Pharmaceutical Sciences
December 2018, 24, 257-263
doi: 10.15171/PS.2018.38
http://ps.tbzmed.ac.ir/

Research Article

Cytoprotective Properties of Carnosine against Isoniazid-Induced Toxicity in Primary Cultured Rat Hepatocytes

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Article Info

Article History:
Received: 1 May 2018
Revised: 25 June 2018
Accepted: 26 June 2018
ePublished: 30 December 2018

Keywords:
-Cytotoxicity
-Drug-induced liver injury
-Hepatotoxicity
-Mitochondria
-Oxidative stress
-Antioxidants

ABSTRACT

Background: Drug-induced liver injury is a critical clinical complication. Hence, finding new and safe protective agents with potential clinical application is of value. Isoniazid (INH) is an antituberculosis agent widely used against Mycobacterium tuberculosis infection in human. On the other hand, hepatotoxicity is a clinical complication associated with isoniazid therapy. Oxidative stress and its associated events are major mechanisms identified for INH-induced liver injury. Carnosine is an endogenously found peptide widely investigated for its hepatoprotective effects. On the other hand, robust antioxidant and cytoprotective effects have been attributed to this peptide.
Methods: The current study designed to evaluate the potential cytoprotective properties of carnosine against INH-induced cytotoxicity in drug-exposed primary cultured rat hepatocytes. Primary cultured rat hepatocytes were incubated with INH (1.2 mM).
Results: INH treatment caused significant increase in cell death and lactate dehydrogenase (LDH) release. On the other hand, it was found that markers of oxidative stress including reactive oxygen species were significantly increased in INH-treated cells. Cellular glutathione reservoirs were also depleted in INH-treated group. Carnosine treatment (50 and 100 µM) significantly diminished INH-induced oxidative stress and cytotoxicity.
Conclusion: These data mention carnosine as a potential protective agent with therapeutic capability against INH hepatotoxicity.

Introduction

Tuberculosis (TB) is one of the leading global health threats infecting about a third of the world's population.[1] The combination therapy of antituberculosis agents including isoniazid (INH), rifampicin, pyrazinamide and ethambutol are currently used prescriptions for TB. However, hepatotoxicity as a well-known side effect associated with antituberculosis drugs restricts their clinical application.[2]

INH as an efficacious and frontline antimicrobial agent to treat tuberculosis was introduced clinically in the 1950s.[3] Despite its efficiency, the INH-induced liver damage is a serious problem which has interrupted treatment regimen during the past decades. Patients have been reported to develop INH therapy-induced elevated serum levels of alanine aminotransferase, liver tissue necrosis, hepatic inflammation and even liver failure which need for liver transplantation.[3-8]

Multiple pathways including oxidative stress, immune response and impairment of energy homeostasis are proposed for hepatotoxicity mechanism of INH.[3] Oxidative stress and its associated complications are well-known underlying mechanisms of hepatotoxicity induced by INH.[9-13] It has been found that INH could elevate cellular level of reactive oxygen species and decrease cellular antioxidant defense system capacity. Depletion of cellular glutathione (GSH) reservoirs which results in oxidative stress and apoptosis induction in hepatocytes has also been reported in INH toxicity models.[12,13] Furthermore, decreased GSH level disrupts cellular removal of superoxide radicals and hydrogen leading to an upsurge in malondialdehyde (MDA) level in the plasma.[9-11] All these data mention the potential therapeutic application of antioxidant molecules against INH hepatotoxicity.

A literature search has fetched oxidative stress to be the primary mechanism of the drug-induced liver toxicity targeted by different antioxidants such as natural or synthetic compounds.[14,15] Carnosine (b-alanyl-L-histidine) (CAR) as a natural dipeptide present in the brain, muscle and other tissues of vertebrates including humans. This peptide plays a variety of biological and

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physiological functions including membrane protecting activity, metal chelating ability, pH-buffering, protection against proteins glycation and macrophage regulating activities.[16-18] Antioxidative and radical scavenging properties are also interesting features of CAR molecule.[19]

Recently, it has been shown that carnosine has promising preclinical and clinical effects in several disease including diabetes, ocular disease, aging, cancer and neurological disorders.[20] Also, several pharmacological and toxicological studies showed that CAR possessed potent antioxidant activities such as scavenging of free radicals and carbonyl species and inhibition of lipid peroxidation. Moreover, CAR efficiently attenuates liver damages with different etiologies.[17,21-26] The current study was designed to evaluate the potential protective properties of CAR against INH-induced toxicity in primary rat hepatocyte culture.

Materials and Methods

Animals

Adult male Sprague Dawley rats were supplied by the Laboratory Animal Center, Tabriz University of Medical Sciences, Iran. They were housed in standard cages under a 12 h light/dark cycle and received a chow diet and tap water. The experiments reported were carried out according to the animal handling protocol of National Institutes of Health Publication (No 85-23, revised 1985) and ethics committee of Tabriz University of Medical Sciences, Tabriz, Iran.

Chemicals

Bovine serum albumin was purchased from the Roche diagnostic corporation (IN). N-acetyl cysteine, carnosine, isoniazid, collagenase (Type II), HEPES, Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and all other reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, USA).

Primary rat hepatocytes culture and treatment

Rat primary hepatocytes were derived from male Sprague Dawley rats (200–250 g) by collagenase perfusion method.[27,28] The viability of hepatocytes was tested to be above 85% before every producer via the trypan blue exclusion assay. DMEM supplemented with 10 % FBS, 1 % antibiotic-antimycotic solution (10 000 units Penicillin, 10 mg Streptomycin, 25 µg Amphotericin B), 0.1 U/ml insulin and 0.01 µM dexamethasone was utilized to incubate isolated hepatocytes for 3 h at 37 °C in a humidified atmosphere of 95 % O2 and 5 % CO2. Cells at a density of 4.8*10 4/ml were plated in 96 well plates to determine cell viability. Hepatocytes were incubated with different concentrations of INH for 4 hours, and in CAR-treated groups, the latter was used 30 minutes before INH addition. For other biochemical assays hepatocytes (2 * 105/ml) were plated in 12 well plates.

Cell viability assays

In order to perform lactate dehydrogenase (LDH) assay, 50 µl of was incubation medium in each treatment was mixed with a freshly prepared β-NADH solution which was then left in the dark for 30 min. The latter solution contains 5.3 mg NADH, 4.5 mg pyruvate and 7.4 mg NaHCO3 in 10 ml 0.05 M phosphate buffer in pH 7.4. Finally, the absorbance of the mentioned mixture was measured at 340 nm by a microplate reader.[27]

In MMT assay, the remaining medium was treated with 15 µl of a 1 mg/ml MTT solution and incubated for 4 h at 37 °C followed by a dissolution of generated formazan crystals with DMSO. An ELISA microplate reader was used to measure the absorbance at 570 nm.[28]

Reactive oxygen species (ROS) determination

The fluorescent probe 2′, 7′- dichlorodihydrofluorescein diacetate (DCFH-DA) was utilized to determine the amount of cellular ROS generation. The dye ate the concentration of 100 µM was added to the treated hepatocytes and incubated for 1 h at 37 °C. The extent of ROS formation was measured as the fluorescence intensity of DCF using spectrofluorometer with excitation wavelength at 485 nm and emission wavelength at 535 nm.[27]

Lipid peroxidation assay

To assess the occurrence of lipid peroxidation, 0.5 ml of the cell lysate was utilized. To prepare cell lysate,primary hepatocytes were centrifuged (5000 g for 5min), washed with ice-cold PBS, pH 7.4 and sonicated with an ultrasonicator (Parasonic 30S, Tehran, Iran) for 3 sets each interval for 0.5 min. The cell lysate was incubated with 1.0 ml of KCl (0.15 M) and 250 µl of 0.2 mM ferric chloride solution at 37 °C for 30 min after which the reaction was terminated by adding ice-cold mixture of 0.25 N HCl containing 0.3 % thiobarbituric acid (TBA), 15 % trichloroacetic acid (TCA) and 0.05 % butylated hydroxyl toluene (BHT) and was heated at 90 °C for 0.5 h. Then, the mixtures were cooled and centrifuged at 7 000 g for 5 min, and the absorbance of the supernatant was measured spectrophotometrically at $\lambda = 535$ nm.[29]

Glutathione level

The level of glutathione in primary rat hepatocytes was estimated via the Poet et al. method.[30]

The method is based on the addition of DTNB reagent to the cell lysate. The reagent is consisted of 12 mM NADPH, 50 U/ml GSH reductase 0.1 mM DTNB, in 0.1 mM sodium phosphate buffer with 1 mM EDTA, pH 7.5. A calibration curve was depicted according to the concentrations of reduced glutathione and results were obtained using an ELISA microplate reader at $\lambda = 415$ nm.

Mitochondrial membrane potential

Hepatocytes were incubated with a culture medium containing 1.6 µM of rhodamine 123 at 37 °C after the end of treatment period. After a 20 min incubation in the dark, the cell plate was washed and sonicated. Finally, the absorbance was read via a spectrofluorometer at 490nm excitation and 520 nm emission wavelengths. The results

were sated as nmol/mg protein.[30]

Results

In order to investigate the occurrence of cell growth inhibition after INH incubation, MTT assay was performed (Figure 1). Increasing concentrations of INH was added to incubation medium, and cellular viability was examined at 12, 24, and 48 hours' time points (Figure 1). As illustrated in Figure 1, INH significantly plummeted hepatocyte growth in a dose and time-dependent manner (Figure 1). The results also indicated that INH (1.2 mM) is capable of induction of cell death in 50% of hepatocytes after 24 hours (Figure 1).

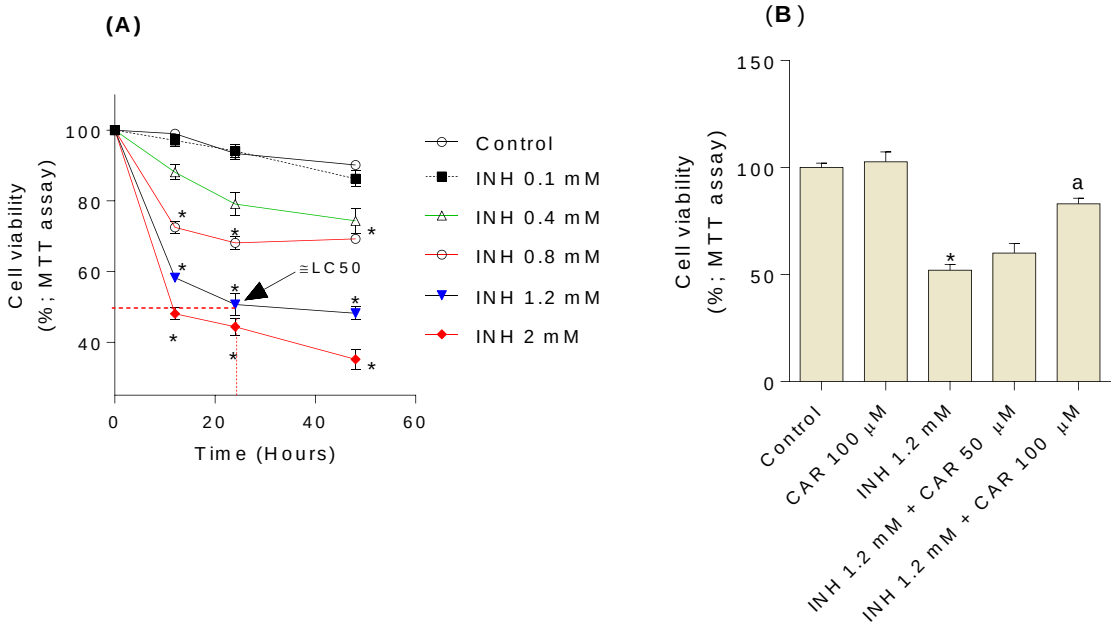

Figure 1. Effect of the isoniazid (INH) on the viability of rat hepatocytes (A). Effect of carnosine (CAR) on the viability of INH-treated hepatocytes (B). Isolated rat hepatocytes were incubated for 4 h with DMEM containing different concentrations of INH. CAR was added 30min before INH. The viability was determined by MTT reduction assay and expressed as a percentage of control. Data are expressed as Mean ± SD of 3 independent experiments.
*Indicates significantly different as compared with control group ($P < 0.05$).
[a] Indicates significantly different as compared with INH–treated hepatocytes ($P < 0.05$).

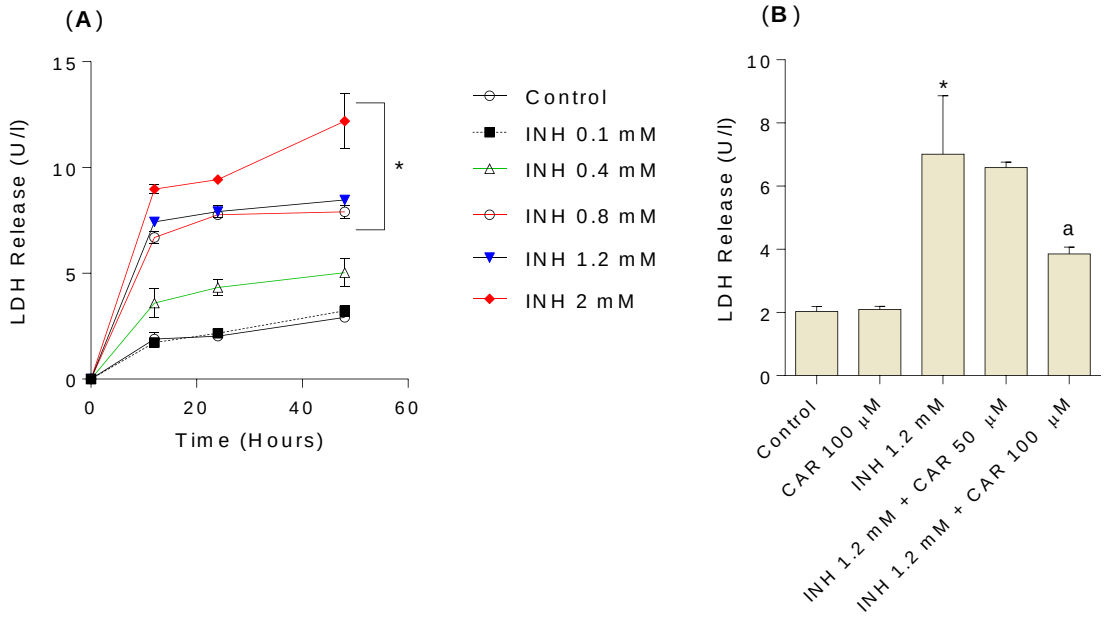

Figure 2. Effect of the isoniazid (INH) on LDH leakage of rat hepatocytes (A). Effect of carnosin (CAR) on the viability of INH-treated hepatocytes (B). Isolated rat hepatocytes were incubated for 4 h with DMEM containing different concentrations of INH. CAR was added 30 min before INH. Data are given as Mean ± SD of 3 independent experiments.
*Indicates significantly different as compared with control group ($P < 0.05$).
[a] Indicates significantly different as compared with INH–treated hepatocytes ($P < 0.05$).

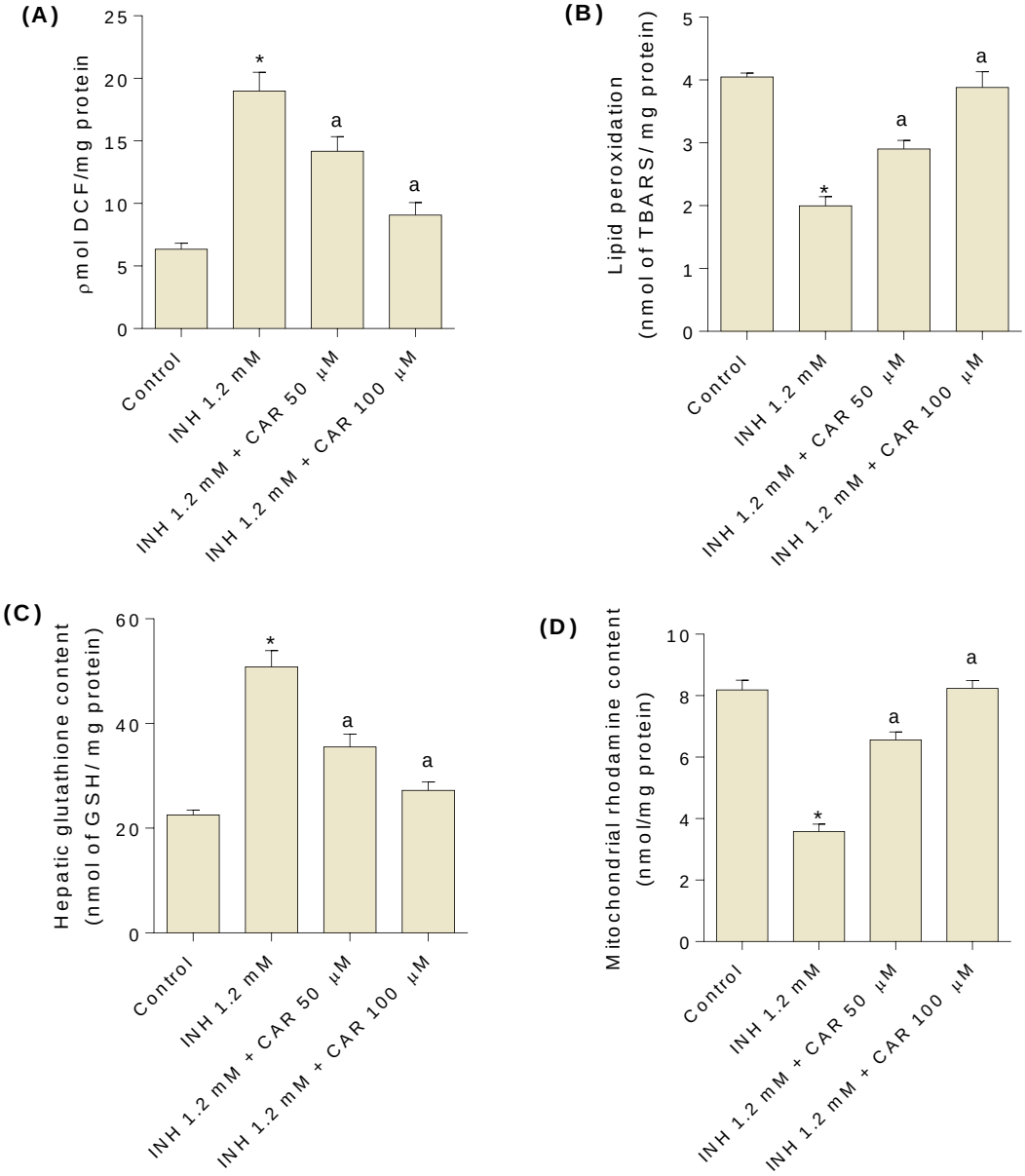

Figure 3. Markers of oxidative stress and mitochondrial function in isoniazid (INH)-exposed primary cultured rat hepatocytes. Data are represented as Mean ± SD of 3 independent experiments.
*Indicates significantly different as compared with control group ($P < 0.05$).
[a] Indicates significantly different as compared with INH–treated hepatocytes ($P < 0.05$).

The integrity of plasma membrane is a vital marker of efficient cellular function. The release of LDH is an indicator of membrane damage which is followed by cell death (Figure 2). In the current study, it was found that the treatment of primary hepatocytes with increasing concentrations of INH led to the release of LDH confirming the role of the drug in the induction of cytotoxicity (Figure 2A). On the other hand, it was found that CAR (100µM) supplementation effectively decreased LDH release in INH-incubated cells (Figure 2B).

It was found that INH (1.2 mM) treatment significantly increased cellular ROS level (Figure 3A). However, the addition of CAR at concentrations of 50 and 100 µM significantly decreased INH-induced ROS formation and oxidative stress in primary cultured rat hepatocytes (Figure 3A).

Peroxidation of membrane lipids is usually a consequent event of oxidative stress occurrence regardless of its reason. As depicted in figure 3, INH (1.2 mM) significantly enhanced lipid peroxidation after 24 hours of incubation. CAR at concentrations used could efficiently reverse this effect (Figure 3 B).

Exhaustion of cellular glutathione, which is a prominent endogenous antioxidant, is another indicator of oxidative hazard. The level of glutathione was measured to evaluate the role of INH in the progression of oxidative damage in hepatocytes. As shown in figure 3C, INH (1.2mM) altered cellular glutathione level in a downward trend while CAR significantly replenished hepatocyte glutathione pools in

INH-treated hepatocytes.

The function of mitochondria as the most crucial subcellular target of ROS was also examined through the measurement of MMP. It was found that INH treatment significantly depolarized hepatocyte mitochondria (Figure 3D). On the other hand, CAR at concentrations of 50 and 100 µM enhanced mitochondrial function via the increment of MMP (Figure 3 D).

Discussion

Xenobiotics undergo extensive metabolism in the liver, resulting in the generation of different metabolites which might be either less or more toxic. On the other hand, individuals always are prone to liver damages since it is the primary site of xenobiotics exposure. Thus, introducing novel protective agents in drug-induce toxicity has been an important agenda to many researchers.[31]

Converging evidence has shown that INH induces hepatotoxicity within the production of active metabolites which bind covalently to cellular biomacromulcules resulting in necrosis and steatosis as a clinical manifestation of toxicity.[32] The INH metabolite, hydrazine, causes diverse liver damages such as hepatocyte necrosis, vacuolation, and inflammation in vivo.[33] Moreover, our research team reported INH-dependent ROS formation, lipid peroxidation and mitochondrial depolarization in isolated rat hepatocytes in vitro.13 Indeed, reduction of cellular antioxidant activity, as well as glutathione depletion, suggests the crucial role of oxidative stress in INH-induced toxicity.[34]

Disruption of endogenous metabolism in the subcellular section which is mainly regulated through mitochondrial function is proposed to be also involved in INH-mediated cytotoxicity.[32] INH has been reported to interfere with mitochondrial biogenesis and dynamics which activates signaling pathways leading to eventual apoptosis. Hence, application of novel chemicals which can counteract these toxicities gain worth in the field of drug toxicology.

CAR is a non-enzymatic free radical scavenger possessing protective effects in chemical-induced toxicities. Besides, the broad spectrum function of CAR has made it a valuable therapeutic agent in various pathologies. For instance, its combination with zinc has successfully been used in gut repair.[35] Also, CAR administration has been suggested in vivo to prohibit the growth of malignant cells while the underlying mechanism remains unclear.36 However, the most prominent mechanism of CAR protection is related to its antioxidant, free radical scavenging and metal chelating properties which will hamper the alterations of cellular biomacromolecules such as proteins and lipids promoted by metal ions to produce reactive radicals. The effect of CAR reduction of oxidative stress can occur in direct and indirect pathways. In addition to directly scavenging ROS, CAR has been shown to counter the deleterious effects of carbonyl reductases like 4-HNE.[37] This has been attributed to the chemical structure of CAR which provides the possible target site for the direct reaction of toxic compounds.[38] Despite the abundance of researches indicating the hepatoprotective effects of CAR, there is no study about its role in INH-induced liver damage. In the current investigation, we found that CAR efficiently plunged INH- induced cell death, oxidative stress and its consequences in rat primary hepatocytes. According to the previous studies, CAR has potential effects on antioxidant detoxification and mitochondrial protection of xenobiotic/drugs which was also confirmed in our experiment. In a study conducted by Artun et al, CAR could significantly palliate liver function enzyme levels, the hepatic malondialdehyde rise in ethanol induced-hepatotoxicity.[39] CAR had beneficial effects in restoring cellular glutathione pools which is again in line with our study and can give us a clue for evaluating the antioxidant impact of CAR.

Conclusion

The data obtained from the current investigation represent CAR as a potential hepatoprotective agent with clinical application against INH hepatotoxicity. On the other hand, antioxidant activity and mitochondria protecting properties are fundamental mechanisms of cytoprotection provided by CAR. The effect of this peptide against oxidative stress and mitochondria-mediated complications could be the subject of further research.

Acknowledgments

The authors would like to thank Drug Applied Research Center and Maragheh University of Medical Sciences, Maragheh, Iran, for moral support.

Conflict of interests

The authors claim that there is no conflict of interest.

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