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Low doses of ethanol decrease the activity of the angiotensin-converting enzyme in the aorta of aging rats and rats treated with a nitric oxide synthase inhibitor and dexamethasone

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ABSTRACT

In the present study, the activity of ACE (angiotensin-converting enzyme) in the aorta of senescent rats and rats treated with the NOS (NO synthase) inhibitor L-NAME (N^G-nitro-L-arginine methyl ester) or dexamethasone and the effect of low doses of ethanol (0.2–1.2 g/kg of body weight, daily for 8–12 days) on this activity were studied. We found that ACE activity increased with age and in response to L-NAME and dexamethasone treatment. Ethanol at a dose of 0.4 g/kg of body weight per day decreased ACE activity in the aorta of aged rats and of rats treated with L-NAME or dexamethasone to the level of activity in young control rats. The optimal ethanol dose (the dose inducing a maximum decrease in ACE activity) increased with increasing doses of dexamethasone: 0.4 g/kg of body weight per day at 30 μg of dexamethasone/kg of body weight and 0.8 g/kg of body weight per day at 100 μg of dexamethasone/kg of body weight. It was also found that optimal doses of ethanol increased the number of cells in the thymus of rats treated with dexamethasone. The optimal dose of ethanol of 0.4 g/kg of body weight per day, which induced a maximum decrease in ACE activity in rat aorta, corresponded to a dose of 30 g of ethanol/day, which, according to epidemiological data, produces a maximum decrease in the incidence of cardiovascular disease in humans. In conclusion, the decrease in ACE activity in vessels may be one of the main mechanisms of the beneficial effects of low doses of ethanol on human health.

INTRODUCTION

At present there is substantial epidemiological evidence indicating that the consumption of low doses of alcohol decreases the risk of CVDs (cardiovascular diseases) and mortality from CVDs [1–9]. Investigations into these effects, which were conducted in different countries on large groups of people (from a few tens of thousands to hundreds of thousands), have revealed a U-shaped dependence of the incidence of CVDs on the amount of alcohol consumed: the incidence of CVDs is increased for non-drinkers and heavy drinkers, whereas the incidence decreases by 20–45 % in people consuming low doses of alcohol (approximately 30 g of ethanol/day). It is known that most CVDs are the result of atherosclerotic changes in vessels. A significant role in vessel atherosclerosis is

Key words: age, alcohol, angiotensin-converting enzyme, glucocorticoid hormone, N^G-nitro-L-arginine methyl ester (L-NAME), thymus.

Abbreviations: ACE, angiotensin-converting enzyme; AngII, angiotensin II; AT_1, angiotensin type 1; CVD, cardiovascular disease; eNOS, endothelial NOS; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; L-NAME, N^G-nitro-L-arginine methyl ester; NOS, NO synthase; PKC, protein kinase C.

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played by AngII (angiotensin II), a product of ACE (angiotensin-converting enzyme). AngII elevates blood pressure, causes cardiac hypertrophy [10], increases vessel wall thickness [11] and activates NADPH oxidase, which enhances the formation of ROS (reactive oxygen species), thereby provoking inflammation and fibrosis in vessels [12–14]. AngII also stimulates cell division and the expression of MCP (monocyte chemoattractant protein) [15]. In a review devoted to the effects of AngII on the cardiovascular system [16], the authors came to the conclusion that AngII acting through the AT_1 (AngII type 1) receptor induces phenotypic changes and growth of cells, regulates the expression of genes of various peptides and proteins (including vasoactive hormones, components of the extracellular matrix and cytokines) and activates different intracellular signalling cascades in cardiomyocytes and fibroblasts, and endothelial and muscle cells. These effects of AngII lead to atherosclerosis of vessels and cardiac insufficiency. The important role of AngII in vessel atherosclerosis gives the impetus for studying the effect of ethanol on the activity of ACE as a possible mechanism underlying the influence of moderate alcohol consumption on CVDs. There is evidence indicating that long-term consumption of high doses of ethanol (above 4 g/kg of body weight) increases the concentration of AngII [17] and ACE activity in rat serum [18], and ACE activity and expression of AT_1 and AT_2 (AngII type 2) receptors in rat lungs [19]. However, there are no results on the effect of low doses of ethanol on ACE activity. Because the risk of CVDs strongly depends on the concentration of AngII and hence the activity of ACE, which produces it, it can be assumed that a decrease in the risk of CVDs may be due to the inhibition of ACE activity during the consumption of low doses of ethanol. To answer this question, we studied the effect of low doses of ethanol on ACE activity in the aorta of aging rats and following the administration of the NOS (NO synthase) inhibitor L-NAME (N^G-nitro-L-arginine methyl ester) and the glucocorticoid hormone dexamethasone. It is known that atherosclerosis of vessels develops in the rat with aging [20] and following treatment with the NOS inhibitor L-NAME [21,22]. The vascular pathology in these cases is determined by an increase in ACE activity in vessels, since the increase in activity precedes the development of the pathology and ACE inhibitors prevent it [20–22]. Glucocorticoid hormones increase the expression of ACE mRNA in muscle [23] and endothelial cells of vessels [24], and ACE activity in the lungs [25]. The long-term intake of glucocorticoids leads to hypertension [26] and an increase in the risk of CVDs [27,28]. It is well known that the immune system and its key organ the thymus are very sensitive to the action of glucocorticoids [29], with high levels of glucocorticoids causing thymus involution and suppressing immunity. Therefore we also studied the effect of low doses of ethanol on thymus involution caused by dexamethasone.

MATERIALS AND METHODS

Animals and aorta preparation

Male Wistar rats weighing 320–490 g at 10 and 32 weeks of age (Animal Collection of the Institute of Theoretical and Experimental Biophysics, Pushchino, Russia) were used. The 10- and 32-week-old rats weighed 330 ± 10 g and 480 ± 10 g respectively. Rats ($n = 139$; $n = 124$, 10-week-old rats and $n = 15$, 32-week-old rats) were maintained in the animal facility with free access to water and standard rat chow (control group). The local ethics committee criteria for care and use of laboratory animals were carefully observed. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animals in the L-NAME experimental groups were given the NOS inhibitor L-NAME (1 mg/ml) and/or ethanol (0.2–2 %) dissolved in the drinking water for 8–12 days. In another experimental group, dexamethasone with a daily dose of 10–100 μg/kg of body weight was injected into the peritoneal cavity for 8 days. When the effect of ethanol on the dexamethasone-treated rats was studied, the rats were given a 0.4–1.2 % ethanol solution to drink. Routine monitoring showed that rats consumed approximately 100 ml of drinking water/kg of body weight each day irrespective of whether or not L-NAME or ethanol were contained in water, and the drinking regime did not change throughout the treatment protocols. Daily water consumption for the experimental groups is shown in Table 1. For the other experimental groups, daily water consumption was the same. At this rate of water drinking, each rat received daily 100 mg of L-NAME and/or 0.2–2 g of ethanol/kg of body weight.

The aorta was prepared as described by Korystov et al. [30] as follows. At the end of the treatment protocols, animals were anaesthetized with diethyl ether and the thorax was dissected before injection of heparin (500 units) into the heart to prevent blood clotting. The procedure from the beginning of the operation to the removal of the aorta took less than 3 min. Rats were under anaesthesia during this period and died shortly after the injection of heparin. Adventitial fat adherent to the aorta was cleaned *in situ*. Then the aorta was removed, rinsed with ice-cold (4 °C) 10 mM Hanks/Hepes solution (pH 7.4) and placed in the same solution. Residuary fat was carefully cleaned; care was taken not to damage the endothelium. The aorta was cut into eight 4–5-mm-thick sections beginning with the point at which the aorta was parallel with the vertebral column. Aortic sections were numbered from 1 to 8 starting with the section adjacent to the aortic arch. Sections 1–5 were in the thoracic aorta, and sections 7 and 8 were in the abdominal aorta in the rats of different ages. Section 6 was in the thoracic aorta in 32-week-old rats and in the abdominal aorta in 10-week-old rats. An aortic section 4–5-mm long (average,

Table 1 Daily water intake for the experimental groups

Values are means ± S.E.M. Group 1, 10-week-old rats; group 2, 32-week-old rats; group 3, 32-week-old rats + 0.4 % ethanol; group 4, 10-week-old rats + 0.4 % ethanol; group 5, 10-week-old rats + 2 % ethanol; group 6, 10-week-old rats + L-NAME; group 7, 10-week-old rats + dexamethasone (30 μg/kg of body weight); group 8, 10-week-old rats + dexamethasone, (100 μg/kg of body weight); group 9, 10-week-old rats + dexamethasone (100 μg/kg of body weight) + 0.8 % ethanol; group 10, 10-week-old rats + L-NAME + 2 % ethanol.

Group	Daily water intake (ml/kg of body weight)
1	98 ± 7
2	94 ± 8
3	102 ± 5
4	108 ± 3
5	97 ± 8
6	96 ± 8
7	95 ± 7
8	104 ± 8
9	112 ± 9
10	99 ± 5

4.4 mm) had a wet weight from 2.8 mg for 10-week-old rats to 4.1 mg for 32-week-old rats. Aortic sections were cut lengthwise, turned inside out with the endothelium on the outside and attached to the tip of a plastic pipette. The linear dimensions of an aortic segment were determined using a slide gage to an accuracy of 0.1 mm.

Measurement of ACE activity in the aorta

ACE activity was determined by measuring the hydrolysis of Hip-His-Leu (hippuryl-L-histidyl-L-leucine) using the method described by Ackermann et al. [31], with a modification of the method by Miyamoto et al. [32]. Briefly, isolated rat aorta sections were placed in 450 μl of 10 mM Hanks/Hepes solution (pH 7.4) and incubated for 10 min at 37 °C with shaking (25 Hz, amplitude, 1 mm) for equilibration before the addition of the ACE substrate. The reaction was started by the addition of 10 mM Hip-His-Leu (50 μl). After 30 min of incubation at 37 °C, the reaction was stopped by the addition of 1 ml of 0.1 M NaOH. After stirring the reaction mixture, the aortic sections were taken out of the solution, and their dimensions and weight were determined. A 200-μl aliquot of the remaining solution was incubated with 50 μl of *o*-phthaldialdehyde (2 mg/ml in DMSO) for 30 min at 37 °C, and the reaction was stopped by the addition of 2 ml of 0.8 M HCl. The samples were centrifuged at 3000 ***g*** at 4 °C for 5 min, and fluorescence was measured using an MF44 PerkinElmer fluorimeter at excitation and emission wavelengths of 360 and 500 nm respectively. For determining ACE activity, a standard curve was generated using His-Leu. The ACE activity was expressed as pmol of Hip-His-Leu hydrolysed/min per mm^2 of the inner aorta surface (the endothelium surface). The mean ACE activity in the aorta was determined by averaging the ACE activities of all (eight) sections for each rat, and then these values were averaged for all of the rats used in the experiment.

Determination of the number of cells in the thymus

The thymus was taken from control or dexamethasone-treated rats before the removal of the aorta. The thymus was squeezed through nylon cloth in 3 ml of 10 mM Hanks/Hepes solution (pH 7.4). The concentration of viable (unstained) thymocytes was determined using a haemocytometer under a microscope after staining with a 0.04 % Trypan Blue solution.

Drugs

L-NAME, DMSO, Hanks solution, Hepes, Hip-His-Leu acetate salt, His-Leu, *o*-phthaldialdehyde and Trypan Blue were obtained from Sigma. Heparin and dexamethasone were pharmaceutical preparations. Rectified ethanol was used in the experiments.

Statistical analysis

Results are means ± S.E.M., with the number of rats used in the experiments given in the Figure legends or in the text. The significant differences in multiple comparisons were determined using ANOVA and post-hoc Tukey tests. P values <0.05 were considered significant.

RESULTS

Effect of low doses of ethanol on ACE activity in the aorta of aging rats

The results shown in Figure 1 indicate that ACE activity in the aorta of 32-week-old rats was 1.5 times higher than in the aorta of 10-week-old rats ($P < 0.05$). The intake of 0.4 % ethanol by 32-week-old rats for 8 days lowered ACE activity to that in 10-week-old rats ($P < 0.05$).

Effect of low doses of ethanol on ACE activity in the aorta of control rats and rats treated with L-NAME

In control rats receiving ethanol, a tendency for aortic ACE activity to increase with increasing concentrations of ingested ethanol was observed (Figure 2). At an ethanol concentration of 2 %, ACE activity increased by 8 %; however, this increase was not significant. In rats receiving the NOS inhibitor, ACE activity increased with the increasing duration of treatment with L-LAME ($P < 0.05$; Figure 2, initial points on curves two and three). The effect of L-NAME decreased with an increasing concentration of ethanol in the drinking water and, at an ethanol concentration in the water of 0.4 %, reached the value observed in control rats ($P < 0.05$). As the ethanol

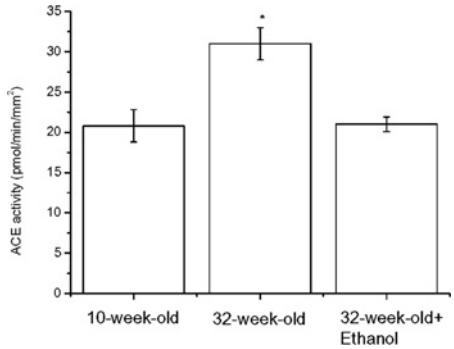

Figure 1 ACE activity in the aorta of 10- and 32-week-old rats, and 32-week-old rats receiving 0.4 % ethanol for 8 days

$^{*}P < 0.05$ compared with the ACE activity in the aorta of 10-week-old rats ($n = 6$–9 for each experimental point).

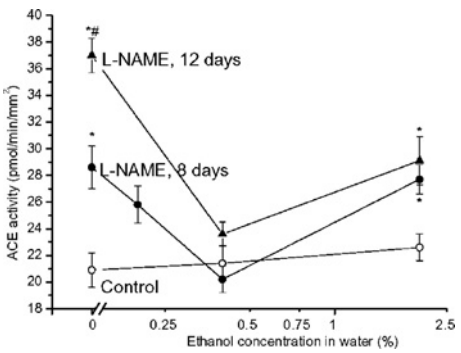

Figure 2 Dependence of ACE activity in the aorta of control rats and rats receiving L-NAME for 8 and 12 days on the concentration of ethanol consumed

Control rats consumed ethanol for 8 days. The age of rats at the beginning of the experiment was 10 weeks. $^{*}P < 0.05$ compared with the ACE activity in the aorta of control rats consuming the same dose of ethanol; $\#P < 0.05$ compared with the ACE activity in rats receiving L-NAME for 8 days ($n = 6$ for each experimental point).

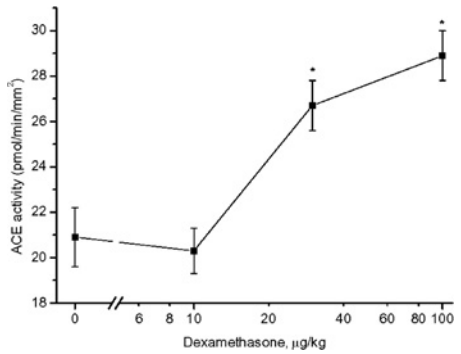

Figure 3 Effect of dexamethasone on ACE activity in the aorta of rats

Dexamethasone was injected for 8 days. The age of the rats at the beginning of the experiment was 10 weeks. $^{*}P < 0.05$ compared with ACE activity in the aorta of rats receiving no dexamethasone ($n = 6$–9 for each experimental point).

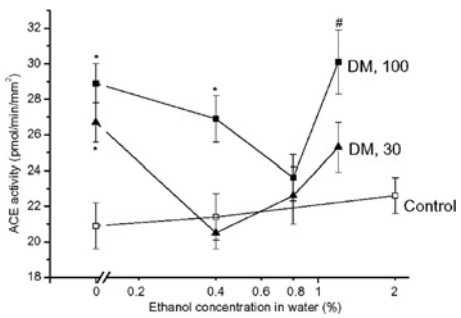

Figure 4 Dependence of ACE activity in the aorta of control rats and rats receiving a dexamethasone dose of 30 or 100 μg/kg of body weight for 8 days on the concentration of ethanol consumed

Control rats consumed ethanol for 8 days. The age of the rats at the beginning of the experiment was 10 weeks. $^{*}P < 0.05$ compared with the ACE activity in the aorta of control rats; $\#P < 0.05$ compared with the ACE activity in rats receiving a dexamethasone (DM) dose of 30 μg/kg of body weight.

concentration increased to 2 %, ACE activity increased above the control level.

Effect of low doses of ethanol on the thymus and ACE activity in the aorta of rats treated with dexamethasone

Figure 3 shows how ACE activity in the aorta of rats treated for 8 days with dexamethasone depended on the dose. At a dexamethasone dose of 10 μg/kg of body weight, ACE activity remained unchanged compared with rats not receiving dexamethasone and, at doses of 30 and 100 μg/kg of body weight, activity increased by 28 and 38 % respectively ($P < 0.05$). Figure 4 shows the effect of ethanol on ACE activity in the aorta of rats treated with different doses of dexamethasone. Ethanol decreased the increase in ACE activity induced by the treatment with dexamethasone. At a dexamethasone dose of 30 μg/kg of body weight, ACE activity decreased to the control level after the intake of 0.4 and 0.8 % ethanol ($P < 0.05$). At a dexamethasone dose of 100 μg/kg of body weight, 0.4 % ethanol had little affect on ACE activity, whereas, after the consumption of 0.8 % ethanol, the decrease in ACE activity was maximal ($P < 0.05$).

It is known that an increase in the concentration of glucocorticoids in the body induced by stress or their exogenous administration leads to the involution of the thymus and suppression of immunity. Figure 5 shows the dependence of the number of cells in the thymus on the dose of dexamethasone. Even at a dexamethasone dose of 10 μg/kg of body weight, thymus cellularity decreased by

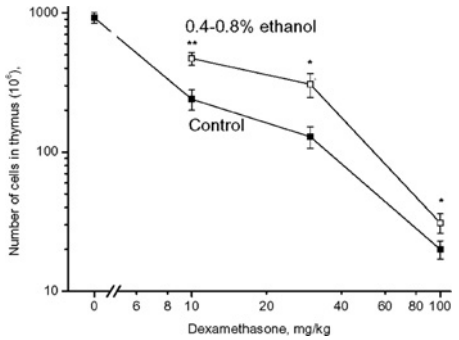

Figure 5 Dependence of the number of cells in the thymus on the dexamethasone dose in control rats or in rats consuming 0.4–0.8 % ethanol

Rats were treated with dexamethasone and ethanol for 8 days. Rats treated with a dexamethasone dose of 10 or 30 μg/kg of body weight were given 0.4 % ethanol to drink, and rats injected with a dexamethasone dose of 100 μg/kg of body weight were given 0.8 % ethanol to drink. The age of rats at the beginning of the experiment was 10 weeks. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the number of cells in the thymus of rats treated with the same dexamethasone dose ($n = 6$–9 for each experimental point).

more than 3 times and continued to decrease as the dose increased further. Ethanol at optimal concentrations for these dexamethasone doses (0.4 % for 10 and 30 μg/ml and 0.8 % for 100 μg/ml) increased thymus cellularity: 2–2.4 times at dexamethasone doses of 10–30 μg/kg of body weight and 1.6 times at a dose of 100 μg/kg of body weight ($P < 0.05$–0.01). The ethanol concentrations of 0.4 and 0.8 % in the drinking water did not affect thymus cellularity in control rats after 8 days of treatment. The number of cells in the thymus of rats consuming 0.4 and 0.8 % ethanol were 920 ± 50 ($n = 6$) and 950 ± 60 ($n = 6$) respectively, which did not differ from the values in the controls (Figure 5).

DISCUSSION

In the present study, we have investigated the effect of low doses of ethanol on ACE activity in the aorta of senescent rats and rats treated with a NOS inhibitor and dexamethasone. The major findings of the study were: (i) ethanol at a dose of 0.4 g/kg of body weight per day decreased the ACE activity induced by L-NAME or dexamethasone (30 μg/kg of body weight) treatment, or with aging to the level in control young rats; (ii) the optimal dose of ethanol increased with the dose of dexamethasone: 0.4 g/kg of body weight per day at a dexamethasone dose of 30 μg/kg of body weight and 0.8 g/kg of body weight per day at a dexamethasone dose of 100 μg/kg of body weight; and (iii) optimal doses of ethanol increased the cellularity in the thymus that had been reduced by treatment with dexamethasone. Of the doses tested in the present study, the optimal dose of ethanol that decreased ACE activity in the aorta of aging rats and of rats treated with a NOS inhibitor and dexamethasone (30 μg/kg of body weight) to that in young rats was 0.4 g/kg of body weight per day. According to epidemiological findings, the optimal dose of ethanol that decreases the risk of CVDs in humans is 30 g/day [1–9]. With the average weight of a human taken as approximately 70 and 80 kg (the average body weight of a U.S.A citizen in 1960–1962 and in 1999–2002) [33], this dose is 0.3–0.4 g/kg of body weight per day, which is close to the optimal dose in our experiments. The increase in the optimal dose of ethanol at a dexamethasone dose of 100 μg/kg of body weight indicates that, in a state of high stress which is characterized by an increased level of glucocorticoids, the optimal dose for humans may be higher than that specified by epidemiological findings.

An increase in ACE activity and in the concentration of its product AngII are one of the major causes of vascular atherosclerosis and most CVDs. Therefore the decrease in ACE activity by low doses of ethanol may be one of the reasons for the reduction in the risk of CVDs upon intake of low doses of ethanol. There is evidence indicating that an alternative mechanism underlying the effect of low doses of ethanol on CVDs may be due to a decrease in the concentration of LDLs (low-density lipoproteins) and an increase in the concentration of HDLs (high-density lipoproteins) [9,34,35]. Other effects of low and high ethanol doses on the cardiovascular system are discussed in detail by Lucas et al. [8]. In that review [8], the authors indicate that NOS activity is stimulated by PKC (protein kinase C) [36]. Given that activation of NOS is reported to inhibit ACE activity [37], it is possible that activation of the PKC pathway might contribute to the decrease in ACE activity observed following low ethanol consumption in the present study. There is 'cross-talk' between eNOS (endothelial NOS) and tissue ACE expression/activity by means of feedback regulation [37]. Therefore the increase in ACE activity observed during high dose ethanol consumption [17–19] may also be caused by a change in NOS activity, since daily consumption of high ethanol doses (4 g/kg of body weight) decreases eNOS activity in rats by up to 50 % [38].

The increase in the number of cells in the thymus upon the intake of low doses of ethanol, which was decreased by treatment with dexamethasone, indicates that low doses of ethanol may improve the immunity in stress, as the thymus is the main organ of immunity and the suppression of immunity in stress is brought about by glucocorticoids [39]. High ethanol doses decrease thymus size [40–42] and increase adrenal gland weight and plasma corticosterone levels [42]. These changes are distinctive indications of Selye stress syndrome [43]. Thus ethanol intoxication induces stress and thymus involution is caused by an increase in plasma corticosterone. Low

doses of ethanol, however, are not toxic and do not induce stress. Indeed, it has been shown that low and moderate doses of ethanol actually suppress stress [44]. Ethanol increases the level of endogenous opioids [45], and opioids block thymus involution [40]. Therefore an increase in endogenous opioids stimulated by low doses of ethanol may be the cause of the increase in the thymus in dexamethasone-treated rats after consumption of a low dose of ethanol.

The dose of ethanol which decreases ACE activity in rats and CVDs in humans is the same: approximately 0.4 g/kg of body weight per day. It is possible that this dose reduces the progression of atherosclerosis in rats and decreases vessel ACE activity in humans. However, this has not been investigated yet. In addition, it is necessary to study factors that may affect the optimal dose of ethanol, including gender, age, stress and different disorders. The individual variability of the dose of ethanol that decreases the risk of CVDs is important because it is similar to the threshold dose for liver damage [46] and therefore a decrease in CVD risk may be accompanied by an increased risk of cirrhosis.

AUTHOR CONTRIBUTION

Antonina Korystova, Ludmila Kublik and Maria Levitman performed the laboratory experiments with the rat aortas. Maksim Emel'yanov determined the ACE activity. Vera Shaposhnikova performed the experiments with the rat thymus. Yuri Korystov supervised the project, analysed the data and wrote the paper.

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