Effects of Hypoxia on Heparan Sulfate in Bovine Aortic and Pulmonary Artery Endothelial Cells

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Newly synthesized heparan sulfates purified from the cell layer of bovine aortic endothelial cells (BAECs) and main pulmonary artery endothelial cells (BPAECs) cultured under either normoxic (21% oxygen) or hypoxic (3% oxygen) conditions were characterized by size, charge, and capacity to bind to antithrombin III. Incorporation of radiolabeled sulfate into cell layer-associated heparan sulfate was reduced by 70% in BAECs and by 45% in BPAECs during exposure to 3% oxygen; degradation of radiolabeled heparan sulfate was not affected by hypoxia. However, the percentage of total radiolabeled heparan sulfate that bound to antithrombin III was increased by 33% for BAECs and by 120% for BPAECs when compared with radiolabeled heparan sulfate synthesized during the 21% oxygen exposure. Both the high- and low-affinity antithrombin III affinity radiolabeled heparan sulfate consisted of two components of different sizes; the low-affinity components (mean sizes, 60 and 40 kd) generated under normoxic conditions were smaller than their respective high-affinity components (mean sizes, 70 and 55 kd) by molecular sieve chromatography. The components of low–antithrombin III affinity heparan sulfate generated during exposure to 3% oxygen were increased in size compared with the corresponding low-affinity components generated during the 21% oxygen exposure for both BPAECs and BAECs. In addition, the amount of the larger high–antithrombin III affinity component was reduced in both cell types exposed to hypoxia. There was no difference in functional heparin-like activity per dish between cells cultured at 3% and 21% oxygen; BAECs had twofold to threefold greater activity per dish than did BPAECs at both levels of oxygen. We conclude that the overall amounts of heparan sulfates synthesized by cultured BPAECs and BAECs are affected similarly but not to the same extent during exposure to 3% oxygen and that hypoxia may differentially influence the chain length and antithrombin III–binding capacity of heparan sulfate species.

(Circulation Research 1992;71:782–789)

Key Words • heparan sulfate • endothelial cells

Multiple procoagulant and anticoagulant functions occur at the luminal surface of vascular endothelium. Mechanisms of anticoagulation focus on both reducing the production of new thrombin and inactivating already formed protein. Thrombin production may be reduced through the action of activated fluid-phase protein C produced as a result of the interaction of protein C, thrombin, and endothelial cell surface thrombomodulin at the vessel wall.1 Formed thrombin may be proteolytically cleaved by antithrombin III; this reaction takes place at the vessel wall and is catalyzed by heparan sulfate from the endothelial cell surface.2–6

It is not known whether heparan sulfates derived from the surface of different endothelial cell populations exposed to low oxygen tensions in vivo have different hemostatic capabilities with respect to this catalytic function. Differences in function might be expected to occur, since hypoxia is known to alter many endothelial cell properties, including barrier and coagulation function, and has been shown to variably alter the production of heparan sulfates synthesized by endothelial cells derived from different vascular tissues in several animal species.7–13 These effects might be important in humans where hypoxia is commonly associated with prothrombotic tendencies.14–16 In this study, primary cultures of bovine aortic endothelial cells (BAECs) and bovine pulmonary artery endothelial cells (BPAECs) derived from similarly aged calves were exposed to either 21% or 3% oxygen, and the amounts, sizes, and antithrombin III binding capacity of newly synthesized, cell-associated heparan sulfates were assessed.

Materials and Methods

Endothelial Cell Cultures

Bovine endothelial cells were obtained from similarly aged calf aortas and pulmonary arteries according to previously published methods.7 Endothelial cells were harvested by lightly scraping the luminal surface of longitudinally split aortas and main pulmonary arteries. Cells were suspended in minimal essential medium (MEM; Flow Laboratories, Inc., McLean, Va.) containing 15% heat-inactivated fetal calf serum (Biocell Laboratories, Carson, Calif.) and were initially seeded onto 25-cm² flasks or 60-cm² plastic dishes (Costar Corp., Cambridge, Mass.). Cultures were incubated at 37°C in 95% air–5% CO₂ and were passed enzymatically
Degradation of Cell-Associated Heparan Sulfate Under Hypoxic Conditions

Pulse-chase experiments were performed to measure the degradation of cell-associated heparan sulfates synthesized by BPAEC and BAEC cultures under hypoxic conditions. Nine confluent monolayers of each cell type maintained at 21% oxygen were rinsed with phosphate-buffered saline and serum-free MEM containing 60 μCi/ml carrier-free Na[35S]O4, and 4 μCi/ml tritiated valine (65 Ci/mmol, New England Nuclear) was added. After 24 hours of incubation, cells were harvested from three of the BAEC and three of the BPAEC cultures. Fresh serum-free MEM without radiolabel was added to each of the remaining cultures. Half of the remaining cultures of each cell type were then placed in 3% oxygen–5% CO2–92% N2; the other half continued to be maintained at 21% oxygen for an additional 24 hours. After this incubation period, the cells were harvested, and radiolabeled heparan sulfate was purified by a modification of the technique of Castor et al.

Cell layers were adjusted to 0.2N with concentrated NaOH. After 24 hours at room temperature, 25 μg hyaluronic acid was added to each sample as a carrier, and solutions were neutralized to pH 6–8 with 2 M HCl and boiled for 5 minutes. GAGs were then precipitated with cold ethanol containing 1.3% (wt/vol) sodium acetate. Samples were stored at −4°C overnight. The GAG precipitate was separated from the supernatant by centrifugation for 30 minutes at 10,000g. An aliquot of the supernatant was counted in a liquid scintillation counter (model 1600TR, Packard Instrument Co., Inc., Downers Grove, Ill.) using a double-label protocol for sulfur and tritium. All pellets were redissolved in the papain–pronase buffer used in the previous experiment and were treated with pronase at 55°C in a shaking water bath for 12 hours. Samples were again boiled for 5 minutes to terminate digestion, and GAGs were reprecipitated with cold ethanol containing 1.3% sodium acetate. Combined tritiated valine counts from both supernatants were used as a measure of valine incorporation into proteins. After centrifugation, GAG precipitates were redissolved in Tris buffer and were treated with two sequential aliquots of chondroitinase ABC added 4 hours apart to digest non-heparan sulfate GAGs. Intact heparan sulfate was separated from digested GAGs by molecular sieve chromatography over a Superdex 200 column in a fast protein liquid chromatographic (FPLC) system (Pharmacia LKB Biotechnology). Sixty-four fractions (2 ml) were collected at a flow rate of 1 ml/min using 20 mM triethanolamine as the buffer. Column fractions were counted by liquid scintillation using a double-label protocol, and peak fraction sulfate radioactivity was totaled. Comparisons of sulfate disintegrations per minute (heparan sulfate) and valine disintegrations per minute (incorporation into protein) were made among cultures maintained under normoxic and those maintained under hypoxic conditions for each cell type.

Preparation and Standardization of Sepharose–Antithrombin III Column

Antithrombin III affinity columns were made by coupling electrothoretically pure human antithrombin III (Helena Laboratories, Beaumont, Tex.) to activated CNBr–Sepharose 4B (Pharmacia LKB Biotechnology) according to the methods of Andersson et al.22 and Hook et al.23 Columns were standardized by separation of radioiodinated bovine kidney heparan sulfate (heparan sulfate, sodium salt, Seikagaku America Inc.,Rockville,
according to antithrombin III affinity as follows. Iodinated bovine kidney heparan sulfate was prepared by phenylation of the commercially obtained standard heparan sulfate mentioned above using N-succinimidyl-3-(4-hydroxyphenyl)propionylate (Bolton Hunter reagent, Pierce Chemical Co., Rockford, Ill.), and derived material was separated from the unreacted reagent by mono Q FPLC using a linear salt gradient (0–1.2 M NaCl). Material eluting at 0.6 M NaCl was found to be phenylated heparan sulfate, which was then radioiodinated according to the method of Greenwood et al. Iodinated [125I]heparan sulfate was loaded onto the Sepharose–antithrombin III column; the column was washed with 0.15 M NaCl (low salt wash), and bound material was eluted using 1 M NaCl (high salt fraction). Aliquots of each fraction were counted in a gamma counter (Beckman Instruments, Inc., Fullerton, Calif.) whose efficiency for 125I was 45%. Approximately 17% of the total counts were bound to antithrombin III, and the recovery was 95%.

Both iodinated fractions, low salt wash and high salt elution, were separately combined, dialyzed, and concentrated by lyophilization. Concentrated fractions were subjected to electrophoresis on cellulose acetate (see conditions below) along with undervailed and unlabeled heparin (sodium heparin, Elkins-Sinn Inc., Cherry Hill, N.J.) and heparan sulfate standards. After staining with 1% Alcian blue overnight and destaining in acetic acid to locate the positions of the heparin and heparan sulfate standards, the cellulose acetate was cut into sections; each section was gamma counted. Material from the low-affinity low salt wash was found to coelectrophores with unlabeled bovine kidney heparan sulfate; material from the high-affinity high salt elution coelectrophoresed with commercial heparin (data not shown). These data indicated that this column could separate heparan sulfate by antithrombin III affinity.

Radiolabeled endothelial cell heparan sulfates were subjected to antithrombin III affinity chromatography under the same conditions as above. Low-affinity low salt wash and high-affinity high salt fractions were separately concentrated, and aliquots of each concentrate were then rechromatographed on Sephacryl-200.

Some aliquots of antithrombin III affinity–purified high- and low-affinity BPAEC heparan sulfates were subjected to one-dimensional cellulose acetate electrophoresis along with heparin and heparan sulfate standards. Samples were run in pyridine formate buffer, pH 3.1 on cooled cellulose acetate paper, for 130 minutes at 130 mA in a cooled horizontal electrophoresis apparatus (Multiphor, Pharmacia LKB Biotechnology). Electrophoresed heparan sulfate samples and standards were visualized by 1% Alcian blue staining at 37°C for 24 hours. After photography, sheets were cut into sections, the sections were dissolved using Filtron X (National Diagnostics Inc., Mannville, N.J.), and radioactivity was measured in an LKB scintillation counter (LKB-Wallac Co., Finland).

Measurement of Heparin-Like Activity

The heparin-like activity of high-affinity heparan sulfates purified from cultures (n=3) from each group was measured using a commercial chromogenic assay based on the ability of heparin (or heparin-like material) to form antithrombin III complexes that interact and bind factor Xa added in excess. Unbound factor Xa is used to release p-nitroaniline from an artificial peptide substrate S-2222 (Coatest heparin kit, acid-stopped technique, Helena Laboratories). Known amounts of standard heparin or purified heparan sulfate from endothelial cell cultures were incubated with 10 μl antithrombin III (1 IU/ml) for 10 minutes at room temperature. Factor Xa (7 nkat S-2222/ml, 200 μl) was then added to mixtures that were incubated at room temperature for 2 minutes. The artificial peptide substrate S-2222 was then added, and the mixture was incubated at room temperature for an additional 3 minutes. The reaction was stopped by the addition of 300 μl of 20% acetic acid, and the absorbance of released p-nitroaniline was read at 405 nm. Standard curves of absorbance versus heparin concentration were constructed. Absorbencies from mixtures containing heparan sulfate were converted into heparin-equivalent international units, and results were normalized to the number of cells per dish.

Data and Statistics

Experiments were performed three times, each time with three separate monolayers in each group. Qualitative results were similar and reproducible in all experiments. Statistical comparisons were made among the four groups (BPAECs at 21% oxygen, BPAECs at 3% oxygen, BAECs at 21% oxygen, and BAECs at 3% oxygen) using a one-way analysis of variance to determine differences. A post hoc Scheffe’s test was used to determine significance at the p<0.05 level for group pairs that exhibited statistically significant differences. This same protocol was followed for the degradation experiments, except that comparisons were made only for the 48-hour results.

Results

Incorporation of Radiolabeled Sulfate Into Glycosaminoglycan and Heparan Sulfate

After normoxic exposures, 60,850±11,016 cpm per dish of radiolabeled sulfate (mean±SD, n=3) were incorporated into the GAGs of BPAECs; significantly more radiolabel (80,670±17,154 cpm per dish) was incorporated into the GAGs of BAECs (p<0.05). Radiosulfate incorporation into GAGs isolated from the cell layer of cultures exposed to 3% oxygen significantly decreased by approximately 33%, to 40,040±4,525 cpm per dish, for BPAECs and decreased by approximately 70%, to 21,635±2,227 cpm per dish, for BAECs (p<0.05, hypoxic relative to normoxic level).

As stated, culturing at 3% oxygen did not alter the number of endothelial cells per dish (9.0×10⁶ cells per dish) for both cell types. However, incorporation of radiolabeled sulfate into heparan sulfates isolated from the cell layer of BPAEC and BAEC cultures exposed to hypoxia significantly decreased from their normoxic levels (Figure 1). For BPAECs, incorporation of radiosulfate into heparan sulfate decreased by approximately
Effect of Hypoxia on the Degradation of Newly Synthesized Heparan Sulfate

Hypoxia had no effect on the degradation of newly synthesized cell-associated heparan sulfate from either BPAECs or BAECs (Figure 2). At 48 hours, radiolabeled heparan sulfate in normoxically maintained BPAECs was reduced to 25% of initial 24-hour values and was reduced to 30% of the initial values in hypoxically maintained cultures. Similarly, radiolabeled heparan sulfate isolated from BAECs was reduced to 17.6% of initial 24-hour values in both normoxically and hypoxically maintained cultures. As measured by tritiated valine incorporation into sodium hydroxide–sensitive and pronase-sensitive material, hypoxia also had no effect on protein degradation in both cell types. Tritiated valine incorporation was reduced to 40–50% of the initial control values for both cell types, irrespective of oxygen tension (Figure 3).

Antithrombin III Affinity Separation of Heparan Sulfates

Although hypoxia resulted in reductions in sulfate incorporation into heparan sulfate, the percentage of binding to antithrombin III was greater for molecules synthesized under the 3% oxygen condition for both types of endothelial cells than for molecules synthesized under the 21% oxygen condition (Figure 4). This effect was greatest for material isolated from the cell layer of hypoxically exposed BPAECs (a twofold increase in the percentage of binding) compared with BAECs (a 30% increase in the percentage of material that was bound to the column).

**Figure 2.** Bar graph showing the effect of hypoxic exposure on previously incorporated radiolabeled sulfate into purified bovine pulmonary artery endothelial cell (BPAEC) and bovine aortic endothelial cell (BAEC) heparan sulfate. 24, BPAEC and BAEC cultures removed after 24 hours of pulsing while exposed to 21% oxygen; 48N, BPAEC and BAEC cultures removed after an additional 24 hours of nonpulsed normoxic exposure; 48H, BPAEC and BAEC cultures removed after an additional 24 hours of nonpulsed hypoxic (3% oxygen) exposure. Cultured cells were doubly pulsed with radiolabeled sulfate and valine for 24 hours in serum-free medium while exposed to 21% oxygen. After removal of medium, cultures were maintained in serum-free medium at either 3% or 21% oxygen for an additional 24 hours. Radiolabeled heparan sulfates were purified from the cell layers of all cultures. Values shown are the mean ±SD of three cultures. *p<0.05 vs. the 24-hour normoxic value (24).

**Figure 1.** Bar graph showing the incorporation of radiolabeled sulfate into purified bovine pulmonary artery endothelial cell (BPAEC) and bovine aortic endothelial cell (BAEC) heparan sulfate. Cells were pulsed for 24 hours in serum-free medium while exposed to either 3% or 21% oxygen. Values shown are the mean ±SD of three cultures. *p<0.05 vs. the corresponding 3% value.

45%, and for BAECs, incorporation decreased by approximately 70% (p<0.05).

Sepharclay-200 Gel Filtration Chromatography of Antithrombin III Affinity–Separated Heparan Sulfates

Chromatography of low– and high–antithrombin III affinity–separated heparan sulfate fractions on Sepharclay-200 demonstrated that both low- and high-affinity heparan sulfates consisted of two populations of molecules (Figure 5). Each component of the high–antithrombin III affinity heparan sulfate from cells exposed to 21% oxygen was larger (70 and 55 kd) than the corresponding low–antithrombin III affinity component (60 and 40 kd) (Figures 5A and 5C). The molecular weights given above are estimates of the molecular weight of the most abundant species of heparan sulfate, given that the column was calibrated with linear dextrans. Exposure to 3% oxygen resulted in a decrease in the amount of radiolabel incorporated into the largest molecular weight peak of high–antithrombin III affinity cell-associated heparan sulfate (the 70-kd peak) in both BPAEC and BAEC cultures (Figures 5B and 5D). Further, the sizes of low-affinity radiolabeled components synthesized at 3% oxygen for each cell type (Figures 5A and 5C and Figures 5B and 5D) were identical to sizes of high-affinity components, which had not changed from sizes of these components synthesized at 21% oxygen (70 and 55 kd).

Cellulose Acetate Electrophoresis of BPAEC High– and Low–Antithrombin III Affinity–Purified Heparan Sulfate Fractions

Cellulose acetate electrophoresis (Figure 6) of low- and high-affinity–purified fractions of BPAEC heparan...
Heparan sulfate indicated that the high-affinity material comprised two portions, one of greater charge density, which coelectrophoresed with the heparan sulfate standard, and one of lesser charge density. The low-affinity fraction was found to contain only one spot that consisted of material of the lesser charge density. Most of the radiolabel in the high-affinity fraction was localized to the spot with the higher charge density.

Functional Heparin-Like Activity of Heparan Sulfates

The functional heparin-like activity of heparan sulfate isolated from BAECs exposed to 21% and 3% oxygen was similar: 0.031±0.005 IU per dish for cells exposed to 21% oxygen and 0.034±0.008 IU per dish for cells exposed to 3% oxygen (mean±SD). The functional heparin-like activity of heparan sulfate isolated from BAECs was one-half to one-third less at both oxygen conditions: 0.012±0.005 IU per dish for cells exposed to 21% oxygen and 0.019±0.008 IU per dish for cells exposed to 3% oxygen (mean±SD).

Discussion

Although endothelial cells from the main pulmonary artery and aorta are derived from the same fetal tissue and exposed to the same oxygen tension in utero, their differentiated functions might vary because of differences in their environment in the postnatal animal. One of the major differentiated functions of endothelial cells in adult animals is to present a nonthrombogenic surface to flowing blood. Because main pulmonary arterial endothelium is exposed to low hydrostatic pressure and relatively low oxygen tension while aortic endothelium is exposed to high hydrostatic pressure and relatively high oxygen tension, cells from these two vascular beds would have different hemostatic capabilities.

Heparan sulfates from the endothelial cell surface contribute to the antithrombotic potential of endothelium by catalytically facilitating thrombin inactivation by antithrombin III; this reaction is mediated via heparin-like sequences within heparan sulfate chains to which both antithrombin and thrombin bind. Previous studies examining the effect of hypoxia on heparan sulfate synthesis in cultured endothelial cells have demonstrated variable results. Humphries et al. reported reduced amounts of heparan sulfate synthesized by cultured hypoxic BAECs as part of a general reduction in GAG synthesis; BAECs were not studied. Levene et al. found increases in the synthesis of heparan sulfate in cultured hypoxic porcine aortic endothelial cells. Neither of these studies related changes in total levels of heparan sulfate to the degree of heparan sulfate–antithrombin III binding, i.e., to alterations in the amount of heparan sulfate that potentially could participate in thrombin inactivation, nor were cells from different vascular beds from the same animal evaluated.

We found that both cultured BAECs and BPAECs responded similarly to an acute 3% oxygen exposure by decreasing sulfate incorporation into GAGs and into heparan sulfate; the BPAEC findings were similar to those previously reported by Humphries et al. Degradation of newly synthesized heparan sulfates was not.
increased by exposure to hypoxia. Thus, these data indicated that hypoxia modulated heparan sulfate synthesis in cultured BPAECs and BAECs.

Although exposure to 3% oxygen resulted in a decrease in the amount of heparan sulfate synthesized by both cell types, the relative synthesis of specific heparan sulfates capable of binding to antithrombin III was increased. BPAECs exposed to 3% oxygen were more effective in this respect than were BAECs exposed to 3% oxygen. Increased amounts of radiolabeled heparan sulfate binding to antithrombin III have recently been found to be synthesized by hamster lung explants in a model of permeable pulmonary edema produced by endotoxin; these edematous lungs were presumably hypoxic.29 These data suggest that increasing synthesis of specific heparan sulfates that bind to antithrombin III may be a generalized response to hypoxia for many cell types. In particular, BPAECs, which are exposed to lower oxygen tensions in vivo than are BAECs but which were maintained at 21% oxygen after isolation before use in this study, may have been more responsive to the acute low oxygen exposure than were the BAECs, in terms of their specific heparan sulfate response.

The amount of the larger-sized component of the high–antithrombin III affinity heparan sulfate synthesized during the 3% oxygen exposure was specifically reduced in both cell types. The size of this component was not altered. However, the sizes of both low-affinity heparan sulfates isolated from cells exposed to 3% oxygen were increased relative to components synthesized from cells exposed to 21% oxygen. Although the relation between high molecular and lower molecular weight components is unclear, it would appear that exposure to 3% oxygen can selectively influence chain lengths of specific heparan sulfate components as well as modulate the overall synthesis of these components. These effects could have been mediated through hypoxia-induced alterations in the transcription or translation of specific heparan sulfate synthetic enzyme mRNAs in a fashion similar to that found for thrombomodulin.13 We speculate that hypoxia may have also upregulated the synthesis of enzymes responsible for post-synthesis modifications of the chains with respect to making them heparin-like and capable of binding to antithrombin III (intracellular membrane bound 6-O and 3-N sulfotransferases).30 Exposure to hypoxia is known to increase the synthesis of some proteins, e.g., the hypoxia-associated proteins recently described in bovine endothelial cells, the α₁-adrenergic receptor proteins, and erythropoietin.5–10,13 Because cultured endothelial cells have been shown to synthesize several structurally different populations of heparan sulfates, some of which are anticoagulantly active, different classes of heparan sulfates may therefore be independently regulated.31–33 Enzymatic processing or other forms of degradation of intact heparan sulfate chains might also have been influenced by hypoxia, with chains with antithrombin III–binding capability being more resistant.34 This is unlikely, given that overall degradation of heparan sulfate was not affected by hypoxia.

The functional assay, which measured the total heparin-like activity of the nonradiolabeled heparan sulfate
as well as the radiolabeled heparan sulfate from the cell layer, indicated that more heparin-like activity per dish was found in BAEC cultures exposed to either 21% or 3% oxygen. Only small, nonsignificant increases in the amount of heparin-like activity per dish were noted for both cell types when cultured at 3% oxygen, probably because the newly synthesized heparan sulfates made up only a small fraction of the total heparan sulfate from the cell layer. It may be that 24 hours of hypoxia was not sufficiently long to significantly increase the number of molecules of heparan sulfate with functional heparin-like domains.

In summary, BAECs and BPAECs cultured at 21% oxygen responded similarly to an acute exposure to 3% oxygen for 24 hours by increasing the synthesis of specific cell-associated heparan sulfates capable of binding to antithrombin III while decreasing the overall synthesis of heparan sulfate. This response of large-vessel endothelial cells, if operative in vivo, would be important in helping to maintain a nonthrombogenic surface under hypoxic conditions.

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FIGURE 6. Cellulose acetate electrophoretograms of bovine pulmonary artery endothelial cell heparan sulfate stained with Alcian blue. Two micrograms of standard heparin (HEP) and bovine kidney heparan sulfate (HS) and radiolabeled bovine pulmonary endothelial cell high-affinity (HI) and low-affinity (LO) purified heparan sulfates were applied at the origin of the paper. Material was electrophoresed in pyridine formate (pH 3.1) buffer for 2.5 hours at 130 mA. The paper was stained with 1% Alcian blue overnight at 60°C and destained using multiple changes of 1% acetic acid and water. After photography, the entire sheet was cut into sections using a standard grid. Each grid section was dissolved using Filtrex X and counted in a liquid scintillation counter. The counts (CPM) represent those contained in the associated spot. High-antithrombin III affinity HS (HI) separated into two distinct spots; the material coelectrophoresing with the HS standard contained most of the counts. Low-affinity material (LO) coelectrophoresed with the majority of less highly charged HI material. Less highly charged HI and LO material contained fewer counts than the more highly charged HI HS.
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Circ Res. 1992;71:782-789
doi: 10.1161/01.RES.71.4.782

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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