niektórych postaciach migotania przedsionków. Antyagregacyjne działanie aspiryny może być niedostateczne u osób pierwotnie na nią wrażliwych, jeżeli zaistnieją szczególne okoliczności, na przykład w trakcie wysiłku fizycznego. U pacjentów z chorobą niedokrwienną serca już w warunkach podstawowych ma miejsce nadmierne pobudzenie układu krzepnięcia. W czasie wysiłku fizycznego zjawisko to może się nasilać i powodować niedokrwienie mięśnia serca. Potwierdzenie tej hipotezy może mieć istotne znaczenie w prowadzeniu terapii przeciwpłytkowej u pacjentów z chorobą niedokrwienną serca.

Cel: Celem pracy była ocena agregacji płytek krwi i stężenia P-selektyny u chorych ze stabilną postacią choroby niedokrwiennej serca (w II stadium wg *Canadian Cardiac Society,* CCS) w czasie kontrolowanego wysiłku fizycznego.

Metoda: Do badania włączono 35 osób (w tym 15 kobiet i 20 mężczyzn, średnia wieku 64,7±10 lat) z chorobą niedokrwienną serca w II stadium wg CCS udokumentowaną koronarograficznie. Chorzy byli leczeni preparatami przeciwdławicowymi zgodnie z obowiązującymi standardami Polskiego Towarzystwa Kardiologicznego, otrzymywali przy tym aspirynę w dawce 75 mg raz dziennie od co najmniej 30 dni. Pacjenci nie stosowali innych leków mogących wpływać na agregację płytek krwi. W grupie badanej wykonywano badanie koronarograficzne z dostępu przez tętnicę udową, w standardowych projekcjach. Stopień zwężenia naczynia wieńcowego 70% i powyżej był uznawany za istotny.

Grupę kontrolną stanowiło 10 osób, w tym 4 kobiety i 6 mężczyzn, średnia wieku 64,4±11 lat, otrzymujących aspirynę w dawce 75 mg raz dziennie od co najmniej 30 dni oraz niestosujących innych leków mogących wpływać istotnie na agregację płytek krwi.

Parametry agregacji płytek krwi badano 2-krotnie: przed wysiłkiem i na jego szczycie. Próbę wysiłkową wykonywano na bieżni ruchomej Burdick T 600. Trzykrotnie: przed próbą wysiłkową, na jej szczycie oraz godzinę po zakończeniu próby pobierano krew, aby ocenić funkcje płytek krwi – ich aktywność spontaniczną i po podaniu trombiny, oznaczając rozpuszczalną P-selektynę, a także stopień ekspresji P-selektyny na błonie płytek w próbkach krwi przed i po stymulacji trombiną.

Wyniki: W grupie kontrolnej wykazano istotne zwiększenie agregacji z ADP na szczycie wysiłku w porównaniu z wartościami przed wysiłkiem ($p < 0,05$). Nie wykazano statystycznie istotnej zmiany agregacji na szczycie wysiłku pod wpływem kolagenu. Ekspresja P-selektyny na błonie płytek bez stymulacji trombiną nie różniła się istotnie między grupą badaną a kontrolną przed wysiłkiem i na szczycie wysiłku, natomiast 1 godz. po zakończeniu wysiłku była istotnie wyższa w grupie badanej ($p < 0,05$). Wydzielanie P-selektyny na błonie płytek po stymulacji trombiną różniło się istotnie między grupą badaną a kontrolną na szczycie wysiłku ($p < 0,03$) i 1 godz. po zakończeniu wysiłku ($p < 0,01$), natomiast przed wysiłkiem różnica była na granicy istotności statystycznej ($p=0,06$).

Stopień agregacji płytek wywołany ADP przed wysiłkiem korelował ze stopniem agregacji wywołanym ADP na szczycie wysiłku ($r=+0,86$). Także stopień agregacji płytek wywołany kolagenem przed wysiłkiem korelował ze stopniem agregacji wywołanym kolagenem na szczycie wysiłku ($r=+0,61$).

Wnioski: 1. Wysiłek fizyczny nie nasila agregacji płytek krwi u osób z chorobą niedokrwienną serca przyjmujących regularnie aspirynę. 2. U osób z chorobą niedokrwienną serca, mimo przyjmowania aspiryny, aktywacja płytek krwi mierzona wydzielaniem P-selektyny na błonie płytek jest zwiększona i ulega dalszemu nasileniu w trakcie próby wysiłkowej. 3. Stężenie rozpuszczalnej P-selektyny nie odzwierciedla ekspresji P-selektyny na błonie płytek krwi u osób z chorobą niedokrwienną serca.

Słowa kluczowe: agregacja płytek krwi, P-seketyna

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