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Pioglitazone inhibits hypertrophy induced by high glucose and insulin in cultured neonatal rat cardiomyocytes

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Received March 2, 2007, accepted April 4, 2007

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Pharmazie 62: 925–929 (2007) *doi: 10.1691/ph.2007.12.7553*

The aim of the present study was to determine whether the antidiabetic agent pioglitazone has a direct inhibiting effect on myocardial hypertrophy induced by high glucose and insulin in primary cultured neonatal rat cardiomyocytes. Culture preparations of ventricular muscle cells newborn rats were utilized. At 72 h of culture age, the cardiomyocytes were incubated for another 48 h with 25.5 mmol/L glucose plus 0.1 μmol/L insulin (group 2), 25.5 mmol/L glucose and 0.1 μmol/L insulin plus 10 μmol/L pioglitazone (group 3), 10 μmol norepinephrine (group 4), respectively. Cells cultured continuously in medium served as control (group 1). Cellular surface area, protein content, atrial natriuretic factor (ANF) mRNA, and cardiotrophin-1 (CT-1) mRNA were assessed after treatment with different agents. All those parameters increased significantly after treatment with high glucose and insulin as compared with control ($P < 0.01$). These effects were inhibited markedly by pioglitazone. The cellular surface area and ANF mRNA in group 3 were decreased as compared with group 2 ($P < 0.01$). The protein content and CT-1 mRNA in group 3 were also decreased as compared with group 2 ($P < 0.05$). We concluded that a the cellular level myocardial hypertrophy induced by high glucose and insulin was inhibited directly by pioglitazone in primary cultured cardiac myocytes. CT-1 may be involved in myocardial hypertrophy induced by high glucose and insulin and inhibiting effects of pioglitazone on myocardial hypertrophy.

1. Introduction

Glucose dose-dependently increases neonatal rat myocyte protein synthesis (Liao et al. 2006), and insulin increases total mass of cellular protein in cultured rat cardiomyocytes (Bell et al. 2005). Left ventricular hypertrophy (LVH) is common in type 2 diabetic patients (Dawson et al. 2005; Hildebrandt et al. 2005), and is an independent risk factor for myocardial ischemia, cardiac arrhythmia, sudden death, and heart failure, all common findings in patients with type-2 diabetes (Sato et al. 2005). Pioglitazone is involved in the inhibition of cardiac hypertrophy induced by angiotensin II (Ang II) in neonatal rat cardiac myocytes *in vitro* and cardiac hypertrophy induced by pressure overload mice *in vivo* (Asakawa et al. 2002). The improvement of impaired cardiac glucose uptake in advanced type-2 diabetes by pioglitazone requires co-administration of insulin (Pelzer et al. 2005). Pioglitazone not only has therapeutic effects on type-2 diabetes but may also have inhibitory effects on cardiac hypertrophy in type-2 diabetes (Rodriguez et al. 2006). The effects and/or mechanisms of pioglitazone on myocardial hypertrophy induced by high glucose and insulin are still unclear. In the current study we hypothesize that pioglitazone may inhibit myocardial hypertrophy induced by high glucose and insulin in isolated neonatal rat primary cardiomyocyte cultures. The mechanisms behind the inhibitory effect of pioglitazone on myocardial hypertrophy were explored.

2. Investigations and results

2.1. Pioglitazone inhibited high glucose and insulin induced increase in surface area of cardiac myocytes

To assess cellular hypertrophy, surface area of cardiac myocytes was quantified. High glucose and insulin induced cardiac myocyte surface area increase 28% ($P < 0.01$) as compared with normal control. Pioglitazone inhibited high glucose and insulin induced increase in cell surface area (a decrease of 9% in cell surface area compared with high glucose and insulin treated cells, $P < 0.01$). NE induced cardiac myocyte surface area increase 34% ($P < 0.01$) as compared with normal control (Fig. 1).

2.2. Inhibitory effect of pioglitazone on high glucose and insulin induced increase in protein content

Total protein content of the cells was also significantly increased by high glucose and insulin (normal control, 13.22 ± 1.36 μg/well; high glucose and insulin, 21.56 ± 2.34, $n = 6$, $P < 0.01$), and this effect was inhibited by pioglitazone (pioglitazone, 18.64 ± 2.24 μg/well, $n = 6$, $P < 0.05$). Total protein content of the cells was also significantly increased by NE in positive control group (23.38 ± 2.41 μg/well, $n = 6$, $P < 0.01$) as compared with normal control (Fig. 2).

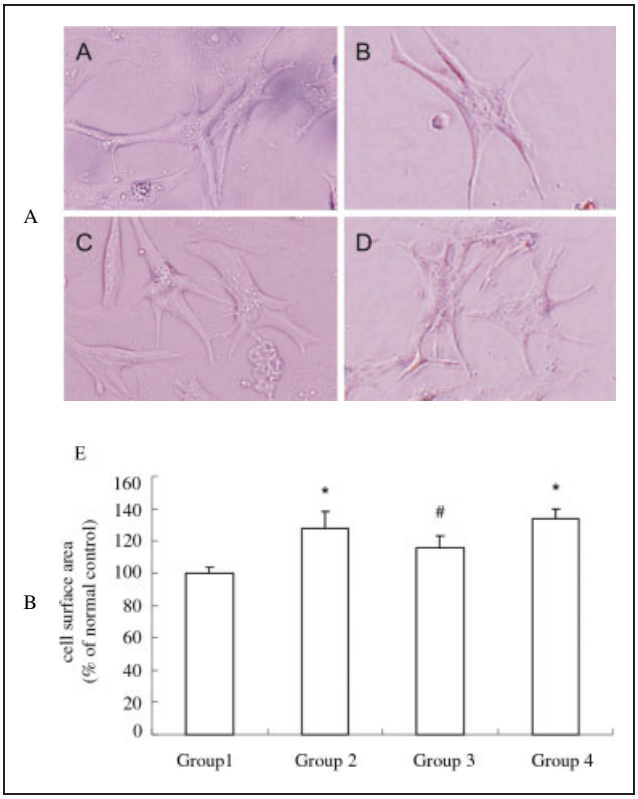

Fig. 1: High glucose and insulin, or NE induced increase in myocyte size and inhibiting effects of pioglitazone: A, B, C, and D represent living cardiac myocytes. (A) Myocytes were not treated with any drugs as normal control (group 1); (B) Myocytes were exposed to 25.5 mmol/L glucose and 0.1 μmol/L insulin for 48 h (group 2); (C) Myocytes were exposed to 25.5 mmol/L glucose, 0.1 μmol/L insulin and 10 μmol/L pioglitazone for 48 h (group 3); (D) Myocytes were exposed to 10 μmol/L NE for 48 h (group 4) as positive control. ×400. E represents the quantitative analysis of the inhibitory effect of pioglitazone on cardiac myocyte enlargement induced by high glucose and insulin. Bar graphs with error bars represent mean ± SD (n = 80). * P < 0.01 vs group 1; # P < 0.01 vs group 2

2.3. Inhibitory effect of pioglitazone on the increase of ANF mRNA and CT-1 mRNA expression induced by high glucose and insulin

We investigated the inhibitory effect of pioglitazone on the increase of ANF mRNA and CT-1 mRNA expression induced by high glucose and insulin in this experiment.

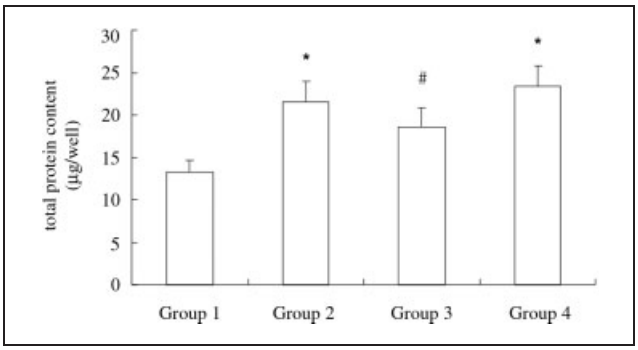

Fig. 2: Inhibitory effect of pioglitazone on high glucose and insulin induced increase in total protein content. Myocytes were not treated with any drugs as normal control (group 1). Myocytes were exposed for 48 h to high glucose and insulin in the absence (group 2) or presence (group 3) of pioglitazone (10 μmol/L). Myocytes were exposed for 48 h to NE (group 4) as positive control. Bar graphs with error bars represent mean ± SD (n = 6). * P < 0.01 vs group 1; # P < 0.05 vs group 2

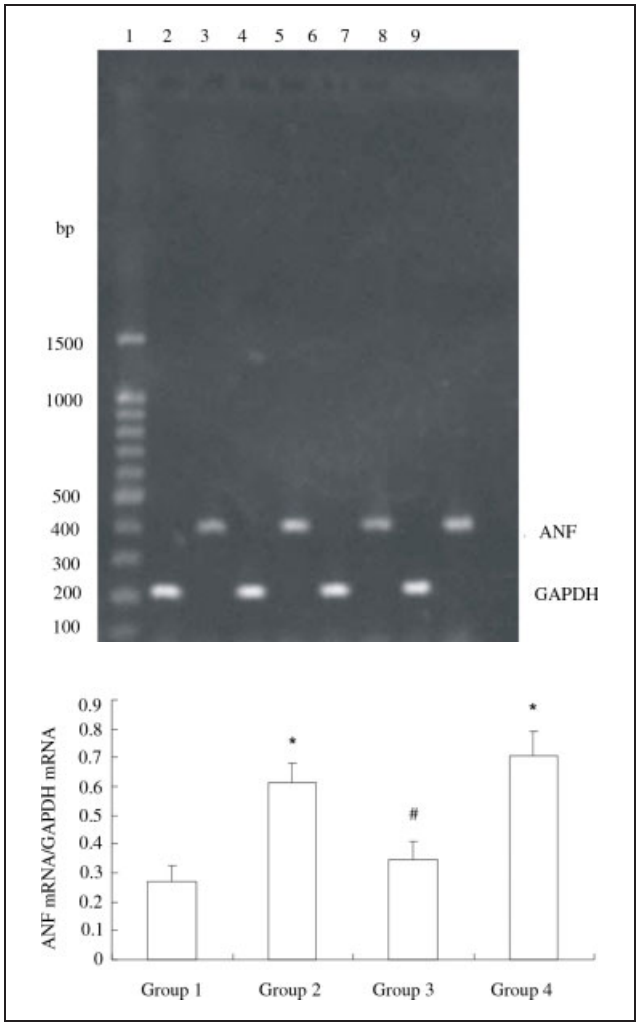

Fig. 3: Inhibitory effect of pioglitazone on high glucose and insulin induced increase in mRNA expression for ANF in cardiac myocytes. Myocytes were not treated with any drugs as normal control (group 1). Myocytes were exposed for 48 h to high glucose and insulin in the absence (group 2) or presence (group 3) of pioglitazone (10 μmol/L). Myocytes were exposed for 48 h to NE (group 4) as positive control. ANF (upper panel) and GAPDH (lower panel) mRNA were analyzed by RT-PCR. Lane 1: DNA marker. Lane 2, 3: group 1. Lane 4, 5: group 2. Lane 6, 7: group 3. Lane 8, 9: group 4. The quantitative analysis of the results is as follows: group 1, ANF mRNA/GAPDH mRNA = 0.2707 ± 0.0553; group 2, ANF mRNA/GAPDH mRNA = 0.6112 ± 0.0700; group 3, ANF mRNA/GAPDH mRNA = 0.3467 ± 0.0627; group 4, ANF mRNA/GAPDH mRNA = 0.7058 ± 0.0835. Bar graphs with error bars represent mean ± SD (n = 5). * P < 0.01 vs group 1; # P < 0.01 vs group 2

As shown in Figs. 3 and 4, high glucose and insulin increased mRNA expression for ANF and CT-1 in cardiac myocytes, while pioglitazone inhibited these effects.

3. Discussion

In the present study, pioglitazone showed inhibitory effects on hypertrophic cardiac myocytes by inhibiting increases in cell size, protein content, ANF mRNA and CT-1 mRNA expression induced by high glucose and insulin. ANF is a representative marker of cardiac hypertrophy (Hara et al. 2005; Song et al. 2006) as well as cell size and protein content of cardiac myocytes. CT-1 is a new cardiomyocyte hypertrophy-inducing factor (Hara et al. 2005; Kurdi et al. 2005) which exerts various hypertrophic effects both *in vitro* and *in vivo* (Takahashi et al. 2005; Tian et al. 2004;

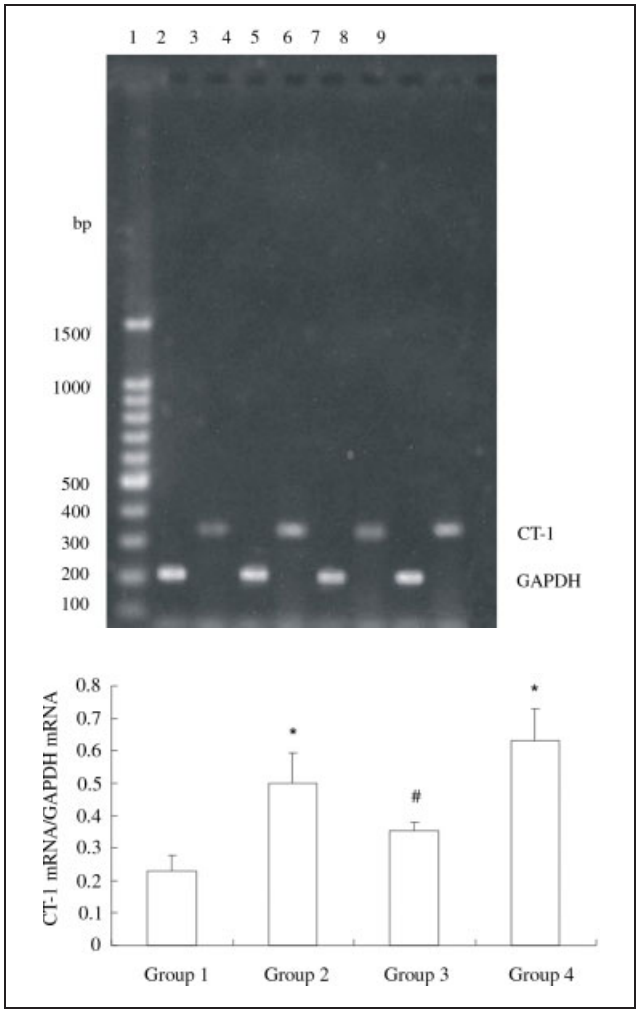

Fig. 4: Inhibitory effect of pioglitazone on high glucose and insulin induced increase in mRNA expression for CT-1 in cardiac myocytes. Myocytes were not treated with any drugs as normal control (group 1). Myocytes were exposed for 48 h to high glucose and insulin in the absence (group 2) or presence (group 3) of pioglitazone (10 μmol/L). Myocytes were exposed for 48 h to NE (group 4) as positive control. CT-1 (upper panel) and GAPDH (lower panel) mRNA were analyzed by RT-PCR. Lane 1: DNA marker. Lane 2, 3: group 1. Lane 4, 5: group 2. Lane 6, 7: group 3. Lane 8, 9: group 4. The quantitative analysis of the results is as follows: group 1, CT-1 mRNA/GAPDH mRNA = 0.2284 ± 0.0478; group 2, CT-1 mRNA/GAPDH mRNA = 0.5001 ± 0.0926; group 3, CT-1 mRNA/GAPDH mRNA = 0.3534 ± 0.0246; group 4, CT-1 mRNA/GAPDH mRNA = 0.6302 ± 0.0970. Bar graphs with error bars represent mean ± SD (n = 5). * $P < 0.01$ vs group 1; # $P < 0.05$ vs group 2

Wu et al. 2006). Pioglitazone, a new antidiabetic agent, is one of the novel insulin-sensitizing agents and high-affinity ligands for peroxisome proliferator-activated receptor (PPAR)-γ (Gumieniczek et al. 2006; Lemoine et al. 2006; Pelzer et al. 2005). PPAR-γ is required for adipogenesis but is also found in the cardiovascular system, where it has been proposed to oppose inflammatory pathways and act as a growth suppressor (Asakawa et al. 2002; Yamamoto et al. 2001). In the cardiomyocyte-specific PPAR-γ-knockout (CM-PGKO) mouse model study, the expression of cardiac embryonic genes (atrial natriuretic peptide and beta-myosin heavy chain) has been increased and Nuclear Factor (NF) kappaB activity elevated in the heart. And the effects of PPAR-γ agonist treatment were not found in CM-PGKO mice. So the cardiomyocyte PPAR-γ suppresses cardiac growth and embryonic gene expression and inhibits NF kappaB activity *in vivo* (Duan et al. 2005). The activation of the NF-kappaB signaling pathway, which is one of the most important signal transduction pathways involved in the hypertrophic growth of the myocardium, may suppress the activity of the PPARs, affording a link between cardiac hypertrophy and the fall in fatty acid oxidation in the hypertrophied heart (Planavila et al. 2006). Therefore, the inhibitory effect of pioglitazone on hypertrophic cardiac myocytes induced by high glucose and insulin in the current study may be mediated through the activation of PPAR-γ activity and/or suppression of NF-kappaB signaling pathway to regulate the molecular responses to hypertrophic stimuli in the cardiac myocytes. These molecular responses may inhibit the expression of CT-1 and finally resulted in the suppression of ANF expression, total protein content, and cell size of cardiac myocytes.

CT-1 is a novel cardiomyocyte hypertrophy-inducing factor belonging to the interleukin-6 (IL-6) family of cytokines (Hara et al. 2005; Kurdi et al. 2005; Pennica et al. 1995). Plasma levels of CT-1 increased in hypertensive patients (Lopez et al. 2005). CT-1 is a unique cardiac cytokine whose release is stimulated by ventricular stretch (Pemberton et al. 2005). There is an association between treatment-induced decrease of plasma CT-1 and LVH regression in hypertensive patients (Gonzalez et al. 2005; Lopez et al. 2005). According to the previous report, CT-1 exerts various hypertrophic effects by activating several signaling pathways, including the Janus activated kinase-signal transducer and activator of transcription (JAK-STAT), extracellular signal-regulated kinases1/2 (ERK1/2), extracellular signal-regulated kinase (ERK5) pathways (Takahashi et al. 2005; Tian et al. 2004; Wu et al. 2006). Different signaling pathways may contribute differently to cardiomyocyte hypertrophy by CT-1, but ERK5 pathway is critical for the hypertrophic responses to CT-1 in cultured cardiomyocytes (Takahashi et al. 2005).

Recently it was demonstrated that pioglitazone has functional influence on myocardium in type-2 diabetic rat models (Rodriguez et al. 2006). There was endothelial-myocyte uncoupling in type-2 diabetic rat hearts as compared to the normal rats, which was ameliorated by pioglitazone. The recovery from ailing to failing myocardium in diabetes by pioglitazone is in part due to decreased matrix metalloproteinase-9 activation, and left ventricular tissue levels of homocysteine (Rodriguez et al. 2006). This may be another mechanism of the effects of pioglitazone ameliorating the status of the myocardium.

To summarize, the discovery of the present study is that the inhibiting effect of pioglitazone on hypertrophic cardiac myocytes induced by high glucose and insulin may be achieved by the activation of PPAR-γ activity and/or other signaling pathways. Furthermore, the inhibiting effect of pioglitazone may be a result of suppression of CT-1 expression by several signaling pathways. This discovery will contribute to a better understanding of the mechanisms of the effects of pioglitazone on hypertrophic cardiac myocytes and its clinical application for patients with type-2 diabetes.

4. Experimental

4.1. Cardiac muscle cell culture preparations

All culture preparations used newborn rats taken from the breeding colony of Wistar rats maintained in the Experimental Animal Center of Shandong University of China. Primary cardiac muscle cell cultures were prepared as previously described (Hu et al. 2006; Simpson 1985; Zhang et al. 2004). Under aseptic conditions, ventricular apices, excluding all valvular and atrial tissue, were removed from the newborn rat hearts, minced with fine

dissecting scissors into fragments approximately 1 mm in diameter, and placed in culture medium. The composition of the culture medium consist of 90% Dulbecco's Modified Eagle Medium (D-MEM, Gibco), 10% newborn bovine serum (Gibco), 20 mmol/L HEPES buffer (pH 7.3), and 16 mmol/L $NaHCO_3$. Following the completion of dissection, the ventricular fragments were dispersed with trypsin into a cell suspension as previously described. The ventricular cell suspension, at a density of 2×10^5 cells/mL, was plated into 24-well clusters or 50 ml flasks. All above clusters or flasks were then incubated at 37 °C in 5% CO_2 incubator. Twenty-four hours after seeding, the cultures were treated with 100 μmol/L 5-bromo-2′-deoxyuridine (5-BrdU) for another 24 h to inhibit the proliferation of non-cardiac myocytes. Forty-eight hours after seeding, the culture medium was changed and the cells were cultured for another 24 h before stimulation.

4.2. Treatment of cell cultures with different drugs

At 72 h of culture age, all the isolated ventricular cell cultures were randomly divided into 4 groups and treated with different drugs. The cultures in group 1 were incubated with serum free D-MEM only as normal control. The cultures in group 2 were incubated with serum free D-MEM added 25.5 mmol/L glucose and 0.1 μmol/L insulin. The cultures in group 3 were incubated with serum free D-MEM added 25.5 mmol/L glucose, 0.1 μmol/L insulin and 10 μmol/L pioglitazone. The cultures in group 4 were incubated with serum free D-MEM added 10 μmol/L norepinephrine (NE) as positive control. All the above cell cultures were incubated at 37 °C in 5% CO_2 incubator for another 48 h.

4.3. Measurement of cardiac muscle cell surface area

At design culture age and treatment conditions, the cardiac muscle cell surface area was measured by the method of Simpson (1985). Cell images captured by video camera (Olympus) were traced and analyzed with Image pro plus (IPP). The area of each cell was then measured to account for the surface portion in contact with the flask. All cells from randomly selected fields in 4 flasks were examined for each condition. Eighty cells were measured in each condition.

4.4. Determination of total protein content of the cells

The preparation, plating, treatment, and grouping of cells were the same as described above. The cells used to determine total protein content (TPC) were washed three times with PBS, dissolved in 0.5 mol/L NaOH (1 ml/well), and incubated at 37 °C for 30 min. TPC was measured by the Coomassie brilliant blue G250 method, using bovine serum albumin as a standard. Duplicate wells were established to count cells per group. TPC was expressed as μg/well.

4.5. RNA extraction and RT-PCR

The cardiac muscle cells in different groups were processed for determination of the mRNA levels of atrial natriuretic factor (ANF) and cardiotrophin-1 (CT-1) by RT-PCR. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also determined as an internal control.

Total cardiac muscle cell RNA of each flask was isolated by TRIzol (MBI, U.S.A.) and the mRNA level was analyzed by RT-PCR, which was performed as described previously (Sakai et al. 2000). The gene-specific primers were synthesized using the published cDNA sequences for ANF, CT-1 and GAPDH. The sequences of the oligonucleotides were as follows: ANF 5′-CTG CTA GAC CAC CTG GAG GA-3′ (sense), 5′-ACC AAG CTG TGT GAC ACA CC-3′ (antisense), 402bp; CT-1 (Aoyama et al. 2000) 5′-TCT ATG GCG AGT GGG TGA GC-3′ (sense), 5′-AGC AAG CAA GCA AAG AAA GA-3′ (antisense), 340bp; 3′-GAPDH 5′-CCT TCA TTG ACC TCA ACT ACA TG-3′ (sense), 5′-CTT CTC CAT GGT GGT GAA GAC-3′ (antisense), 206bp.

The cycle profile of PCR included denaturation for 45 s at 95 °C, annealing for 60 s at each suitable temperature, and extension for 45 s at 72 °C. PCR was performed within the range that demonstrates a linear correlation between the amount of cDNA and the yield of PCR products.

The amplified products on agarose gels were stained with ethidium bromide, visualized by a UV transilluminator and photographed. The photographs were scanned (Pharmacia Biotech, German), and quantification was done with a Totallab.

4.6. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA followed the Student-Newman-Keuls test for significance was used to compare the 4 groups. A value of $P < 0.05$ was considered significant.

Acknowledgements: This project was supported by Foundation for University Key Teachers from the Ministry of Education of China and Foundation for Natural Sciences in Shandong Province of China (No. Z2006D05)

[1] These authors contributed equally to this article.

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