Microvascular Endothelial Dysfunction and Enhanced Thromboxane and Endothelial Contractility in Patients with HIV

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Abstract

Background: The prevalence of cardiovascular disease is increased with human immunodeficiency virus (HIV) infection, but the mechanism is unclear. We hypothesized that HIV increases microvascular reactive oxygen species, thereby impairing endothelial function and enhancing contractility.

Method: Subcutaneous microvessels were isolated from gluteal skin biopsies in premenopausal, African American, HIV positive women receiving effective anti-retroviral therapy, but without cardiovascular risk factors except for increased body mass index (n=10) and healthy matched controls (n=10). The arteries were mounted on myographs, preconstricted and relaxed with acetylcholine for: endothelium-dependent relaxation, endothelium-dependent relaxation factor (nitric oxide synthesize-dependent relaxation), endothelium-dependent hyperpolarizing factor (potassium-channel dependent relaxation) and endothelium-independent relaxation (nitroprusside). Contractions were tested to endothelium-dependent contracting factor (acetylcholine contraction with blocked relaxation); phenylephrine, U-46,619 and endothelin-1. Plasma L-arginine and asymmetric dimethylarginine were measured by high performance capillary electrophoresis.

Results: The micro-arterioles from HIV positive women had significantly (% change in tension; P<0.05) reduced acetylcholine relaxation (-51 ± 6 vs. -78 ± 3%), endothelium-dependent relaxation factor (-25 ± 4 vs. -39 ± 3%), endothelium-dependent hyperpolarizing factor (-17 ± 4 vs. -37 ± 4%) and decreased nitric oxide activity (0.16 ± 0.03 vs. 0.70 ± 0.16 Δ unit) but unchanged nitroprusside relaxation. They had significantly enhanced endothelium-dependent contracting factor (+21 ± 6 vs. +7 ± 2%) and contractions to U-46,619 (+164 ± 10 vs. +117 ± 11%) and endothelin-1(+151 ± 12 vs. +97 ± 9%), but not to phenylephrine. There was enhanced reactive oxygen species with acetylcholine (0.11 ± 0.02 vs. 0.05 ± 0.01 Δ unit; P<0.05) and endothelin-1 (0.31 ± 0.06 vs. 0.10 ± 0.02 Δ unit; P<0.05). Plasma L-arginine: asymmetric dimethyl arginine rates was reduced (173 ± 12 vs. 231 ± 6 μmol·μmol⁻¹, P<0.05).

Conclusion: Premenopausal HIV positive women had microvascular oxidative stress with severe endothelial dysfunction and reduced nitric oxide and arginine: asymmetric dimethylarginine ratio but enhanced endothelial, thromboxane and endothelin contractions. These microvascular changes may herald later cardiovascular disease.

Keywords: Cardiovascular disease (CVD); Endothelial dysfunction; Nitric oxide (NO); Endothelium-dependent relaxing factor (EDRF); Reactive oxygen species (ROS); Asymmetric dimethylarginine (ADMA); Thromboxane-prostanoid receptors (TP-Rs); Endothelin-1 (ET-1)

Introduction

Highly active antiretroviral therapy (HAART) has prolonged the life of those infected with the human immunodeficiency virus (HIV), but sadly, they suffer from an increased burden of many diseases usually encountered in older subjects, such as myocardial infarction [1] and stroke [2], accompanied by carotid artery remodeling and accelerated arteriosclerosis [3]. These complications have been related both to HAART [4] and to HIV infection [3]. Small vessel disease contributes to renal glomerulopathy [5], microvascular dementia [6], congestive cardiac heart failure (CHF) [7] and pulmonary hypertension [8], all of which are commoner in HIV-infected individuals. Subjects with HIV infection have vascular inflammation [9] and endothelial dysfunction as assessed indirectly from brachial artery flow-mediated vasodilatation (FMD) [10]. This has been related in some [11], but not all [10] studies, to HAART or to increased systemic markers of reactive oxygen species (ROS) [12].

We reported that subdermal microvessel dissected from a gluteal skin biopsy from patients with stage I chronic kidney disease (CKD), but without overt cardiovascular disease (CVD), had severe microvascular endothelial dysfunction and impaired nitric oxide synthase (NOS) activity [13]. These effects were not detected by brachial artery endothelium-dependent flow-mediated dilation [14]. Thus, we have studied subcutaneous microvessels directly to investigate the hypothesis that HIV infection in premenopausal women largely free of CVD risk factors is accompanied by microvascular ROS, endothelial dysfunction and reduced nitric oxide (NO) and enhanced contractility. We investigated: acetylcholine (ACh)-induced endothelium-dependent relaxation (EDR); endothelium-dependent relaxation factor (EDRF; nitric oxide synthase (NOS)-dependent relaxation) and endothelium-dependent hyperpolarizing factor (EDHF; potassium-channel dependent relaxation) and endothelium independent relaxation (EIR; sodium nitroprusside (SNP) relaxation) and related these to microvascular NO generation and to the plasma ratio of L-arginine to...
asymmetric dimethylarginine (ADMA) which is the NOS substrate and inhibitor respectively. We also investigated the endothelium-dependent contracting factor (EDCF; contractions under spontaneous tone with relaxation pathways inhibited [15]).

Patients with HIV have enhanced arterial and venous thromboembolic disease [2,16], coagulation [18] and pulmonary hypertension [8] which have been related to thrombomoduline-prostanoid receptor (TP-Rs) and/or endothelin 1 (ET-1) signaling [13,19-23]. ROS-dependent activation of vascular TP-Rs contributes to vasculopathy, inflammation [24] and atherosclerosis [25], but the roles of ET-1 and TP-R signaling in the vascular disease of patients with HIV infection has not been explored. Therefore, we also studied microvascular contractile responses to the stable TP-R agonist, U-46,619 and to ET-1 and compared these to phenylephrine (PE) which does not cause prominent vascular oxidative stress [15,26].

Materials and Methods

Study population

Self-identified African American premenopausal women (n=10) enrolled in the Metropolitan Washington Women’s HIV Study Group (WHIS) who were free of CVD risk factors except for increased body mass index (BMI) and had well-controlled HIV were the test group. All received HAART and all had an HIV viral load<500 copies/ml within 3 months. The control group (n=10) was selected from matched healthy African-American premenopausal subjects participating in the WHIS. Exclusion criteria for both groups included: prior stroke, myocardial infarction, kidney or liver disease, dementia, hypertension, diabetes mellitus or endocrine disease, anemia, dyslipidemia, alcoholism or current substance abuse, post-menopausal or ovariectomised state or receiving female sex hormones, a smoker within the last 6 months, abnormal liver function tests, urinalysis or serum creatinine, inability to comprehend the informed consent or requirement for treatment other than HAART.

Subject was given a written consent as approved by the Georgetown University Institutional Review Board. Their seated blood pressure (BP) was measured with an automated apparatus after 15 minutes of rest with a mean of 3 readings. No subject had a BP ≥140/90 mmHg or a serum creatinine ≥ 1.3 mg·ml⁻¹ or an abnormal urinalysis (except for one trace of protein).

Preparation of small subcutaneous vessels

A subcutaneous gluteal fat biopsy (approximately 3 × 0.6 × 2 cm³) was placed without delay in physiological saline solution (PSS) [27] at 4°C. Small arteries (200 to 250 μm in diameter) were mounted in two isometric, four chamber Mulvany-Halpern small-vessel myographs (Danish MyoTech, Aarhus, Denmark) [13].

Microvascular protocols

The resistance vessels were warmed to 37°C, equilibrated for 30 min, and the internal circumference set to give a wall tension of 0.2 mN·mm⁻² [15]. The myograph chambers were bubbled with 5% CO₂ and 21% O₂, and maintain at pH of 7.4 [15,27]. Vessels were contracted three times with norepinephrine (NE) (10⁻⁵ mol·l⁻¹), followed by one exposure to high-potassium (123 mmol·l⁻¹) solution (KPSS) and finally a repeat exposure to KPSS containing NE (10⁻³ mol·l⁻¹, NAK). NAK provided the reference contraction [15]. Contractions were maintained for 3 min before rinsing with PSS.

EDR, EDRF, EIR and EDCF responses

Two vessels were preconstricted with 10⁻⁴ mol·l⁻¹ NE and relaxed with acetylcholine (ACH; 10⁻⁴ to 10⁻³ mol·l⁻¹, EDR) or sodium nitroprusside (SNP; 10⁻⁴ to 10⁻³ mol·l⁻¹, EIR). The EDRF response was the relaxation to Ach in PSS (vehicle) minus the same response with 10⁻⁵ mol·l⁻¹ N⁵-nitroarginine methyl ester (L-NAME) to inhibit NOS. The EDHF response was the relaxation to Ach in L-NAME pre-treated vessels minus the response after blockade of calcium activated potassium channels with 10⁻⁴ mol·l⁻¹ ¹apamin (AP) plus 10⁻⁵ mol·l⁻¹ charybdotoxin (CTX 10⁻⁴ mol·l⁻¹ [15]). This combination reduced relaxations to 10⁻⁴ mol·l⁻¹ ACh to 2 ± 6% which was similar to endothelium removed (3 ± 4%). EDCF responses were taken as the contractions to Ach of vessels under spontaneous tone incubated with L-NAME, AP and CTX to block vasodilator pathways.

Contraction to PE, U-46,619 and ET-1

Two vessels were contracted with graded concentrations of phenylephrine (PE; 10⁻⁴ to 10⁻³ mol·l⁻¹), U-46,619 (10⁻⁴ to 10⁻³ mol·l⁻¹) or endothelin-1 (ET-1; 10⁻⁶ to 10⁻³ mol·l⁻¹).

Time control studies

NE contractions were similar at the beginning (85 ± 4%) and end (78 ± 4%) of the experiments and Ach relaxation were unchanged during incubation in PSS for 30, 60 and 120 min [27] and remained stable over 10 hours (before: 75 ± 3 vs. after 10 hours: 70 ± 5%; NS).

Vascular NO and ROS

The assessment of NO activity of microvessels was the same as previously described. Vessels were preloaded with 5 × 10⁻⁶ mol l⁻¹ of 4-amino-5-methoxyamino-2',7'-dihlorofluorescein diacetate (DAF-FM DA; Invitrogen, Carlsbad, CA) and 10⁻⁷ mol·l⁻¹ of L-arginine [28].

For ROS activity, vessels were preloaded with 4-(9-acridinecarboxyanilino)-2,2',6,6'-tetramethylpiperidin-1-oxyl free radical (TEMPO-9-AC; 10⁻⁷ mol·l⁻¹, Invitrogen, Carlsbad, CA) [29,30]. One set was prepared for EDCF studies and stimulated with 10⁻⁴ mol·l⁻¹ ACh and another set with 10⁻³ mol·l⁻¹ ET-1. Excitation was set at 360 nm and emission was isolated at 460 nm. Incubation with PEG superoxide dismutase (125 units ml⁻¹) prevented 92% of the fluorescence to Ach in vessels from Ang II-infused rats [30].

Plasma L-arginine, ADMA and symmetric dimethylarginine (SDMA)

These were measured by Beckman Coulter PACE/MDQ High Performance Capillary Electrophoresis (Fullerton, CA) with Laser Induced Fluorescence Detection (HPCE-LIF) in plasma as previously described [31]. The intra- and inter-assay coefficient of variations was 3.8% and 4.6% for ADMA, 4.2% and 5.2% for SDMA, and 6% and 6.5% for arginine.

Statistical analysis

Data are presented as mean ± SEM. Cumulative dose-response experiments were analyzed by nonlinear regression (curve fit) and differences assessed by two-way, repeated-measures ANOVA followed, if appropriate, with Bonferroni post hoc t-tests for multiple comparisons. A probability value<0.05 was considered statistically significant.

Result

The two groups were well matched (Table 1). The CD4 count of patients...
with HIV averaged 448 ± 40 cell·ml⁻³. They had been receiving HAART for ≈10 years. Three HIV positive participants had hypertension, which was fully controlled with a single medication that was withheld 14 days before. No subject had blood pressure (BP)>140/90 mmHg on the day of study or more than trace proteinuria and none had an abnormal serum creatinine concentration. The subjects in both groups were free of other cardiovascular risk factor, except for increased body mass index (BMI).

A representative tracing of tension developed by gluteal subdermal microvessels from a control and an HIV-infected patient is shown in Figure 1. NE increased the tension in both vessels. ACh induced a relaxation at 10⁻⁷ mol·l⁻¹ in the control vessel and at 10⁻⁶ mol·l⁻¹ in the vessel from the HIV-infected subject that also had a diminished maximum relaxation.

The group studies of relaxation responses are shown in Figure 2 and Table 2. All relaxation responses to ACh (EDR, EDRF and EDHF) were reduced significantly in subjects with HIV but the EIR to SNP was unchanged.

The DCF contractions to ACh (Figure 3) and the contractions to U-49,619 and ET-1 (Figure 4 and Table 2) were enhanced in patients with HIV, but the response to PE was unchanged.

The DAF-FM-DA fluorescence (NO activity) with ACh was reduced by >3 fold (P<0.001) in vessels from HIV-infected women (Figure 5A), whereas the ACh- and ET-1-induced increases in tempo-9-AC fluorescence (ROS activity) were enhanced by >2 fold (P<0.01) (Figure 5B and 5C).

Table 1: Clinical characteristics of study groups (mean ± SEM).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls (n=10)</th>
<th>HIV (n=10)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>37 ± 2</td>
<td>41 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>Body Mass Index (kg·m⁻²)</td>
<td>36 ± 4</td>
<td>40 ± 3</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of HIV (years)</td>
<td>10 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of HAART (years)</td>
<td>11 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ count (cell·ml⁻³)</td>
<td>1254 ± 50</td>
<td>448 ± 40</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Viral load (copies·ml⁻¹)</td>
<td>328 ± 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>117 ± 3</td>
<td>106 ± 3</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>77 ± 2</td>
<td>65 ± 2.3</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma BUN (mg·dl⁻¹)</td>
<td>12 ± 1</td>
<td>11 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma creatinine (mg·dl⁻¹)</td>
<td>0.80 ± 0.02</td>
<td>0.75 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol (mg·dl⁻¹)</td>
<td>179 ± 6</td>
<td>173 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol (mg·dl⁻¹)</td>
<td>47 ± 2</td>
<td>51 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol (mg·dl⁻¹)</td>
<td>109 ± 6</td>
<td>96 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>Triglyceride (mg·dl⁻¹)</td>
<td>117 ± 34</td>
<td>124 ± 18</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma glucose (mg·dl⁻¹)</td>
<td>81 ± 2</td>
<td>90 ± 4</td>
<td>NS</td>
</tr>
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</table>

Table 2: Maximum changes in vascular tension

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (%)</th>
<th>HIV (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDR</td>
<td>-78 ± 3</td>
<td>-51 ± 6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>EDRF</td>
<td>-39 ± 3</td>
<td>-28 ± 4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>EDHF</td>
<td>-37 ± 4</td>
<td>-17 ± 4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>EIR</td>
<td>-88 ± 4</td>
<td>-87 ± 3</td>
<td>NS</td>
</tr>
<tr>
<td>EDCF</td>
<td>+7 ± 2</td>
<td>+21 ± 6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PE</td>
<td>+88 ± 6</td>
<td>+102 ± 7</td>
<td>NS</td>
</tr>
<tr>
<td>U-46,619</td>
<td>+117 ± 11</td>
<td>+164 ± 10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ET-1</td>
<td>+97 ± 9</td>
<td>+151 ± 12</td>
<td>&lt;0.01</td>
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Mean ± SEM values. Data were obtained from experiments described in Figures 2-4.
Table 3: Plasma levels of L-arginine, ADMA and SDMA (mean ± SEM values).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls</th>
<th>HIV</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arginine (µmol l⁻¹)</td>
<td>101 ± 7</td>
<td>85 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>ADMA (µmol l⁻¹)</td>
<td>0.44 ± 0.02</td>
<td>0.48 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>SDMA (µmol l⁻¹)</td>
<td>0.57 ± 0.05</td>
<td>0.46 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>L-arginine:ADMA (µmol·µmol⁻¹)</td>
<td>231 ± 6</td>
<td>178 ± 12</td>
<td>&lt;0.05</td>
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Discussion

This is the first direct assessment of microvascular ROS, endothelial function and NO and TP-R and ET-1 contractility in patients with HIV. The main new findings are that these microvessels had reduced ACH-induced endothelium-dependent relaxation (EDR, EDRF and EDHF responses) and plasma arginine: ADMA concentration. The defects in relaxation were endothelium-dependent as the response to the direct acting SNP was not perturbed and the NO activity was reduced. There was also enhanced EDCF responses and enhanced contractions to U-46,619 and to ET-1 and enhanced ROS generation with the EDCF and the ET-1 contraction. The enhanced contractions were selective since those to PE were preserved.

All patients were premenopausal, African American women without renal, hepatic, or overt cardiovascular disease except for increased BMI. The infection was well controlled with HAART over 10 years. The two groups were well matched for many factors influencing endothelial function including age, high blood pressure, cholesterol, BMI, blood glucose and serum creatinine. Thus, the abnormal endothelial function was not likely related to confounding conditions.

Microvascular ROS and endothelial dysfunction are associated with hypertension [26], but the prevalence of hypertension in HIV has been reported to be reduced [18], unchanged [32] or increased [33]. The absence of hypertension in our subjects, despite endothelial dysfunction, microvascular ROS and enhanced contractility, may relate to depletion of specific CD subsets of T-cells since these patients, although in remission, had diminished CD4 T lymphocyte counts which prevents the development of hypertension in experimental animals [34].

HIV leads to systemic oxidative stress from three sources: HIV infection, shed HIV-related proteins, and HAART [12]. Indirect, brachial artery endothelial-dependent flow-mediated dilation studies concluded that patients with HIV had impaired [10] or intact [35] endothelial function associated with protease inhibitors [11], or independent of HAART [10]. However, conduit artery function [14] does not predict microvascular dysfunction consistently [13,36]. HAART [37] and HIV-1 proteins [12] both caused endothelial dysfunction and oxidative stress in cells or animals. Thus, the enhanced microvascular ROS and endothelial dysfunction in the patients were likely the outcome of prolonged HIV infection and circulating HIV proteins [12], complicated by vascular inflammation and HAART [11].

We confirmed that plasma arginine: ADMA was reduced in HIV-infected patients but our relatively small study did not detect the very modest increase in plasma ADMA reported previously [38]. This reduced arginine: ADMA ratio might impair NOS activity by reducing the substrate: inhibitor ratio, uncoupling NOS to generate O2⁻, or enhancing endothelial uptake of ADMA [39] and might therefore have contributed to the enhanced microvascular ROS and reduced NO in these vessels. ROS increased ADMA in the vessels of angiotensin II-infused rats without changing plasma levels because of impaired cellular ADMA export [30]. Arginine is increased by inflammation [40]. Thus, the normal plasma

The plasma levels of L-arginine, ADMA and SDMA were not different between groups but the L-arginine: ADMA ratio was reduced in women with HIV (Table 3).
ADMA and the decreased plasma arginine: ADMA of the HIV-infected subject are consistent with microvascular ROS and inflammation.

Markers of ROS [26,41], the level of thromboxane metabolites [42,43], and ET-1 [42,44] and expression of TP-R x [45] all are associated with risk factors for chronic renal disease, CVD and vasculopathy. EDCF is generated by ET-1 which stimulates thromboxane A2 (TxA2) biosynthesis [46] whereas activation of TP-Rs releases ET-1 [47] and generates ROS [48,49]. In turn, ROS are required for microvascular EDCF responses mediated by TP-R [30,50], can enhance responses to TP and ET receptor stimulation [15] and can stabilize TP-Rs in active form [51]. Thus, increases in vascular ROS in HIV may initiate positive feedback interactions that may under lie the enhanced EDCF, TP-R and ET-1-responses. Further studies will be required to evaluate these possibilities.

This study has some limitations. First, the sample size is small, but was sufficient to demonstrate statistically robust conclusions. Second, the source of microvascular ROS was not identified. The vessels were fresh vessels dissected from a gluteal biopsy and study of both vascular function and the ROS source would require more blood vessels than were available from a single biopsy. Third, while elective microvascular NO can account for the defective EDRF response, NO does not mediate EDHF response, whose cause was not identified. Fourth, all the women with HIV were treated with HAART which can contribute to endothelial ROS and endothelial dysfunction [12,37]. Few HIV-infected subjects decline HAART, and those that do often have many complications. Thus, the selection of an appropriate untreated control group will be difficult.

In conclusion, this is the first direct demonstration of severe microvascular oxidative stress, endothelial dysfunction and NO deficiency coupled with enhanced endothelium-dependent contractions and contractions to thromboxane and ET-1 in HIV positive subjects without overt CVD risk factors except for increased BMI.

Perspective

HIV increases thromboxebolism [12,16] and pulmonary hypertension [8] both of which are related to TP-R signaling [21,22]. ET-1 can cause pulmonary hypertension [19] which is treated by ET receptor blockade [20]. ET-1 is released by ROS or hypoxia from pulmonary endothelial cells [23] and by HAART from blood vessels [52]. Therefore, the increased responsiveness to ET-1 and activation of TP-Rs likely derives from oxidative stress and could contribute to the development of an EDCF and to the increased risk for thromboxebolism and pulmonary hypertension in HIV.

CVD likely originates with small-vessel disease [42] which underlies CHF, microvascular dementia, and proteinicronic chronic kidney disease and the subsequent development of large vessel diseases that also are prevalent in patients with HIV. Correction of oxidative stress reduced vascular ADMA [30]. Our findings suggest potential therapeutic roles for TP-R or ET-R antagonists for prevention of pulmonary hypertension, thromboxebolism, CVD and microvascular diseases in HIV-infected subjects. This could not be obtained by aspirin [42,53]. Two current studies suggest this to be urgent. First, in the caliber of the small retinal vessels of HIV infected subjects predicted mortality independent of HAART but no mechanism was identified [54]. Second, prophylactic antiretroviral administration to normal subjects with high risk behavior was recommended for HIV prophylaxis [55]. Since increasing numbers of normal subjects may be exposed to prophylactic antiretroviral therapy for prolonged periods, it is important to develop preventative strategies.

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