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Intrahepatic cholesterol influences progression, inhibition and reversal of non-alcoholic steatohepatitis in hyperlipidemic mice

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ABSTRACT

Hepatic inflammation is the key factor in non-alcoholic steatohepatitis (NASH) and promotes progression to liver damage. We recently identified dietary cholesterol as the cause of hepatic inflammation in hyperlipidemic mice. We now show that hepatic transcriptome responses are strongly dependent on cholesterol metabolism during diet-induced NASH and its inhibition by fenofibrate. Furthermore, we show that, despite doubling hepatic steatosis, pharmacological LXR activation reverses hepatic inflammation, in parallel with reversing hepatic cholesterol levels. Together, the results indicate a prominent role of cholesterol during the development, inhibition and reversal of hepatic inflammation in NASH and reveal potential new therapeutic strategies against NASH.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by lipid accumulation in the liver (steatosis) and has become a major health problem. NAFLD may progress towards a more harmful condition, i.e. non-alcoholic steatohepatitis (NASH), in which inflammation is present. Ultimately, NASH can lead to further liver damage such as fibrosis, cirrhosis and liver failure [1,2].

We have previously shown that hyperlipidemic mice, like the humanized apolipoprotein E2 knock-in (APOE2ki) mice and the low density lipoprotein (LDL) receptor-deficient mice, develop NASH after only a few days of high fat, high cholesterol (HFC) feeding [3,4]. In APOE2ki mice, the human APOE2 allele replaces the murine variant and is expressed under the control of endogenous promoter sequences in a tissue specific manner and at physiological levels. APOE2 has a markedly reduced affinity for the LDL receptor, leading to a plasma lipoprotein profile resembling human type III hyperlipoproteinemia [5]. NASH development in APOE2ki mice could be inhibited by co-administrating fenofibrate (FF), a synthetic ligand of peroxisome proliferator-activated receptor alpha (PPARα) [3]. PPARα is a nuclear receptor with fatty acids as its natural ligands [6,7]. Upon activation, it initiates transcription of genes involved in lipid metabolism resulting in increased fatty acid oxidation [8].

Abbreviations: NASH, non-alcoholic steatohepatitis; LXR, liver X receptor; APOE2ki, humanized apolipoprotein E2 knock-in; NAFLD, non-alcoholic fatty liver disease; LDL, low density lipoprotein; HFC, high fat, high cholesterol; FF, fenofibrate; PPARα, peroxisome proliferator-activated receptor alpha; T09, T0901317; TG, triglyceride; Acox1, acyl-CoA oxidase 1; WT, wild type; SREBP, sterol regulatory element-binding protein ½; IPA, ingenuity pathway analysis; Cept1, choline/ethanolaminephosphotransferase 1; FFA, free fatty acid; ABC, ATP binding cassette; Acaa, acetyl-CoA acyltransferase; Acta1, actin alpha; Arg, arginase; Acl, argininosuccinate lyase; Cyp7a1, cytochrome P450 7a1; Cyp8b1, cytochrome P450 8b1; Fdft1, farnesyldiphosphatefarnesyltransferase 1; Fdps, farnesyldiphosphate synthase; Gpdh, glyceraldehyde-3-phosphate dehydrogenase; Hmgcs, HMG-CoA synthase; Insig2, insulin stimulated gene 2; Lpl, lipoprotein lipase; SAT, spermidine/spermine n(1)-acyltransferase 1; Sc5dl, sterol 55-desaturase-like; Scd, stearoyl-CoA desaturase

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Table 1
Activation of hepatic transcription factors. Table shows the regulation of genes according to IPA®. Regulation determined by literature mining regulation is shown as ↑ (up-regulated by the transcription factor), or ↓ (down-regulated by the transcription factor). In numbers, the actual regulation of these genes in our dataset is shown by either diet only (HFC) or the diet supplemented by fenofibrate (HFCff); non-significant regulation ($P > 10^{-6}$) is shown by "ns".

Gene	Reported effect transcription factors			Measured response			
	LXR	PPARa	SREBP	HFC 2 days	HFC 7 days	HFCff 2 days	HFCff 7 days
ABCA1	↑			ns	1.9	ns	ns
ABCC3	↑			−1.7	−1.5	2.7	2.3
ABCG5	↑			ns	1.5	1.7	ns
ACAA1		↑		1.4	ns	3.1	4.1
ACAA2		↑	↑	−1.8	−3.2	−2.7	ns
acta1		↑		ns	−9.3	ns	24
arg		↓		−1.5	−1.6	ns	1.4
asl		↓		−1.8	−2	ns	ns
Cyp7a1	↑	↓	↓	ns	−2.4	ns	ns
CYP8B1		↑	↑	−1.9	−2.5	ns	ns
fdft1	↑		↑	ns	−3.1	ns	1.9
fdps			↑	ns	−2.8	−2.3	ns
gpdh		↑		ns	1.8	ns	ns
HMGCS			↑	−1,5	ns	ns	2,9
Insig2				−1,5	ns	1,8	1,6
LpL	↑	↑	↑	ns	2.8	2.2	3.3
SAT		↓		ns	2	ns	ns
Sc5dl		↑	↑	ns	−1.4	ns	ns
scd	↑		↑	1.2	ns	ns	1.9
scd2	↑		↑	ns	2.6	ns	ns
SREBP	↑			ns	2.1	ns	ns

ATP binding cassette (ABC), acetyl-coa acyltransferase (Acaa), actin alpha (Acta1), arginase (Arg), argininosuccinate lyase (Acl), cytochrome P450 7a1 (Cyp7a1), cytochrome P450 8b1 (Cyp8b1), farnesyldiphosphatefarnesyltransferase 1 (Fdft1), farnesyldiphosphate synthase (Fdps), glyceraldehyde-3-phosphate dehydrogenase (Gpdh), HMGCoA synthase (Hmgcs), insulin stimulated gene 2 (Insig2), lipoprotein lipase (Lpl), spermidine/spermine n(1)-acyltransferase 1 (SAT), sterol 55-desaturase-like (Sc5dl), stearoyl-CoA desaturase (Scd), sterol regulatory element-binding protein ½ (SREBP).

In the current study, the hepatic transcriptional responses induced by feeding APOE2ki mice a HFC diet were investigated in detail. We identified the cholesterol metabolism pathway to be of major importance during the early development of NASH. Subsequently, we aimed to identify how cholesterol metabolism influences NASH development and to investigate the role of intrahepatic cholesterol and triglycerides during inhibition and reversal of NASH by using pharmacological ligands of PPARα and liver X receptor (LXR).

2. Materials and methods

2.1. Mice and diet

*APOE2*ki mice were housed under standard conditions. Experiments were performed according to Dutch and French laws, approved by the Committee for Animal Welfare of Maastricht University, Maastricht, The Netherlands, and the Pasteur Institute review board, Lille, France. Groups of 10 homozygote female mice were fed either chow or HFC [3] (diet 1635, SAFE, Villemoisson-sur-orge, France) for 2 or 7 days with or without FF 0.2% (F6020, Sigma–Aldrich, Zwijndrecht, the Netherlands). Additionally, mice were given HFC for 2 months, followed by 5 days of gavage treatment with an LXR agonist, T0901317 (T09), (30 mg/kg) or vehicle. Mice were sacrificed by cervical dislocation. Tissues were isolated, snap-frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA analysis

Hepatic mRNA extraction, cDNA synthesis, and microarray data generation has been described previously [3]. In short, microarray analysis was performed by selecting genes according to their *P*-value of the diet/treatment effect for each delay coefficient (four *P*-values per probe set). For the multi-testing problem, Bonferroni correction was used. Genes with a *P*-value lower than 10^{-6} were selected as significantly regulated. Biological pathway analysis was done with ingenuity pathway analysis (IPA®) (Ingenuity Systems, Redwood City, USA) software. Microarray data were

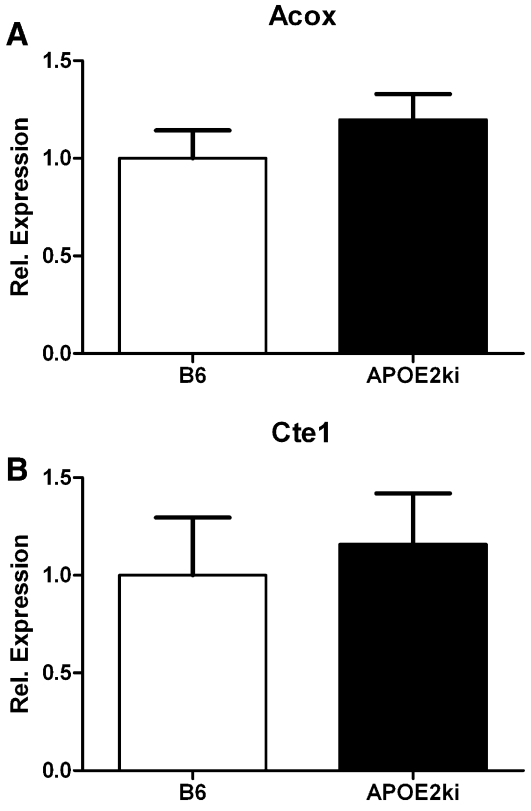

Fig. 1. Acox1 and Cte1 gene expression. Relative gene expression of Acox1 (A) and Cte1 (B) in hepatic mRNA of APOE2ki female mice (black bar) and C57Bl/6 (B6) female mice (white bar) on a chow diet.

validated previously on individual samples by QPCR [3]. Quantification of gene-expression by quantitative PCR was done as described previously [3], using cyclophillin A as reference gene. Primers:

5′-GCAGCCACCCCGAGGTAAA-3′ (Cte1 forward), 5′-GCCACGG AGCCATTGATG-3′ (Cte1 reverse), 5′-TGTGACCCTTGGCTCTGTTCT-3′ (Acox1 forward), 5′-GTAGTAAGATTCGTGGACCTCTG-3′ (Acox1 reverse), 5′-TTCCTCCTTTCACAGAATTATTCCA-3′ (cyclophillin A forward), 5′-CCGCCAGTGCCATTATGG-3′ (cyclophillin A reverse).

2.3. Lipid analysis

Hepatic cholesterol and triglyceride contents were measured as described previously [3] or liver specimens were dried to constant weight in a Speedvac (Savant®); (oxy)sterols were extracted overnight by chloroform/methanol (2:1) at 4 °C. Cholesterol and its precursors were determined after alkaline hydrolysis and derivatization to the corresponding trimethylsilyl-ethers by gas chromatography-flame ionization detection and gas chromatography-mass spectrometry as described before [9].

2.4. Histology

Frozen liver sections (7 μm) were fixed in acetone and stained with Mac1 (M1/70) antibodies. Pictures were taken with a Nikon® digital camera DMX1200 and ACT-1 v2.63 software (Nikon® Corporation, Tokyo, Japan).

2.5. Statistical analysis

Groups were compared using two-tailed non-paired *t*-tests using Graphpad Prism® 4.0. Data are expressed as means ± S.E.M. and considered significant at $P < 0.05$.

3. Results

As shown previously, HFC feeding results in an increase of both plasma and liver triglyceride (TG) and cholesterol levels, both of which were normalized by FF treatment [3]. Additionally, transcriptome analysis has shown that PPARα-dependent transcriptional responses in hyperlipidemic APOE2ki mice do not play a prominent role in early HFC-induced changes in hepatic gene expression [3]. Therefore, we compared basal expression levels of two established PPARα target genes, i.e. acyl-CoA oxidase 1 (Acox1) and cytososlic acyl-coa thioesterase 1 (Cte1) in APOE2ki livers with levels in C57BL6 wild type (WT) livers (Fig. 1). The data showed no difference in expression, indicating that basal hepatic PPARα activity in APOE2ki mice is equal compared to WT animals.

Interestingly, of the genes down-regulated by the diet and up-regulated by FF (Supplementary Table 1), the group of genes involved in cholesterol metabolism was over-represented (15% of the regulated genes in this group). These genes included: Hmgcs1 and Fdft1, involved in cholesterol biosynthesis; AbcC3, involved in hepatic bile acid transport [10]; and Insig2, a regulator of intracellular cholesterol levels [11]. To acquire insight into the potential impact of cholesterol metabolism-related transcription factors, a literature search was performed on transcription factors involved in cholesterol metabolism: LXR and sterol regulatory element-binding protein ½ (SREBP). Changes in expression levels of target genes present in our dataset were compared to their regulation known in literature, as reported by IPA® (Table 1). Genes known to be induced by SREBP activation (acaa2, cyp8b1, fdft1, fps, hmgcs, sc5dl) were rather inhibited upon HFC feeding, indicating an inhibition of SREBP activity. In addition, LXR target genes (abca1, abcg5, lpl, scd, scd2, srebp) were mostly regulated upon HFC in the sense of LXR activation. The analysis thus suggests that these cholesterol sensors (i.e. SREBP inhibition and LXR activation) are major factors controlling regulation of lipid genes upon HFC feeding.

We hypothesized that the observed regulation of LXR and SREBP arises from increased hepatic cholesterol [11]. To assess this hypothesis in vivo, intrahepatic levels of cholesterol and one of his precursors, lathosterol, were measured in the liver. The levels of both compounds were markedly increased upon HFC feeding (Fig. 2).

Addition of FF to the HFC fed mice was shown previously to inhibit NASH development [3]. In parallel, FF treatment inhibited the accumulation of hepatic cholesterol and its precursor lathosterol (Fig. 2) as well as reversing the HFC-induced regulation of many cholesterol metabolism-associated genes (Table 1, Supplementary Table 1).

To distinctively elucidate the impact of intrahepatic cholesterol on hepatic inflammation, we aimed to specifically decrease hepatic cholesterol levels. Hereto, APOE2ki mice were given HFC for up to 2 months to establish NASH. Hereafter, animals were treated with T09, a synthetic agonist of LXR. This agonist specifically lowers cholesterol levels while increasing steatosis [12]. As expected, treatment resulted in a tendency to decrease of plasma cholesterol (1123 mg/dl ± 479 vs. 826 mg/dl ± 270), while inducing hypertriglyceridemia (200 mg/dl ± 139 vs. 1221 mg/dl ± 818). Furthermore, T09 lowered intra-hepatic cholesterol dramatically (Fig. 3A) while doubling steatosis (Fig. 3B). In accordance with hepatic cholesterol levels, and despite doubling hepatic TG, inflammation was strongly

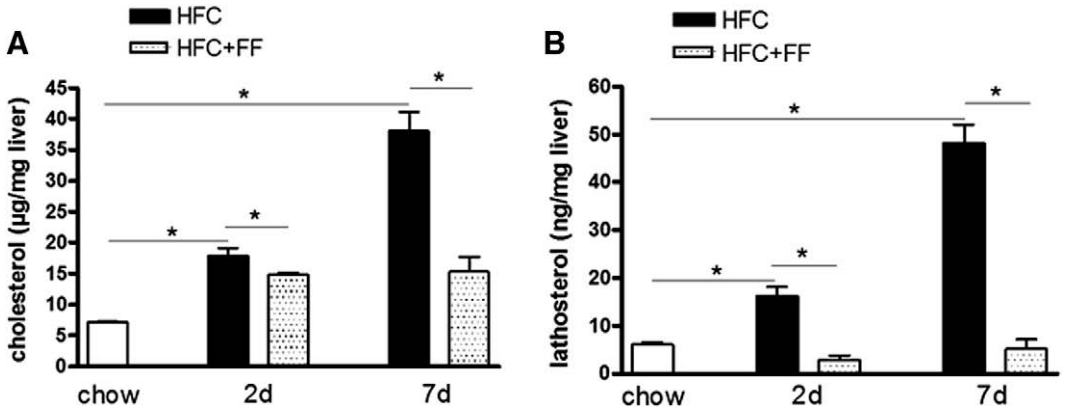

Fig. 2. Hepatic cholesterol and lathosterol levels. Measurement of cholesterol (A) and its precursor lathosterol (B) in the livers of female APOE2ki mice after 2 and 7 days of HFC diet (black bars) and HFC + FF (grey bars) feeding compared to livers of animals fed a chow diet (white bars). * indicates significant differences between groups.

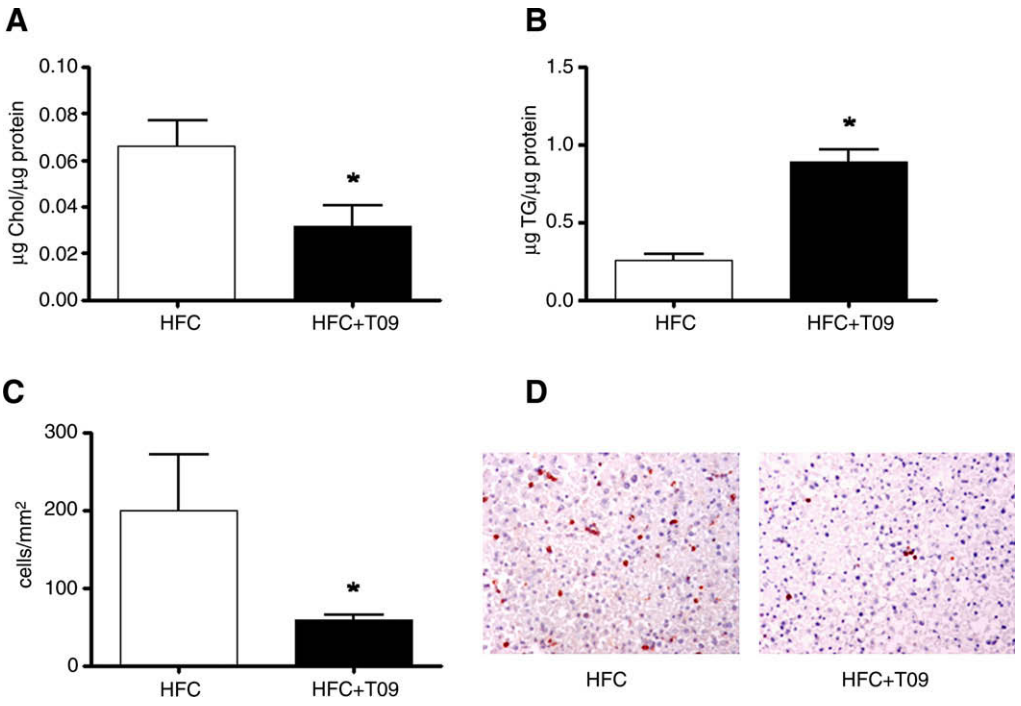

Fig. 3. Reversal of NASH by T09. Measurement of hepatic cholesterol (A) and TG (B) and Mac1-positive cell count (C) in the livers of female APOE2ki mice after 2 months of HFC (white bars) and after T09 treatment (black bars). Panel (D) shows representative pictures of Mac1 staining. * indicates significant differences between groups.

reversed (Fig. 3C and D), as can be clearly seen by the decrease in (infiltrating) Mac1-positive inflammatory cells. Parallel with its effects on hepatic cholesterol, LXR activation thus reversed inflammation in a model of severe established NASH.

4. Discussion

Previously, we have shown that HFC feeding induces NASH very rapidly in hyperlipidemic mice [3,4]. Current data show that the lack of a PPARα signature in the hepatic transcriptional response was not the consequence of an increased basal activity of this transcription factor due to the increased basal lipid levels found in APOE2ki mice. This supports the notion that other transcription factors are likely to be more dominant. Although amongst the dietary components there were some putative PPARα ligands, it is now becoming more clear that in vivo, PPARα activation relies more on the generation of de novo ligands, such as 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine by fatty acid synthase and choline/ethanolaminephosphotransferase 1 (Cept1) [13].

Current study also suggests that transcriptional responses in the liver are mainly due to increased hepatic cholesterol levels, reflected by the hepatic transcriptional response to HFC feeding. In fact, we have previously found 30% of genes regulated by HFC to be associated with cholesterol metabolism [3]. Current literature-based search suggested that SREBP activity was generally inhibited, as several genes regulated by this transcription factor were down-regulated [14,15]. This is probably a consequence of negative feedback mechanisms resulting from the hepatic cholesterol accumulation [16]. However, some SREBP target genes were up-regulated, despite the presumed inhibition of SREBP by the elevated cholesterol levels [15]. It is possible that for these genes, the activation of LXR by oxysterols [14,17] overrides SREBP inhibition (Table 1).

Both LXR and SREBP have been reported as important players in steatosis and NASH via the induction of lipogenesis [12,18]. In fact, hepatic free cholesterol and SREBP levels have been shown to increase in human NASH [19]. Moreover, a lipidomic analysis of human livers has shown a progressive increase of free cholesterol during different stages of NASH [20]. Additionally, mitochondrial free cholesterol accumulation has been shown to sensitize the liver for developing inflammation in rats through mitochondrial glutathione depletion [21]. In addition, we have previously shown that dietary cholesterol can provoke hepatic inflammation, possibly due to direct Kupffer cell activation upon scavenging of remnant lipoproteins [4]. Together with our current results, these observations underline the role of hepatic cholesterol as a probable cause of hepatic inflammation.

In parallel with inhibiting NASH development in mice, FF supplementation led to a decrease of intra-hepatic cholesterol levels. Furthermore, a significant fraction of the genes reversed by FF are known to be involved in cholesterol metabolism and contain known or putative SREBP target genes [14], suggesting that the inhibition of cholesterol accumulation in the liver contributed to the inhibition of NASH. However, in mice, FF robustly inhibits hepatic TG accumulation [3], which is thought to be the critical first hit during the development of NASH [22]. Consequently, to explore the exact contribution of cholesterol metabolism on inflammation in the absence of TG accumulation, we reversed an established NASH with T09, an LXR agonist. LXR is an oxysterol sensor which induces a set of genes that modulate cholesterol uptake, transport, and efflux [17,23,24]. Additionally, treatment with this compound induces steatosis and hypertriglyceridemia by increased lipogenesis [12,16] and free fatty acid (FFA) uptake via increased expression of the FFA transporter CD36 [25] in mice. Increased lipogenesis has also been shown in mice and murine and human cell lines [12,16]. Our results show that the accumulation of hepatic TG did not counteract the ability of LXR activation to reduce inflammation and underline the importance of the hepatic cholesterol/LXR pathway in NASH. However, it has to be kept in mind that LXR activation can modulate inflammation directly [16], which also may have played a role.

Previously, we reported that male hyperlipidemic mice develop hepatic inflammation even without any accumulation of hepatic lipids and that, rather than hepatic triglyceride accumulation, dietary cholesterol consumption was causal for hepatic inflammation

[4]. Taken these findings together with the current results, we further dissociate the development of steatosis and hepatic inflammation in these mouse models of NASH and, in addition to dietary cholesterol, substantiate the importance of hepatic cholesterol levels.

Some important clinical implications arise from our studies. First, it has to be noted that in humans, the beneficial effects, if any, of FF treatment on steatohepatitis development are not completely understood. It has been shown that FF improves metabolic syndrome parameters and liver tests, but effects on liver histology, including steatosis scores, remained minimal [26]. However, it has been suggested that FF may be of use in improving NASH with an improved efficacy when combined with statin treatment [27]. Second, although the clinical use of LXR agonists is debated, they show promise as potential targets against cardiovascular disease [16]. However, the clinical use of such agonists is severely doubted due to their pro-steatotic properties, which has refrained them until now to be used in human subjects. Steatosis itself is generally considered a relatively benign and reversible condition, and detrimental outcome is often associated with the presence of inflammation [2,22]. In fact, one of the physiological roles of the liver is to accumulate TG in response to prolonged fasting, which is necessary for the energy homeostasis in the body and is mainly regulated by this organ [28,29]. Additionally, TG may even be a protective pool of FFA in the liver [30]. Moreover, LXR-induced steatosis does not lead to worsening of hepatic insulin sensitivity in mice [31]. Therefore, the arguments against LXR agonists as a potential treatment against atherosclerosis in humans may have to be reconsidered. Additionally, these results reinforce the central role of cholesterol during the development of hepatic inflammation.

LXR agonists may thus well be considered as a potential treatment for NASH, at least to treat its most harmful component, i.e. inflammation; as lowering liver inflammation may be the first concern in the treatment of NASH, despite a possible increase in steatosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.01.046.

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