The anemia of the newborn induces erythropoietin expression in the developing mouse retina

N. Scheerer,1 N. Dünker,2 S. Imagawa,3 M. Yamamoto,4 N. Suzuki,3 and J. Fandrey1

Institutes of 1Physiology and 2Anatomy, University of Duisburg-Essen, Essen, Germany; 3Graduate School of Comprehensive Human Sciences, 4Center for Tsukuba Advanced Research Alliances, University of Tsukuba, Tsukuba, Japan

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Scheerer N, Dünker N, Imagawa S, Yamamoto M, Suzuki N, Fandrey J. The anemia of the newborn induces erythropoietin expression in the developing mouse retina. Am J Physiol Regul Integr Comp Physiol 299: R111–R118, 2010. First published May 12, 2010; doi:10.1152/ajpregu.00108.2010.—The hematopoeitic hormone erythropoietin (Epo), regularly produced by the kidneys and the liver, is also expressed in neuronal tissue, where it has been found to mediate paracrine neuroprotective effects. In most studies exploring the rescue effects of Epo, apoptosis was exogenously induced by different cell death stimuli. Herein, we set out to study the expression and function of Epo in physiologically occurring apoptosis in a model of retinal development. We made use of an organotypic ratinal wholemount culture system that resembles the physiological in vivo situation with cell connections still retained. Epo mRNA expression in the retina, liver, and kidney showed a significant increase during early development, coinciding with the anemia of the newborn. In the retina of Epo-green fluorescent protein transgenic mice, Epo-expressing cells were identified and found to be distributed in the retinal ganglion cell layer. Treatment of retinal wholemount cultures with recombinant Epo resulted in a significant decrease of apoptotic ganglion cells as well as photoreceptor cells throughout retinal development. Moreover, transforming growth factor-β-induced apoptosis was completely antagonized by Epo when both factors were simultaneously applied. Investigations on the signaling pathway revealed a decrease in Bax mRNA levels in Epo-treated retinal cells. We conclude that Epo exerts wide and prolonged neuroprotective activity in physiologically occurring apoptosis and thus contributes to proper retinal development.

apoptosis; erythropoietin receptor; neuroprotection; retinal ganglion cells

APOPTOSIS IS A WIDESPREAD physiological process during development of the nervous system in general and in the retina in particular (4). During postnatal development of the retina, cells unable to form proper synapses and communication are eliminated by apoptosis (40). Therefore, apoptosis is essential for adequate murine retinal development and postnatally occurs in three waves or peaks at postnatal day (P) 2, 9, and 15 (31). At P2, cells of the inner neuroblastic layer are affected by apoptosis. This cell type later develops into ganglion cells and undergoes apoptosis again at P15, together with photoreceptor cells. At P9, most apoptotic cells are localized in the inner nuclear layer and are most likely amacrine cells (31).

Several factors have either stimulating effects, like transforming growth factor-β (TGF-β) (1, 14), or repressing effects on apoptosis, like insulin (39) and among others brain-derived neurotrophic factor (BDNF) (5). The interaction of these factors results in the control of retinal apoptosis (10).

The hormone erythropoietin (Epo) was first described as the main regulator of red blood cell production, inhibiting apoptosis of erythroid progenitor cells by binding to its specific membrane receptor EpoR (13, 23). Both Epo and Epo receptor (EpoR) null mutant mice exhibit reduced primitive erythropoiesis and die in utero because of severe anemia (42). However, EpoR was also found to be expressed by nonerythroid cells, e.g., neuronal cells (24, 34, 43). Experimental evidence suggested that Epo also exerts anti-apoptotic activity on developing neuronal tissue (7) and limits ischemic damage of the adult brain in stroke models (3). Moreover, Epo was found to rescue retinal photoreceptor cells from light-induced apoptosis (17, 18) as well as retinal ganglion cells from neurotrophic factor deprivation (41).

In most studies exploring the rescue effects of Epo on retinal cells, apoptosis was exogenously induced by different cell death stimuli in adult retinal cells (26). However, the expression and function of Epo during physiological development with naturally occurring apoptosis is still unknown.

Primary cell cultures provide an excellent opportunity to examine the effects of hormones and extracellular molecules in a controlled environment. Herein, we made use of an organotypic retinal wholemount culture system (9) to investigate anti-apoptotic effects of Epo during postnatal murine retinal development. In our experimental system, which closely resembles the physiological in vivo situation with cell connections still retained, we set out to identify the Epo-expressing cells and investigate which retinal cells are rescued from apoptosis by Epo. Furthermore, we studied whether Epo interacts with other factors like TGF-β and which pathways are activated by Epo. This study contributes to characterizing the importance of Epo for adequate retinal development.

MATERIALS AND METHODS

Retinal cultures. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC), following the Guidelines of the National Institutes of Health regarding the care and use of animals for experimental procedures. The study protocol was approved by the institutional animal ethics committee of the Universitätsklinikum Essen, Universität Duisburg-Essen. Eyes were dissected from specific pathogen-free C57BL/6J mice (Jackson Laboratory) on postnatal days (P) 2, 9, and 15. Lens, vitreous body, and pigment epithelium were removed, and the neural retinas were preincubated with 0.5 mg/ml hyaluronidase for 15 min at 37°C. Retinas were cultured as organotypic wholemounts in 2 ml chemically defined medium as described previously (9, 10, 20). In dose-response experiments, cultures were treated with 0.2, 0.5, 1, 2, 5, 10, and 20 U/ml recombinant human Epo (Boeringer Ingelheim, Ingelheim, Germany) or with 2, 5, 10, and 20 ng/ml recombinant human TGF-β1 (R&D Systems, Wiesbaden, Germany) to determine the optimal concentration of the factors to prevent or to induce apoptosis. Where
indicated, in all further experiments, a concentration of 0.5 U/ml recombinant Epo or 2 ng/ml recombinant human TGF-β1 (R&D Systems, Wiesbaden, Germany) were added to the serum- and insulin-free basal medium (Dulbecco’s modified eagle medium F-12; Sigma-Aldrich). Cultures were maintained for 24 h at 37°C in a 5% CO₂ atmosphere.

Detection of apoptosis. Apoptotic cell death was determined by counting 4′,6-diamidino-2-phenylindole (DAPI)-stained pycnotic nuclei of retinal single cell suspensions after dissociating cultured wholemount retinas. For cell scoring of DAPI-stained nuclei, retinas were dissociated into single cell suspension by incubation at 37°C with 50 U/ml collagenase (Sigma-Aldrich, Munich, Germany), 1.5 mg/ml trypsin (Sigma-Aldrich), 0.7 mg/ml hyaluronidase (Sigma-Aldrich), and 0.1 mg/ml DNase (Roche, Penzberg, Germany), followed by passes through a siliconized Pasteur pipette. Digestion of the tissue was stopped by addition of EDTA, and cells were fixed for 1 h in 8% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). An aliquot of the cell suspension was spotted on a slide using a cytopsin at 30 rpm for 7 min. Cells were stained and mounted with DAPI (2 µg/ml; Sigma-Aldrich).

The effect of Epo and TGF-β treatment, respectively, was scored by counting at least 1,000 cells and at least 10 pycnatic nuclei as well as at least 10 fields of view using a NIKON ECLIPSE 1000 microscope.

Immunocytochemistry. To find out which cell types are rescued by recombinant Epo, we stained cytopsins of retinal wholemount explants, cultured for 24 h in medium containing 0.5 U/ml recombinant Epo, with the ganglion cell marker Brn3a or the photoreceptor marker opsin (1).

The cytopsins were preincubated with BGT1 (3 mg/ml BSA, 100 mM glycine, and 0.25% Triton) and with a Biotin-Avidin Endogenous Blocking Kit (DAKO) for 1 h. Before incubation with the polyclonal mouse Brn3a antibody (1:100; Chemicon, Schwalbach, Germany) or the polyclonal mouse opsin antibody (1:10,000; Sigma-Aldrich) at 4°C overnight, the cytopsins were blocked with 15% normal goat serum in BGT1 for 30 min at room temperature. The reaction was visualized using goat biotinylated anti-mouse (1:400; Molecular Probes) and further incubated with horseradish peroxidase-conjugated anti-rabbit IgG polyclonal antibody (1:1,000; Molecular Probes) at 4°C overnight. After being washed with PBS three times, the slides were further incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:500; Biosource, Bethesda, MD) for 30 min at room temperature. Specific staining was visualized by incubation with 3′,3′-diaminobenzidine (DAB; Dako Cytomation). Hematoxylin (Shandon Therma) was used for counterstaining.

Statistics. Statistical significance of differences was calculated by one-way ANOVA and Newman-Keuls posttest or Student’s t-test.

RESULTS

Hypoxia-induced Epo mRNA expression during early postnatal retinal development coincides with the anemia of the newborn. RT-PCR measurements revealed that Epo mRNA levels in the retina significantly increased during early postnatal development, peaked at P9, and later decreased, reaching levels similar to P0 (Fig. 1A). During this period, mice also experience the anemia of the newborn, a condition we confirmed by measuring hemoglobin and hematocrit levels. Hemoglobin and hematocrit levels significantly decreased during the first 3 wk after birth (Fig. 1, D and E), suggesting that the increase in Epo mRNA levels in the retina might be due to developing tissue hypoxia.

Kidney and liver of these mice also showed a significant increase in Epo mRNA levels and thus likewise reflect the hypoxic effects caused by the anemia of the newborn (Fig. 1, B and C).

Identification of Epo-expressing cells in the retina. To identify Epo-expressing cells in the retina, Epo-GFP transgenic mice were used. These mice express transgenic constructs in which GFP was either linked to 17-kb upstream sequences of the mouse Epo gene in the 17K-Epo-GFP transgene, whereas the 22K-Epo-GFP transgene contains 22-kb upstream and 162-kb downstream sequences from the Epo gene (37).

Eyes were fixed in 4% paraformaldehyde-PBS at 4°C overnight, washed two times with PBS, incubated in 20% sucrose at 4°C overnight, and then frozen in embedding medium (Serva, Amstetten, Austria) at −80°C. Frozen sections of these eyes were incubated for 1 h in 3% hydrogen peroxide-PBS, washed three times with PBS, and then incubated with blocking buffer (5% normal goat serum-1% BSA in 20% skim-powdered milk in PBS with 1% Triton; 1:5) for 1 h at room temperature. The slides were then incubated with rabbit anti-GFP polyclonal antibody (1:1,000; Molecular Probes) at 4°C overnight. After being washed with PBS three times, the slides were further incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:500; Biosource, Bethesda, MD) for 30 min at room temperature. Specific staining was visualized by incubation with 3′,3′-diaminobenzidine (DAB; Dako Cytomation). Hematoxylin (Shandon Therma) was used for counterstaining.

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Table 1. Primer sequences for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>5′-Actin</td>
<td>ctc tga acc cta agg cc</td>
</tr>
<tr>
<td>3′-Actin</td>
<td>gga csa cac aac tct gat gg</td>
</tr>
<tr>
<td>5′-Epo</td>
<td>ctc gga gct cac agg gaa tgg stg</td>
</tr>
<tr>
<td>3′-Epo</td>
<td>cgg gaa gtt gtc ggc gtc gag</td>
</tr>
<tr>
<td>5′-EpoR</td>
<td>tca ccc acc gct tgg aag ac</td>
</tr>
<tr>
<td>3′-EpoR</td>
<td>tga ctc acc ctc gag ctt gt</td>
</tr>
<tr>
<td>5′-Bax</td>
<td>tct ctc gct acc ctc g</td>
</tr>
<tr>
<td>3′-Bax</td>
<td>cgg aac tca aag aag gcc ag</td>
</tr>
<tr>
<td>5′-Bax</td>
<td>ggc tcc acc aag aag ctt ag</td>
</tr>
<tr>
<td>3′-Bax</td>
<td>tgc ctt cag cca aca tgt ca</td>
</tr>
</tbody>
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Epo, erythropoietin; EpoR, Epo receptor.
DAB-positive cells were localized in the ganglion cell layer of 17K-Epo-GFP and 22K-Epo-GFP transgenic mouse retina. Strikingly, those cells were without exception found in the outermost periphery of the retina of 6- to 20-wk-old transgenic mice, where the pars optica meets the pars ceca (Fig. 2, A–C). In contrast, retinas of P9 and P12 transgenic mice showed DAB-positive cells in the center of the retina, where the optic nerve leaves the retina (Fig. 2, D and E). No signal was detected in wild-type mice.

EpoR mRNA is continuously expressed in the retina during postnatal development. We detected EpoR mRNA expression in the retina during all development stages and found no significant changes in expression levels, suggesting that EpoR could mediate Epo activity throughout postnatal development (Fig. 1 F).

Epo rescues retinal ganglion and retinal photoreceptor cells from apoptosis. Both ganglion and photoreceptor cells undergo apoptosis at P15 (31). To find out which cell type is rescued by Epo, we double-stained cytospins of retinal wholemounts, treated with 0.5 U/ml Epo, with DAPI and the ganglion cell marker Brn3a or the photoreceptor cell marker opsin. Analysis of the data revealed a significant decrease of ganglion (Fig. 4 A) and photoreceptor (Fig. 4 B) cell apoptosis after application of Epo. Thus the rescue effect of Epo is not limited to a single cell type in the retina but affects ganglion as well as photoreceptor cells, emphasizing the importance of Epo for proper retinal development.

Epo prevents apoptosis by inhibiting Bax mRNA expression. To investigate the signaling pathways underlying the neuroprotective activity of Epo, we concentrated on downstream signaling molecules involved in the mitochondrial apoptosis pathway. Our results revealed significantly lower Bax mRNA expression in Epo-treated retinas compared to controls. These findings suggest that Epo inhibits apoptosis by downregulating Bax expression through a yet unknown signaling pathway.

Epo protects P15 retinal ganglion cells from TGF-β-mediated apoptosis. TGF-β is one of the principal mediators of retinal cell death during postnatal development (1) and mainly affects retinal ganglion cells at P15. We therefore focused on investigating the interaction between Epo and TGF-β in P15 retinas. Previously, we have shown that application of TGF-β alone resulted in a significant increase of apoptotic cells compared with untreated controls with maximum effects at a concentration of 2 ng/ml (1). Simultaneous application of TGF-β and Epo did not only prevent the proapoptotic effect of TGF-β but even reduced the number of pycnotic nuclei compared with untreated controls (Fig. 5). Thus TGF-β-induced apoptosis was completely antagonized by Epo when both factors were applied simultaneously, suggesting that Epo can compensate for the well-described proapoptotic role of TGF-β during retinal development.

Fig. 1. Quantitation of erythropoietin (Epo) mRNA expression in retina (A), kidney (B), and liver (C), hemoglobin (D) and hematocrit (E) levels, and quantitation of EpoR mRNA expression in the retina (F) during murine development at postnatal days 0–20 (P0–P20) and in adult mice. mRNA levels were measured by RT real-time PCR. Experiments were performed in triplicates (n = 3). Data are means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001; statistical differences compared with adult group, calculated by one-way ANOVA test and Newman-Keuls Posttest comparing all experimental groups.
Fig. 2. Immunohistochemical staining of Epo-green fluorescent protein (GFP) mRNA-expressing cells with an anti-GFP antibody of frozen retinal sections from 6- to 20-wk-old mice expressing transgenic constructs in which GFP was linked to 17-kb upstream sequences (17K-Epo-GFP, A) and mice expressing transgenic constructs in which GFP was linked to 22-kb upstream and 162-kb downstream sequences (22K-Epo-GFP, B and C) and from P9 (D) and P12 (E) 22K-Epo-GFP mice. Staining was visualized by 3’,3’-diaminobenzidine (DAB) reaction (brown precipitate); counterstaining was done with hematoxylin.
levels after application of Epo compared with the controls (Fig. 6A). Interestingly, Bcl-2 mRNA levels were not affected (Fig. 6B). These data suggest that Epo may shift the balance of pro- and anti-apoptotic factors toward the anti-apoptotic members of the Bcl-family by inhibiting the mRNA expression of the proapoptotic member Bax.

DISCUSSION

Epo is the primary regulator of red blood cell production. Besides its hematopoietic function, Epo is neuroprotective in the retina. However, this anti-apoptotic effect has usually been found in experiments where Epo prevented cell death induced by exogenous stimuli. Examples are light-induced photoreceptor degeneration (17, 18), axotomy (26, 41), glutamate-induced toxicity (28), or ocular hypertension (16). Epo was also found to support proper brain development by preventing apoptosis in the embryonic brain (43) and to promote neurite outgrowth of rat retinal ganglion cells after axotomy (2). Mice with a specific knock out for Epo or EpoR in the brain suffered from embryonic and adult neurogenesis defects (38), indicating an important role of Epo for proper brain development. However, the role of Epo in physiologically occurring retinal apoptosis,
Basal group, calculated by Student’s t-test. *P < 0.05; n = 3; statistical differences compared with basal group, calculated by Student’s t-test.

a process contributing to normal postnatal development, remained mostly unknown. Likewise, knowledge about Epo expression in the developing retina is missing. In this study, we investigated the physiological role of Epo during retinal development in an organotypic retinal wholemount culture system that maintains tissue organization and interaction between cells and may therefore provide a model that recapitulates the in vivo situation.

In mammalian species, hemoglobin levels decrease after birth because of the switch from fetal to adult hemoglobin causing the anemia of the newborn, which is accompanied by an exponential rise of serum Epo levels (22). Epo expression was first detected in the murine retina during the first 3 wk after birth and reached a significant increase in Epo mRNA levels with peak values at P9. Peak expression coincided with the reduction in hemoglobin. Epo mRNA levels also increased in liver and kidney of these mice, reflecting the body’s response toward the anemia. Interestingly, the retina appeared to respond earlier when hemoglobin levels started to decline, whereas peak expression in kidneys and liver coincided with the nadir of hemoglobin concentration. Whether this is the result of different oxygen sensitivity in the respective tissues or can be attributed to tissue specific transcription factors remains to be studied (13). Because Epo expression in astrocytes (27) and neuronal cells (36) has been found to be hypoxia-inducible, we conclude that the developing anemia also causes hypoxic Epo expression in the retina during this particular early postnatal period of development.

Our data on the retinal Epo mRNA expression pattern during postnatal development are in accordance with the study of Chen and colleagues (6), who also found a decrease of Epo mRNA levels of P10 to P17 murine normoxic retinas. Elevated Epo expression caused by relative hypoxia caused pathological neovascularization in their model of retinopathy of prematurity (6). Thus changes in the physiological retinal Epo expression pattern contribute to developmental aberration.

In the retina, Epo was localized to the ganglion and inner nuclear cell layer by immunohistochemistry using an anti-Epo antibody (29). However, this method may identify both Epo-secreting cells and cells on which Epo is bound to the EpoR. In this study, we made use of adult transgenic mice expressing GFP under the control of Epo gene regulatory elements (30). Consequently, Epo-expressing cells in the retina were localized in the ganglion cell layer by means of immunohistochemical staining with an anti-GFP antibody. In retinas of 6- to 20-wk-old GFP transgenic mice, those cells were without exception found in the edge of the retina at the passage of the pars optica to the pars ceca where the PO2 is very low. Based on their morphology, these Epo-expressing cells appear to be neurons with the typical neuron specific shape and an axon leaving the soma. In contrast, at P9 and P12, the period of the anemia of the newborn and highest Epo mRNA expression, DAB-positive cells could also be localized in the center of the retina. These findings are supported by Chen et al. (6), who showed in their model of retinopathy of prematurity that hypoxia in the retina was predominantly located in the central part of the retina. In addition, the data from transgenic mouse analysis provided information on regulatory mechanisms of the Epo gene expression in retina, although further experiments are required. Because GFP expression is detectable in the 17K-Epo-GFP transgenic mouse retina, it is likely that retinal cis-regulatory domains are included in the 17-kb upstream sequences of the Epo gene. This means that the regulatory mechanism of Epo gene expression in the retina is different from that in hepatocytes, which is controlled by the proximal downstream region containing a hypoxia-inducible transcription factor binding site (33, 35).

The fact that we detected continuous EpoR mRNA expression in the retina throughout neonatal life suggests that Epo may exert its activity throughout postnatal development. These results correspond with other studies that provided evidence for EpoR expression in the retina (18) and in the embryonic brain (7). Application of recombinant Epo led to a significant decrease of apoptotic cells in murine organotypic retinal wholemounts during all three cell death periods at P2, P9, and P15. These data for the first time prove that neuroprotection by Epo is not limited to artificially induced retinal cell death but that it is also effective in a model of physiologically occurring apoptosis, indicating the importance of Epo throughout retinal development. Furthermore, we were able to demonstrate that retinal ganglion cells and photoreceptor cells can be rescued by Epo during development. Therefore, neuroprotection by Epo is not restricted to one single cell type but appears to have a wide influence on cell survival during retinal development. Because of the fact that the effect of Epo during retinal development is long lasting and affects a great number of retinal cells, we may...
conclude that Epo plays an important role for proper retinal development.

The highest Epo mRNA levels were detected during the cell death period at P9. Thus the high Epo levels immediately preceded the subsequent reduction in apoptosis potentially due to the anti-apoptotic action of Epo. This interpretation is supported by our former findings that proapoptotic factors exert their highest activity shortly before the onset of the cell death periods (1). Because apoptosis during retinal development is known to be induced by TGF-β (1, 11, 12), we performed cell culture experiments in which TGF-β and Epo were simultaneously applied to determine the efficacy of Epo-mediated neuroprotection. Our results show that Epo is not only able to completely compensate for TGF-β-mediated apoptosis but even lowers apoptotic rates below those of untreated controls. We may, thus, conclude that Epo expression coincides with periods of apoptosis in the developing retina of the mouse and can efficiently counteract proapoptotic stimuli.

Former studies addressing the signaling pathways activated by Epo in models of neuronal cell survival did not provide unequivocal data. In a model of axotomy-induced degeneration, extracellular signal-regulated kinase (ERK)-1/-2 but not protein kinase B (Akt) was found to be involved in retinal ganglion cell survival (26), whereas a dual activation of ERK-1/-2 and Akt was reported in Epo-mediated protection from focal cerebral ischemia (25). By contrast, other studies using a model of axotomy of rat retinal ganglion cells identified the phosphatidylinositol 3-kinase/Akt pathway as the predominant pathway activated by Epo, and the authors did not find any evidence for the involvement of ERK-1/-2 (41). More recent investigations upon Epo signaling in retinal cells supported the hypothesis that the individual expression pattern is mainly death stimulus dependent. This was concluded from the finding that the activation of Jak2 only led to a neuroprotective effect in induced but not in inherited retinal cell death (32).

In our study, we concentrated on signaling molecules that are known to be of great importance for the regulation of retinal apoptosis during development like the Bcl-2 family members Bax (21, 31) and Bcl-2 (10, 19). Because our study did not investigate induced but naturally occurring cell death, we were prepared to get results differing from those models using cell death stimuli. Whereas application of Epo in our model resulted in a significant decrease of mRNA for the proapoptotic Bax, mRNA levels of anti-apoptotic Bcl-2 remained unchanged. Consequently, our data suggest that Epo prevents apoptosis in the developing murine retina via the inhibition of the mitochondrial pathway by shifting the balance in favor of the anti-apoptotic members of the Bcl-2 family.

**Perspectives and Significance**

The data presented in this study clearly demonstrate that Epo exerts wide and prolonged neuroprotective activity during postnatal retinal development, rescuing retinal ganglion and photoreceptor cells from naturally occurring apoptosis.

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Present addresses: M. Yamamoto, Dept. of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan; and N. Suzuki, Dept. of Cell and Molecular Biology, Karolinska Institute, Stockholm, Sweden.

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**DISCLOSURES**

No conflicts of interest are declared by the authors.

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