Resveratrol Attenuates Adenosine Diphosphate-Induced Platelet Activation by Reducing Protein Kinase C Activity

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Abstract: Inappropriate platelet activation is the key point of thrombogenesis. The aim of the present study was to investigate the effects of resveratrol (RESV), a compound extracted from the Chinese medicinal herb Polygonum cuspidatum sieb et Zucc, on the platelet activation induced by adenosine diphosphate (ADP) and its possible mechanism. The percentage of platelet aggregation and surface P-selectin-positive platelets, and the activity of protein kinase C (PKC) of platelet were observed with platelet aggregometer, flow cytometry and phosphorimaging system, respectively. RESV at 25, 50 and 100 µM showed anti-platelet aggregation and inhibition of surface P-selectin-positive platelets in a concentration-dependent manner. RESV (50 µM) inhibited the activity of PKC in the membrane fraction of platelets and decreased the percentage of membrane associated PKC activity in total PKC activity. Moreover, DL-erythro-1,3-Dihydroxy-2-aminooctadecane, an elective protein kinase C inhibitor (PKCI), and RESV had additive effects of inhibiting the percentage of platelet aggregation and surface P-selectin-positive platelets. It is suggested that RESV may inhibit platelet aggregation, the percentage of surface P-selectin-positive platelets and subsequent thrombus formation. The mechanisms may be partly relative to the decrease of the activity of PKC of platelets.

Keywords: Resveratrol; Traditional Chinese Medicine; Platelet Aggregation; Flow Cytometry; Protein Kinase C.

Introduction

Resveratrol (RESV), a well-known polyphenolic compound, was extracted from Polygonum Cuspidatum sieb et Zucc, also named veined rabdosia herb (Xiang et al.,

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2004). It was quoted originally in the book *Transactions of Famous Physicians* in the Han Dynasty in China and has been efficaciously used in traditional Chinese medicine to treat several diseases, including thromboembolic diseases for over hundreds of years. In recent years, pharmacological studies have found that RESV possesses multifaceted cardiovascular benefits, including anti-platelet aggregation (Pace-Asciak *et al*., 1995), inhibition of proliferation of vascular smooth muscle cells (Zhou *et al*., 2004) and cardiac fibroblast (Olson *et al*., 2005). However, the mechanisms of these actions are not fully understood, further research may provide convincing and exact evidence that RESV would be invaluable for medical treatment of cardiovascular diseases.

Protein kinase C (PKC), an ubiquitous serine/threonine protein kinase found in a variety of animal tissues, has been implicated in the regulation of many cellular processes, including growth, differentiation, sensory transduction, hormone and neurotransmitter release, gene expression, and cellular metabolism (Kikkawa and Nishizuka, 1986; Huang, 1990). In platelets, the PKC is an important signaling mediator required for activation, secretion of granule contents, and aggregation (Yacoub *et al*., 2006).

In this study, we investigated whether the molecular mechanism of cardiovascular protective effect of RESV was through the inhibition of the activity of platelet PKC. Therefore, we observed the effects of RESV and DL-erythro-1,3-Dihydroxy-2-aminoocetyldecane, an elective protein kinase C inhibitor (PKCI) on ADP-induced platelet aggregation, the percentage of surface P-selectin-positive platelets, and the activity of platelet PKC in healthy volunteers with platelet aggregometer, flow cytometry and phosphorimaging system, respectively.

**Materials and Methods**

**Reagents**

RESV (trans-3, 4′, 5-trihydroxystilbene) was purchased from ZheJiang Pharmacological Institute in China. Stock solution of RESV was made in dimethyl sulfoxide (DMSO) at a concentration of 12.5 mM and kept frozen. When used in platelet preparations, it was diluted with DMSO and used at final concentrations of 25, 50 and 100 uM. When used in PKC activity assay, it was diluted with DMSO and used at final concentration of 50 uM. When used in flow cytometry, it was diluted with 20% DMSO (DMSO:HEPES = 1:4) and used at the final concentrations of 6.25, 12.5, 25, 50, 100. Unless otherwise indicated, all reagents used in platelet aggregations and PKC activity assay were dissolved in 100% DMSO. All the concentrations expressed in this study were final concentrations. Adenosine diphosphate (ADP) and apyrase (grade VII) were purchased from Sigma (St. Louis, MO, USA). PKCI was purchased from Tocris Cookson Inc. (Ellisville, Missouri, USA). \( \gamma^{32} \text{P}\)ATP (5000 Ci/mmol) was purchased from YaHui Biomedical Engineering Company (Beijing, China). R-Phycocerythrin (R-PE)-Conjugated mouse anti-human CD62p monoclonal antibody was purchased from BD Biosciences. PKC Signa TECT® protein kinase C (PKC) assay system was purchased from Promega Corporation (Madison, WI, USA).
Platelet Preparations

Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as described previously (Dogne et al., 2000; 2001). Whole blood was obtained by venous-puncture from healthy non-smoking human volunteers (ages 23–38 years), who had not consumed any medication known to affect platelet function in the last 2 weeks, using standard blood drawing procedures (normal blood flow and no pressure). Informed consent was signed by all subjects. Blood was drawn into tubes containing one-sixth volume of ACD (2.5 g sodium citrate, 1.5 g citric acid, and 2 g glucose in 100 ml deionized water) and then centrifuged (Optima L-XP centrifuge, Beckman Coulter, USA) at 230 g for 20 min at room temperature to obtain PRP. PPP was prepared from the remaining volume of blood by centrifugation at 1600 g for 10 min. Platelet in PRP was adjusted to 2.5 and 3.0 × 10^8 cell/ml (Shah and Saeed, 1995) by dilution of PPP with F-820 automatic blood analysator (Sysmex, Japan). Both PRP and PPP were used within 1.5 hours from blood collection.

For the isolation of washed platelets, apyrases (1 U/ml) were added to whole blood to obtain the PRP. After diluted 3-fold by ACD containing apyrases, the PRP was then centrifuged for 10 min at 980 g at room temperature to pellet the platelets. Platelets were resuspended in Tyrode buffer (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl, 3 mM NaH2PO4, 5 mM glucose, 10 mM HEPES, pH 7.4, 0.2% bovine serum albumin) containing 0.01 U/ml apyrase and adjusted to 2.5 and 3.0 × 10^8 cell/ml. Washed platelets were used for PKC activity studies within 3 hours after isolation.

Platelet Aggregations

Platelet aggregation was studied as described previously by Born (1962) in a four-channel platelet aggregometer (Chronolog, Chicago, USA). Briefly, samples of PRP (250 µl) as prepared above in cuvettes were mixed with HEPES buffer (Rest Group and Control Group), or with 25, 50 and 100 µM of RESV (RESV 25, RESV 50 and RESV 100 Groups), or with 10^-6 M of PKCI (PKCI Group), or with 10^-6 M of PKCI and 50 µM of RESV (PKCI + RESV 50 Group), or with DMSO (DMSO Group, RESV at 0 µM). The final concentration of DMSO was 2% (v/v) in all groups except rest group and control group. After reacting for 6 min at 37°C, aggregation in all groups except rest group was started by using 10^-5 M of ADP (dissolved in HEPES buffer, as follows). Aggregation was allowed to proceed with constant stirring at 1000 rpm. Results were evaluated and calculated using maximal percentage of platelet aggregation which expressed as the percent increase in light transmission after 6 min.

Flow Cytometry

Except specifically stated, all reagents used in flow cytometry were dissoled in 20% DMSO. The flow cytometric analysis of platelets in whole blood has been described previously (Chronos et al., 1994). RPE-CD62P fluorescence was monitored to obtain
the percentage of P-selectin-positive platelets. Within 3 min of collection, 5 µl of whole blood was added to prewarmed cuvettes containing 5 µl of RPE-CD62P, and incubated in the dark at room temperature for 10 min with HEPES buffer (rest group and control group), RESV at 6 different final concentrations (6.25, 12.5, 25, 50, 100, and 200 µM), 10⁻⁶ M of PKC1 (PKCI Group), 10⁻⁶ M of PKC1 and 50 µM of RESV (PKCI + RESV 50 Group), or with 20% DMSO (DMSO Group, RESV at 0 µM). Total volume was 45 µl by added HEPES in all cuvettes except rest group cuvettes which was 50 µl and final concentration of DMSO was 2% (v/v) in all groups except rest group and control group. Later, 10⁻⁵ M of ADP was added in all cuvettes except the rest group, and samples were further incubated in the dark at room temperature for 10 min without stirring. Thereafter, the blood samples were labeled for flow cytometric analyses.

**PKC Activity Assay**

The PKC Signa TECT®, protein kinase C (PKC) assay system was used according to the manufacturer’s instructions. Briefly, to assay membrane associated, cytosolic, and total PKC activities (membrane associated PKC activity + cytosolic PKC activity) in platelet of healthy volunteers, washed platelets (250 µl) as prepared above were mixed with HEPES buffer (rest group and control group), or RESV (final concentration was 50 µM, RESV 50 Group), or DMSO (DMSO Group, RESV at 0 µM). The final concentration of DMSO was 2% (v/v) in RESV Group and DMSO Group. After reacting for 5 min at 37°C, aggregation in all groups except rest group was started by using 10⁻⁵ M of ADP. After 1.2 ml buffer A (0.5 mM EDTA, 0.5 mM EGTA, 25 mM Tris-Hcl PH 7.4, 10 mM β-mercaptoethanol, 1 µg/ml leupeptin and 1 µg/ml aprotinin) was added, samples of washed platelets were split by JY92-II ultrasonic cell destrustor (XinZhi Biotechnology Institute, Shanghai, China) and centrifuged for 2 hours at 4°C, 13000 g to yield a cytosolic supernatant and a membrane associated pellet. The membrane associated pellet was resuspended in buffer B (containing 0.05% Triton X-100 in Buffer A). After centrifugation for 1 hour at 4°C, 13000 g, the supernatant, representing the membrane fraction, was isolated. The cytosolic supernatant was used to detect the cytosolic PKC activity in platelet and the supernatant of membrane was used to detect the membrane associated PKC activity in platelet. Cuvettes of specimen and control were prepared to detect PKC activity in each supernatant. The cuvette of specimen contained 5 µl of supernatant, 5 µl of PKC coactivation 5 × buffer, 5 µl of PKC activation 5 × buffer, 5 µl of PKC biotinylated peptide substrate and 5 µl of [γ⁻³²P]ATP mix (500 µl of 0.5 mM ATP with 5 µl of 10 μci/µl [γ⁻³²P]ATP). The cuvette of control contained 5 µl of supernatant, 5 µl of PKC coactivation 5 × Buffer, 5 µl of 5 × control buffer, 5 µl of PKC biotinylated peptide substrate and 5 µl of [γ⁻³²P]ATP mix (500 µl of 0.5 mM ATP with 5 µl of 10 μci/µl [γ⁻³²P]ATP). After 5-min incubation at 30°C, reactions were terminated by adding 12.5 µl of termination buffer to each action. Ten microlitres from each terminated reaction was spotted on SAM²® membrane. After all samples had been spotted, the SAM²® membrane was washed
and air-dried. Radioactivity was measured by a phosphorimaging system (Typhoon 9200, Amersham, Sweden). The enzyme activity of PKC was calculated as pmol ATP/min/µg protein by using the following formula:

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\text{Enzyme activity} = \frac{(\text{cpm}_\text{reaction with phospholipids} - \text{cpm}_\text{reaction without phospholipids}) \times (37.5)}{(10) \times (\text{time}_{\text{min}}) \times (\text{amt of protein in reaction}_{\mu\text{g}}) \times (\text{specific activity of } [\gamma^{32}\text{P}]\text{ATP})}.
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The amount of protein in reaction samples was calculated by Bradford’s dye-binding assay. The specific activity of \([\gamma^{32}\text{P}]\text{ATP}\) in cpm/pmol of ATP = \((37.5/5) \times (X)/2500 = 0.003 (X)\), \(X\) is the average counts/min of the 5 µl samples from terminated reaction spotted on SAM\(^{20}\) membrane and not washed. The sum of the reaction volume (25 µl) + the termination buffer volume (12.5 µl) is 37.5 µl. Ten is the volume in microliters of the sample spotted on SAM\(^{20}\) membrane. The number of pmol of ATP in the reaction is 2500.

**Statistical Analyses**

Chi Square tests were employed to compare categorical variables, while Mann Whitney U tests were used, where appropriate, in the univariate analysis. Platelet aggregation changes were evaluated by repeated-measures analysis of variance for intra-group comparisons. \(p < 0.05\) were considered significant. SPSS 10.0 statistical software was used in the statistical analysis.

**Results**

**Effect of RESV on Platelets Aggregation**

Compared to the rest group, the percentage of platelet aggregation induced by ADP was significantly higher in control group (77.56 ± 7.93 vs 7.37 ± 3.29, \(p < 0.01\)). Since RESV is almost insoluble in saline that is normally used as a solvent in the assay method, it was dissolved in DMSO. As shown in Fig. 1, stimulation of DMSO-treated PRP (DMSO Group, RESV at 0 uM,) with ADP resulted in slightly decreased aggregation in comparison with no-treated PRP induced by ADP (62.37 ± 6.95%, \(p > 0.05\)), whereas treatment of PRP with RESV at 25, 50 and 100 µM (55.83 ± 7.41%, 45.45 ± 8.47%, and 40.88 ± 9.29%, respectively) showed a concentration-dependent increase in inhibiting platelet aggregation and more profound inhibition than DMSO-treated PRP (\(p < 0.05\)). PKCI (\(10^{-6}\) M, 50.36 ± 10.02%) was not a potent inhibitor of aggregation induced by ADP. However, when \(10^{-6}\) M of PKCI and 50 µM of RESV were added together, their effect in inhibiting platelet aggregation (35.64 ± 9.84%) was significantly lower as compared to DMSO group (\(p < 0.01\)). PKCI and RESV showed additive inhibition of ADP-induced platelet aggregation.
Effect of RESV on the Percentage of Surface P-Selectin-Positive Platelets

ADP-inducing the percentage of surface P-selectin-positive platelets was investigated in the absence or presence of RESV. The percentage of surface P-selectin-positive platelets increased from 2.77 ± 1.36% (rest group, unstimulation) to 62.31 ± 11.46% (control group, stimulation with $10^{-5}$ M of ADP, $p < 0.01$), and DMSO (DMSO Group, RESV at 0 µM) reduced the percentage of surface P-selectin-positive platelets to 38.14 ± 9.57% as compared to the control group. RESV (50 µM) had no influence on the percentage of surface P-selectin-positive platelets in unstimulated samples (data not shown), but inhibited ADP-induced percentage of surface P-selectin-positive platelets dose-dependently (from 31.42 ± 7.46% to 20.96 ± 6.34% at $10^{-5}$ M ADP; PRP were pretreated with HEPES for 5 min in the control group or without ADP in the rest group. Final concentration of DMSO was 2% (v/v) in all groups except rest and control groups. Aggregation reactions were allowed to proceed and the maximal aggregation ratios of platelets were determined. Standard error bars were shown. Numerical data was means ± SE of 6 independent experiments; $^* p < 0.05$ compared to the DMSO group and $^5p < 0.05$ compared to the RESV 50 and the PKCI groups, respectively.

Figure 1. RESV inhibited the percentage of platelet aggregation induced by ADP. PRP (250 µl) were pretreated with DMSO (RESV at 0 µM, DMSO), or with 25, 50 and 100 µM of RESV (RESV 25, RESV 50, and RESV 100), or with $10^{-6}$ M of protein kinase C inhibitor (PKCI), or with $10^{-6}$ M of PKCI and 50 µM of RESV (PKCI + RESV 50) for 5 min prior to the addition of $10^{-5}$ M ADP; PRP were pretreated with HEPES for 5 min in the control group or without ADP in the rest group. Final concentration of DMSO was 2% (v/v) in all groups except rest and control groups. Aggregation reactions were allowed to proceed and the maximal aggregation ratios of platelets were determined. Standard error bars were shown. Numerical data was means ± SE of 6 independent experiments; $^* p < 0.05$ compared to the DMSO group and $^5p < 0.05$ compared to the RESV 50 and the PKCI groups, respectively.

Effect of RESV on the Percentage of Surface P-Selectin-Positive Platelets

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RESVERATROL, ADP, PLATELET ACTIVATION AND PROTEIN KINASE C

Figure 2. RESV attenuated the percentage of surface P-selectin-positive platelets induced by ADP. Whole blood (5 µl) was preincubated at 37°C without stirring for 10 min in the presence of varying concentrations of RESV. Samples were then further incubated for 10 min in the presence of 10^{-5} M ADP. The percentage of surface P-selectin-positive platelets was measured by whole blood flow cytometry (A). The percentage of inhibition of surface P-selectin-positive platelets by RESV were also presented (B). Values were means ± SE of 5 independent experiments. Whole blood (5 µl) were mixed with DMSO (RESV at 0 µM, DMSO), or with 50 µM of RESV (RESV 50), or with 10^{-6} M of protein kinase C inhibitor (PKCI) and or with 10^{-6} M of PKCI plus 50 µM of RESV (PKCI + RESV 50) for 10 min prior to the absence (rest) or presence (control) of 10^{-5} M ADP. The final concentration of DMSO was 2% (v/v) in all groups except rest and control groups. Standard error bars were shown. Numerical data was means ± SE of 5 independent experiments; *p < 0.05 compared to the DMSO group and $p < 0.05$ compared to the RESV 50 and the PKCI groups, respectively (C).

Effect of RESV on PKC Activity of Platelets

Phosphorimaging system was used to study the effect of RESV on the cytosolic PKC activity, the activity of PKC in membrane fraction of platelets and the percentage of membrane associated PKC activity in total PKC activity in healthy volunteers. As shown in Fig. 3, there were no significant differences of total PKC activity among the rest group, the control group, RESV group, and DMSO group (p > 0.05). The cytosolic PKC activity was significantly higher in rest group (4.22 ± 0.39) than those in control group, RESV group, and DMSO group (1.95 ± 0.33, 3.35 ± 0.35, and 2.57 ± 0.27, p < 0.01, p < 0.05, p < 0.05, respectively), whereas the activity of PKC in membrane fraction of platelets
and the percentage of membrane associated PKC activity in total PKC activity were significantly lower in rest group (1.73 ± 0.36, 27.29 ± 6.03%) than those in other groups (3.93 ± 0.31, 2.44 ± 0.28, 3.44 ± 0.25 and 67.25 ± 7.06%, 41.88 ± 6.36%, 57.41 ± 5.62%, p < 0.01, p < 0.05, p < 0.05, respectively). When control group, DMSO group and RESV group were compared, the RESV group had significantly higher cytosolic PKC activity, lower membrane associated PKC activity and the percentage of membrane associated PKC activity in total PKC activity than those in control group and DMSO group (p < 0.01, p < 0.05, respectively). No statistical difference was found between control group and DMSO group in the cytosolic PKC activity, the membrane associated PKC activity and the percentage of membrane associated PKC activity in total PKC activity.

Discussion

Thromboembolic diseases are the most common lethal and mutilation factors, and coronary heart disease, a representative thromboembolic disease, remains a primary contributor to morbidity and mortality in both developing and developed countries (Stehbens, 2001). A great need now arises to develop both efficacious and pharmaceutical medicines to combat these diseases. Thrombosis is the basic pathological change of such disease, and platelet activation is the key point of thrombogenesis. Therefore, platelet activation and consequences in vivo remains a subject of scrutiny (McNicol and Gerrard, 1997), with specific interest in platelets as targets for anti-thrombotic agents (Clutton et al., 2001;

Figure 3. Effect of RESV on activity of PKC in platelets. Washed platelets (250 µl) were preincubated at 37°C without stirring for 10 min with 50 µM of RESV (RESV 50) or DMSO (RESV at 0 µM, DMSO) prior to the addition of 10⁻⁵ M of ADP; Washed platelets were preincubated with HEPES for 10 min prior to the presence (control) or absence (rest) 10⁻⁵ M of ADP. The final concentration of DMSO was 2% (v/v) in all groups except rest and control groups. Phosphorimaging system were used to study the membrane associated, cytosolic, and total PKC activities in platelets as described in “Materials and Methods” (A). The percentages of membrane associated PKC activity in total PKC activity were also presented (B). Standard error bars were shown. Numerical data was means ± SE of 5 independent experiments; *p < 0.05 compared to the DMSO group.
RESVERATROL, ADP, PLATELET ACTIVATION AND PROTEIN KINASE C

Bhatt and Topol, 2003). RESV has recently become a prime target in experimental and clinical cardiovascular research (El-Mowafy, 2002). This study was undertaken to ascertain whether RESV possesses antiplatelet activation and anti-thrombosis properties. As shown in Fig. 1, RESV exerted a potent inhibiting effect of platelet aggregation induced by ADP in a concentration-dependent manner. These results clearly suggested that RESV was an effective inhibitor of platelet function.

P-selectin, normally stored in alpha-granules of platelets or Weibel-Palade bodies of endothelial cells, can be rapidly translocated to the cell surface 10 min after stimulation by inflammatory mediators such as thrombin, histamine, or oxygen free radicals (Geng et al., 1990; Zimmerman and Granger, 1990), which results in triggering multiple intracellular events within leukocytes to promote vascular inflammation and facilitate atherosclerosis and thrombosis (Furie et al., 2001; Burger and Wagner, 2003; Furie and Furie, 2004). In clinic, an increased percentage of surface P-selectin-positive platelets has been found in patients with atherothrombotic diseases, including those with acute coronary syndrome (Yip et al., 2005). However, few pharmacological studies have been reported about the effects of RESV on the surface P-selectin-positive platelets in healthy volunteers. In addition, the mechanism by which RESV affects platelets is not fully clarified. In the present study, we confirmed that ADP-activated platelets increased the percentage of surface P-selectin-positive platelets (Fig. 2C). This effect was significantly diminished when platelets were exposed to RESV at concentrations as low as 6.25 μM (Fig. 2A). RESV decreased the percentage of surface P-selectin-positive platelets in a concentration-dependent manner. Since P-selectin on the activated platelet surface is a key adhesion molecule for initiating thrombus formation in vivo, the present findings provide new insights into the antithrombotic mechanisms of RESV.

PKC, the serine/threonine-specific kinase activated by second messenger action, plays a central role in signal transduction. It has both cytosolic and plasma membrane-bound forms and the former is the most abundant under resting conditions (Kikkawa and Nishizuka, 1986). Furthermore, the cytosolic enzyme can translocate to the plasma membrane upon cell stimulation and elevation of cellular Ca²⁺ (Yung and Hui, 1993). One particular and important aspect of PKC activation is the intracellular redistribution of the enzyme from the cytosol to the cell membrane (Yacoub et al., 2006). In the present study, this mechanism was confirmed by phosphorimaging system. As shown in Fig. 3A, PKC distributed mostly across the cytosol of platelets in resting platelets and redistributed to the membrane later to be activated by ADP. If pretreated by RESV, PKC translocation to the membrane was partially inhibited in the platelets activated by ADP. These results suggested that RESV inhibited the PKC-mediated signal transduction pathway in platelets since the symbol of PKC activation was the translocation of PKC from the cytosolic form to the plasma membrane (Farago and Nishizuka, 1990).

Although the precise intracellular signaling pathway involving P-selectin translocation is not understood clearly, PKC activators have been shown to significantly increase the percentage of surface P-selectin-positive in endothelial cells (Geng et al., 1990). Moreover, thrombin, a well-known platelet stimulator, has also been shown to stimulate the PKC-mediated signal transduction pathway in platelets (Sano et al., 1983). Thus, a
PKC-mediated signal transduction pathway may be at least partially involved in the up-regulation of P-selectin on platelets. This was supported by our results that PKCI, just as RESV, inhibited platelet aggregation and attenuated the percentage of surface P-selectin-positive platelets of healthy volunteers, and that PKCI and RESV showed additive inhibition of ADP-induced platelet aggregation and the percentage of surface P-selectin-positive platelets. Furthermore, ADP-induced PKC activity was decreased in the platelets pretreated with RESV.

In conclusion, our data showed that RESV attenuated ADP-induced platelet aggregation and the percentage of surface P-selectin-positive platelets by reducing PKC activity. RESV might act as an inhibitor on PKC activity in platelets and serve as a novel antithrombotic agent.

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