Sprint-Interval Training-Induced Alterations of Myosin Heavy Chain Isoforms and Enzyme Activities in Rat Diaphragm: Effect of Normobaric Hypoxia

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Abstract: The purpose of this study was two-fold: (i) to investigate if sprint-interval training (SIT) alters myosin heavy chain (MyHC) isoform composition and bioenergetic properties within the rat diaphragm, and (ii) to determine if mild normobaric hypoxia would enhance the effects of SIT-induced diaphragmatic adaptation. Male Wistar rats (8 weeks old) were randomly assigned to one of four groups (n = 7/group): (i) normoxic control (NC); (ii) normoxic training (NT); (iii) hypoxic control (HC); or (iv) hypoxic training (HT). The NT and HT groups were engaged in SIT (1 min sprint and 2–5 min rest, 6–10 sets/day, 5–6 days/week) on a treadmill for 9 weeks. Animals in the HC and HT groups were exposed to normobaric hypoxia (14.5% O₂) during an SIT program from the 4th week of the training period. After completion of the training program, MyHC composition, citrate synthase (CS) activity, and lactate dehydrogenase (LDH) activity in the diaphragm and plantaris muscle were analyzed. An analysis of diaphragmatic MyHC composition demonstrated increased type IIa and decreased type IId/x for both training groups (P < 0.05), with the HT group producing greater changes than the NT group (P < 0.05). The plantaris muscle, however, showed increased Type IIa and IId/x and decreased Type IIb for both the NT and HT groups (P < 0.05). CS activity increased only for the training groups (P < 0.05), and this change was greater for the HT group in the diaphragm and for the NT group in the plantaris muscle (P < 0.05). Further, diaphragmatic LDH activity in HT was significantly lower (P < 0.05) than in HC and NT. These findings demonstrated that SIT could induce alterations in MyHC composition from fast to slow within type II isoforms and also improve the oxidative capacity in the diaphragm and plantaris muscles. It is of importance that our data revealed that SIT-induced diaphragmatic adaptations were enhanced when SIT was performed in normobaric hypoxia.

Key words: respiratory muscle, treadmill running, intermittent hypoxia, bioenergetics.

The diaphragm is the primary inspiratory muscle in mammals and is capable of metabolic and contractile adaptations in the face of increased functional load, such as exercise training. Indeed, numerous publications have reported that prolonged endurance training shifts myosin heavy chain (MyHC) isoform composition in the diaphragm from type IIb to type IIa [1, 2] and promotes diaphragmatic oxidative capacity [1–5]. However, the magnitude of endurance-training-induced changes in the diaphragm appears smaller in comparison to changes typically seen in locomotor muscles [3]. Although the reason for this different alteration in MyHC and bioenergetic adaptation between diaphragm and locomotor muscle is not clear,
a possible explanation is that endurance exercise may place a smaller metabolic load on the diaphragm than on locomotor muscles [3, 5].

Many investigations have focused on the diaphragmatic adaptation to prolonged endurance training, but little is known about how sprint-type training (e.g., supramaximal high-intensity and intermittent) affects the muscle fiber composition and bioenergetic properties of the diaphragm. Sprint-type training improves the anaerobic metabolic capacity of locomotor muscles [6]. This type of training also increases workload on the diaphragm via elevated ventilation not only during the exercise, but also during the recovery period, because of the excess postexercise oxygen consumption (EPOC). Such extra work on the diaphragm seen during the recovery may produce a somewhat different adaptation from that of locomotor muscles. This prolonged loading on the diaphragm may thus cause aerobic-type adaptation even after sprint-type training. Therefore, the primary purpose of this study was to investigate whether sprint-interval training (SIT) could induce the transformation of MyHC composition and augment bioenergetic properties of the diaphragm.

Recent studies have shown that endurance exercise training under hypoxia enhances training effects on the human skeletal muscle compared to such training under normoxia [7–10]. Further, it has been shown that pulmonary ventilation was increased at a given exercise intensity under hypoxia [11, 12] and that ventilation after exhaustive exercise under hypoxia was also higher in comparison with normoxia [13]. Thus the hypoxia also provides an additional workload on the diaphragm during not only exercise, but also the recovery period, and it could provide an additional aerobic adaptation in the diaphragm. Thus the secondary purpose of this study was to investigate the effect of intermittent hypoxic exposure on MyHC composition and the enzyme activities of the rat diaphragm. Based on the aforementioned reasoning, we hypothesized that SIT could improve the diaphragmatic oxidative properties in MyHC composition and bioenergetics and that hypoxia could enhance the SIT-induced adaptation in the rat diaphragm. To identify any differences in adaptation between locomotor muscles and the diaphragm, the plantaris muscle was also analyzed.

MATERIALS AND METHODS

Animal care. Twenty-eight male Wistar rats (5 weeks old) were used in this study. They were obtained from a licensed laboratory animal vendor (Japan SLC, Shizuoka, Japan). This experiment was approved by the Juntendo University Animal Care and Use Committee and conducted according to Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences by the Physiological Society of Japan. On arrival in our laboratory, all animals were fed standard rat chow and water ad libitum and maintained on a 12:12 h light-dark photoperiod in an environment-controlled room (22.0 ± 0.5°C, 55.0 ± 5.0% relative humidity). At the age of 7 weeks, all animals were familiarized with treadmill running for one week, then randomly assigned to one of four groups (n = 7/group): (i) normoxic control (NC; 193.8 ± 11.2 g); (ii) normoxic training (NT; 186.7 ± 6.9 g); (iii) hypoxic control (HC; 192.0 ± 11.9 g); or (iv) hypoxic training (HT; 188.9 ± 10.5 g).

Sprint-interval training (SIT). From 8 weeks old, the NT and HT groups underwent sprint training on treadmills 5 to 6 days a week (KN-73-5S, Natsume, Tokyo, Japan) for 9 weeks. According to the sprint-interval training protocol described by Takekura and Yoshioka [6], the running speed was gradually increased to 75–80 m/min with the number of sprint sets and resting periods being adjusted to enable all animals to run at the target speed. The exercise protocol is summarized in Table 1. The initial treadmill speed was 30–45 m·min⁻¹ in the first week and was increased gradually to 75–80 m·min⁻¹ for the final week. Each training session consisted of 6–10 sets of 1 min of sprinting, and the sprint sets were separated by 2–5 min recovery periods. The training protocol was identical for NT and HT. As an index of physiological training intensity, blood lactate concentration from a tail vein was evaluated every two weeks after the 3rd week (weeks 4–5, 6–7, and 8–9) by using an automated lactate analyzer (Biosen 5040L, Industrie electronik, Germany).

Oxygen conditions. The oxygen concentration of normobaric normoxia and hypoxia were 20.9% (room air) and 14.5% (equivalent to oxygen concentration at about 3,000 m above sea level), based on the previous study of high-intensity training under hypoxic conditions [14]. From the 4th week of training, two hypoxic groups (HC and HT) were exposed to the normobaric hypoxic environment only during the training session (about 30–60 min). The oxygen concentration control apparatus (YHS-C10, YKS, Nara, Japan) was used to generate normobaric hypoxic air from ambient air, and the oxygen concentration was monitored by a calibrated oxygen analyzer (TEU-10, Tabai ESPEC, Tokyo, Japan) during the experimental period.
Muscle preparation. Forty-eight hours after the last training bout, all animals were anesthetized with pentobarbital sodium (50 mg·kg⁻¹). After they reached a surgical plane of anesthesia, the costal region of the diaphragm was quickly removed and frozen in liquid nitrogen. The costal region accounts for 70% of the total diaphragmatic mass [15] and demonstrates the greater adaptability to exercise training than the crural region [1, 16, 17]. The plantaris muscle, which has MyHC composition similar to that of the diaphragm, was also removed as a locomotor muscle. Muscle samples were stored at –85°C until biochemical analyses.

After thawing, muscle samples of the diaphragm were minced and homogenized in ice-cold homogenization buffer (10 mM Tris-HCl, 10 mM NaCl, 0.1 mM EDTA, pH 7.4). Homogenates were then centrifuged at 400 × g for 15 min, and the supernatants were used for measurements of enzyme activities. The total protein concentrations of supernatants were determined with the Bradford technique [18]. The sediments were adjusted to a concentration of 2.5 mg wet muscle weight/ml with MyHC sample buffer (30% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 0.05% bromophenol blue, 62.5% Tris-HCl, pH 6.8) and boiled at 60°C for 10 min for MyHC separation [19]. Each sample was stored at –85°C until analysis.

MyHC composition. MyHC composition was determined by using one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a modified method of Sugiura and Murakami [19]. Twenty-five micrograms of prepared MyHC samples were applied to the SDS-PAGE gel (stacking gel: 4% acrylamide, 34.7% glycerol, 125 mM Tris-HCl pH 6.8; separating gel: 8% acrylamide, 33% glycerol, 375 mM Tris-HCl pH 8.3). The electrophoresis was started at 60 V in stacking gel at 8°C. When the tracking dye had completely entered the separating gel, the voltage was set at 150 V and run for 18 h at 8°C. After the separation, each gel was stained by using Coomas-sie Brilliant Blue (Biosafe G250, Bio-Rad, CA). To determine the percentages of MyHC isoforms, each gel was scanned, then analyzed by NIH image software (ver. 1.63, NIH).

Enzyme activities. Citrate synthase (CS: EC 4.1.3.7) activity, an index of oxidative capacity, and lactate dehydrogenase (LDH: EC 1.1.1.27) activity, an index of glycolytic capacity, were measured at 25°C using the procedure described by Srere [20] and by Bergmeyer et al. [21].

Statistics. All assays were performed in duplicate, and the mean values were used for statistical analysis. The data are presented as means ± SD. The group differences in MyHC and bioenergetic data were analyzed by using two-way ANOVA (oxygen condition × training). Blood lactate concentration was also analyzed by using two-way ANOVA (time × oxygen condition). When a significant F ratio was represented, a Fisher’s PLSD test was applied as a post hoc analysis. The statistical significance was set at P < 0.05.

RESULTS

Blood lactate concentrations

Blood lactate concentrations during SIT are shown in Table 2. The ANOVA revealed that both time (P < 0.05) and oxygen condition (P < 0.05) have significant effects on the blood lactate concentration. The blood lactate concentration in HT was significantly (P < 0.05) higher than NT in week 6–7 and 8–9.

Body weight

Body weights at the end of the training period were NC: 323.7 ± 12.0 g; NT: 290.9 ± 16.2 g; HC: 323.9 ± 10.7 g; HT: 286.2 ± 11.9 g. Body weights of trained rats in the NT and HT groups were significantly (P < 0.05) lower than in the untrained NC and HC groups.
**MyHC composition**

Figure 1 shows a representative SDS-PAGE gel and MyHC composition in the diaphragm of all four groups after the training intervention. No differences were observed between groups for either Type I or Type IIb isoforms. Hypoxia alone did not affect the MyHC composition, but SIT yielded increased Type IIa isoforms ($P < 0.05$) and decreased Type IId/x isoforms ($P < 0.05$). The effect of hypoxia was present, however, when combined with training, and the above changes seen in the training groups were greater for the HT group than for the NT group ($P < 0.05$).

**Table 2. Blood lactate concentration during sprint-interval training program (mM).**

<table>
<thead>
<tr>
<th>Week</th>
<th>Familiarization</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4–5</th>
<th>6–7</th>
<th>8–9</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.4 ± 1.0</td>
<td>3.5 ± 1.0</td>
<td>4.5 ± 2.0</td>
<td>5.5 ± 3.1</td>
</tr>
<tr>
<td>HT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.9 ± 1.0</td>
<td>4.9 ± 1.1</td>
<td>9.7 ± 3.0*</td>
<td>9.9 ± 3.7*</td>
</tr>
</tbody>
</table>

NT and HT mean normoxic training group and hypoxic training group, respectively. Values are means ± SD. HT was trained under normobaric hypoxic environment (14.5% $O_2$) from the 4th week. * Significantly different from NT ($P < 0.05$).

Fig. 1. (a) Electrophoretic identification of myosin heavy chain (MyHC) isoforms in the rat diaphragm. To identify MyHC bands, a mixture of myofibrillar protein (P+S) from the plantaris and soleus muscle was used. (b) MyHC composition in the rat diaphragm. NC, NT, HC, and HT mean normoxic control, normoxic training, hypoxic control, and hypoxic training groups, respectively. Values are means ± SD. * significantly different from NC ($P < 0.05$), § significantly different from NT ($P < 0.05$), † significantly different from HC ($P < 0.05$).

Fig. 2. Citrate synthase (CS; a) activity, lactate dehydrogenase (LDH; b) activity and the ratio of CS to LDH × 100 (CS/LDH; c) in the rat diaphragm. Values are means ± SD. NC, NT, HC, and HT mean normoxic control, normoxic training, hypoxic control, and hypoxic training groups, respectively. * significantly different from NC ($P < 0.05$), § significantly different from NT ($P < 0.05$), † significantly different from HC ($P < 0.05$).
The effects of SIT and normobaric mild hypoxia on the rat diaphragm. The key findings in this study were (i) SIT induced a fast-to-slow shift within Type II isoforms in the diaphragmatic MyHC composition and improved the oxidative capacity in the costal region of the rat diaphragm; and (ii) an exposure to normobaric mild hypoxia during exercise enhanced the biochemical adaptation in the diaphragm induced by SIT. A brief discussion of these findings follows.

**Enzyme activities**

Figure 2 shows CS and LDH activities of the diaphragm in all groups. CS activities in NT and HT were significantly \((P < 0.05)\) higher than NC and HC. Type IIb isoforms in NT and HT were significantly \((P < 0.05)\) lower than NC and HC. The effect of hypoxia was not observed.

Table 3 shows the MyHC composition in the plantaris muscle. Type IIa and IId/x isoforms in NT and HT were significantly \((P < 0.05)\) higher than NC and HC. Type IIb isoforms in NT and HT were significantly \((P < 0.05)\) lower than NC and HC. The effect of hypoxia was not observed.

**DISCUSSION**

To our knowledge, this is the first experiment to study the effects of SIT and normobaric mild hypoxia on the rat diaphragm.

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**Table 3. Biochemical properties of the plantaris muscle.**

<table>
<thead>
<tr>
<th>Variables</th>
<th>NC</th>
<th>NT</th>
<th>HC</th>
<th>HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin heavy chain composition (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.8 ± 0.3</td>
<td>1.3 ± 0.5</td>
<td>0.8 ± 0.3</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>IIa</td>
<td>8.6 ± 1.9</td>
<td>14.7 ± 2.9†</td>
<td>8.2 ± 1.4</td>
<td>14.2 ± 2.1†</td>
</tr>
<tr>
<td>IId/x</td>
<td>41.8 ± 3.3</td>
<td>52.6 ± 4.6†</td>
<td>45.0 ± 2.1</td>
<td>56.6 ± 4.9†</td>
</tr>
<tr>
<td>IIb</td>
<td>48.8 ± 3.5</td>
<td>31.4 ± 5.7†</td>
<td>45.9 ± 2.1</td>
<td>28.3 ± 4.4†</td>
</tr>
<tr>
<td>Enzyme activities (µmol·min⁻¹·mg protein⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate synthase (CS)</td>
<td>0.192 ± 0.008</td>
<td>0.282 ± 0.019†</td>
<td>0.197 ± 0.007</td>
<td>0.266 ± 0.014§†</td>
</tr>
<tr>
<td>Lactate dehydrogenase (LDH)</td>
<td>5.2 ± 0.2</td>
<td>5.1 ± 0.1†</td>
<td>5.4 ± 0.3</td>
<td>4.8 ± 0.2§†</td>
</tr>
<tr>
<td>CS/LDH × 100</td>
<td>3.7 ± 0.2</td>
<td>5.6 ± 0.5†</td>
<td>3.7 ± 0.3</td>
<td>5.5 ± 0.3†</td>
</tr>
</tbody>
</table>

**DISCUSSION**

To our knowledge, this is the first experiment to study the effects of SIT and normobaric mild hypoxia on the rat diaphragm. The key findings in this study were (i) SIT induced a fast-to-slow shift within Type II isoforms in the diaphragmatic MyHC composition and improved the oxidative capacity in the costal region of the rat diaphragm; and (ii) an exposure to normobaric mild hypoxia during exercise enhanced the biochemical adaptation in the diaphragm induced by SIT. A brief discussion of these findings follows.

**SIT-induced alterations in MyHC isoform compositions and enzyme activities in the diaphragm.** It is well known that increased muscular activities with endurance exercise training promote a fast-to-slow shift in MyHC composition or muscle fiber type from type IIB to type IIA in the locomotor and respiratory muscles [1, 2, 6, 22]. It seems very likely that these changes will contribute to improving a resistance to fatigue and enhancing muscular endurance ability with an increased aerobic enzyme activity as required. The main purpose of sprint-type training is generally an improvement of the anaerobic capacity in locomotor muscles [6, 23]; however, such training also produces the fiber-type shift from fast to slow [24, 25]. Current results in the plantaris muscle were thus in line with these previous studies [24, 25]. However, no study has focused on the effects of the sprint-type training on the diaphragm. Our results demonstrated that SIT resulted in a significant increase in MyHC IIA isoform with a concomitant decrease in MyHC IId/x isoform in the costal diaphragm. This direction of MyHC isoform changes, or the fast-to-slow shift within fast MyHC, of the costal diaphragm was similar to that seen after endurance training [1, 2]. Moreover, the isoform change into MyHC IIA after SIT itself was ~4%, and this seems to be larger than the change after endurance training (~1%) [1, 2].
regard to bioenergetic adaptations, CS activities were elevated by SIT in the costal diaphragm and plantaris muscle as well as the ratio of CS to LDH, an index of the aerobic metabolic capacity. This SIT-induced increase in CS activities (about 20%) was similar to an increase in mitochondrial enzyme activities within the costal diaphragm in rats following 8 to 10 weeks of endurance training [3, 4, 16, 26]. The SIT in this study was high in intensity and short in duration (~80 m·min⁻¹, 1 min·sprint⁻¹), and it increased blood lactate concentration more than 4 mM. Thus it is possible that the greater requirement of anaerobic energy supply during each sprint running in working locomotor muscles resulted in increased diaphragmatic work during the recovery period to provide respiratory compensation for the exercise-induced metabolic acidosis. As a result, the diaphragmatic work would be increased not only during the high-intensity exercise bout, but also during the recovery period. Therefore the collective effect of diaphragmatic work during exercise and during the recovery period between sprint bouts may have contributed to the aerobic adaptations in the diaphragm.

Our data also revealed that SIT exercise did not alter the proportion of MyHC IIb isoform in the diaphragm. In contrast, previous studies have shown that both prolonged swimming [2] and treadmill running [1] resulted in a significant decrease of MyHC IIb in the costal diaphragm. The reason for this different adaptation in the diaphragm is not clear. However, from the standpoint that increased neuromuscular activity/overloading elicits the transformation of muscle fiber type from fast-to-slow [27], this result may imply that the recruitment of MyHC IIb fiber by SIT, especially during the resting period, was not enough to cause such an alteration.

Effects of hypoxia. There was no difference in any parameters between NC and HC in the diaphragm, indicating that the exposure to normobaric mild hypoxic environment for about 1 h a day at rest has no influence on the MyHC compositions and bioenergetic characteristics, or on the plantaris muscle. This is not surprising, since the diaphragm has relatively high oxidative capacity in spite of type II fiber dominant skeletal muscle [4, 15, 28], and overload for the diaphragm in this condition may not be large enough to change the MyHC isoforms and bioenergetic properties.

Both an increase of MyHC Ila isoforms and a decrease of MyHC IId/x isoforms in the diaphragm, however, were greater in HT than in NT. Furthermore, diaphragmatic CS activities and the ratio of CS/LDH were also significantly higher in HT than in NT. These data show that the relative contributions of the aerobic energy pathway during SIT might be enhanced by hypoxic exposure, thus improving the oxidative capacity of the diaphragm as compared to normoxia. However, SIT-induced alterations in the plantaris muscle were not enhanced by hypoxia. Although this study could not clarify the reason why the hypoxia enhanced the SIT-induced alteration in only the diaphragm, we postulate following two possible explanations.

One is that SIT under hypoxia may raise the contribution of anaerobic energy supply in working locomotor muscles, especially from the glycolytic energy pathway, because of the increase in oxygen deficit as compared with normoxia. As a result, a greater lactic oxygen debt would be induced under hypoxia in comparison to normoxia. Weyand et al. [29] have reported that an oxygen deficit increased by as much as 18% during 60 s of sprinting under normobaric hypoxia (13.0% O₂), compared to normoxia. McLellan et al. [30] have suggested that 45 s of supramaximal cycle exercise under severe normobaric hypoxia (10.8% O₂) resulted in a twofold increase in muscle lactate concentration in comparison to normoxia. Indeed, in our study (Table 2), the blood lactate concentrations after SIT tended to be higher in HT as compared with NT after week 6–7, which would increase the diaphragmatic work to amortize the further oxygen debt in the recovery period when the plantaris muscle was not working.

We did not directly measure the ventilation (\(V'_E\)) of the rat during the exercise and recovery periods. Although the blood lactate and inspired gas concentrations certainly contribute to the additional increased \(V'_E\), it seems to be difficult to estimate the increased \(V'_E\) by using only these parameters. However, Gargaglioni et al. [31] have reported that the infusion of dichloroacetate, which is an inhibitor of lactate production, resulted in the reduction of hypoxia-induced hyperventilation by about 50%. Further, Kato et al. [13] have shown that compared with normoxia, exercise under hypoxia (12.0% O₂) resulted in an increased \(V'_E\) not only during the exercise (54.7%), but also 30 min after the exercise (31.3%) in human study. Taken together, it is suggested that the prolonged additional work will be greatly applied on the diaphragm under hypoxia in comparison to normoxia during the SIT program and thus lead to an improved oxidative capacity. This could also explain why the alteration in MyHC isoforms within Type II isoforms was greater for HT than NT in the diaphragm.
Another explanation of the improvement of diaphragmatic oxidative capacity by hypoxia is that oxygen supplies to the skeletal muscle are limited by hypoxia because of decreased arterial hemoglobin oxygen saturation or arterial-venous oxygen difference during exercise [32]. For example, Melissa et al. [8] have shown that a combination of endurance training and interval training (≥25 min/day) under normobaric hypoxia (13.5% O\textsubscript{2}) resulted in a greater increase of \( \text{CS} \) activity in the human vastus lateralis muscle than normoxia. Terrados et al. [9] have also shown that one-legged training (30 min/day) under hypobaric hypoxia (572 Torr) resulted in a greater increase of \( \text{CS} \) activity in the human leg than under normobaric normoxia, and they proposed that a stimulus for the enzyme synthesis seemed to be related to the blood oxygen content or tension. What needs to be emphasized is that the duration of training under hypoxia in both studies was ≥25 min/day [8, 9]. Although blood with a low oxygen content would be equally supplied to both the diaphragm and the plantaris muscle in this study, the plantaris muscle was recruited only during intermittent running (≤10 min/day), and it might be too short to cause the greater improvement of aerobic properties under hypoxia. Therefore it is suggested that a prolonged (28–36 min/day) increase of work under less oxygen availability in the diaphragm might upregulate an oxidative enzyme activity and downregulate an anaerobic enzyme activity.

**Conclusion.** These findings revealed that SIT could induce the alterations of MyHC composition within the fast MyHC isoforms and improve the oxidative capacity in the costal diaphragm. Moreover, SIT-induced diaphragmatic adaptations were enhanced when SIT exercise was performed in normobaric hypoxia. A limitation of this study was that functional improvements such as fatigue resistance or whole body endurance performance was not directly assessed. Therefore an experimental confirmation would be required to connect the biochemical adaptations in the diaphragm with the physiological improvements in future study.

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