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Cathepsin B inhibition by Z-FA.FMK blocks TNF-α/D-GalN-induced oxidative damage through increasing antioxidant defence in the brain of mice

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Abstract

Cathepsin B is a cysteine lysosomal protease which takes place in many inflammatory diseases. Inflammatory processes within the brain represent a potential pathogenetic factor in neurodegenerative diseases and inhibiton of cathepsin B, which is an inflammation-related enzyme, can be a potential therapeutic utility in neuroinflammatory diseases. Thus, we researched the effect of benzyloxycarbonyl-phenilalanine-alanine fluoromethylketone (Z-FA.FMK), which is a pharmacological inhibitor of cathepsin B, on tumour necrosis factor-α (TNF-α) and D-galactosamine (D-GalN)-induced brain damage. Because oxidative damage accompanies inflammatory processes we aimed to research the alteration in some markers of oxidative damage and antioxidant defence system. For this investigation mice were treated with 700 mg/kg D-GalN and 15 μg/kg TNF-α one hour after administration with 8 mg/kg Z-FA.FMK. Treatment with Z-FA.FMK before TNF-α/D-GalN injection resulted in decreased lipid peroxidation levels, while catalase, superoxide dismutase, glutathione peroxidase, paraoxonase 1 activities and glutathione levels were increased in the brain tissue of mice. These results showed that cathepsin B inhibition by Z-FA.FMK could be a potential therapeutic utility in neuroinflammatory diseases because of its ability to block TNF-α/D-GalN-induced oxidative damage by increasing antioxidant defence in the brain of mice.

Keywords: Benzyloxycarbonyl-Phenyl-Alanine-fluoromethylketone (Z-FA.FMK), Cathepsin B, D-galactosamine, Oxidative damage, Tumour necrosis factor-α (TNF-α).
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Z-FA.FMK aracılığı ile katepsin B inhibisyonu fare beyninde TNF-α/D-GalN ile oluşturulan oksidatif hasarı antioksidan savunmayı artırarak engeller

Özet

Katepsin B birçok inflamatuvar hastalığın patolojisinde rol alan bir sistein proteazdır. Nörodejeneratif hastalıklarda beyindeki inflamatuvar süreçler potansiyel bir patojenik faktör olarak kabul edildiğinden, inflamasyon ile ilişkili bir enzim olan katepsin B'nin inhibisyonu nöroinflamatuvar hastalıklarda potansiyel bir tedavi yaklaşımı olabilir. Bu nedenle, katepsin B'nin farmakolojik bir inhibitörü olan olan benziloksikarbonil-fenilalanin-alanin-fluorometilketon (Z-FA.FMK)'un tümor nekroz faktor-α (TNF-α) ve D-galaktozamin (D-GalN) ile uyarılan beyin hasarı üzerine olan etkilerini araştırdık. Oksidatif hasar

inflamatuvar süreçlere katıldığından, bazı oksidatif hasar ve antioksidan savunma göstergelerindeki değişimleri araştırmayı amaçladık. Bu araştırma için farelere 8 mg/kg Z-FA.FMK uygulanmasından 1 saat sonra 700 mg/kg D-GalN ve 15 µg/kg TNF-α enjekte edildi. TNF-α/D-GalN enjeksiyonundan önce Z-FA.FMK uygulanması fare beyin dokularında lipid peroksidasyon seviylerinin azalmasına karşın katalaz, superoksit dismutaz, glutatyon peroksidaz ve paraoksonaz 1 aktiviteleri ve glutatyon seviyelerinde artış ile sonuçlandı. Bu bulgular, Z-FA.FMK aracılığıyla katepsin B inhibisyonunun, fare beyninde TNF-α/D-GalN ile uyarılan oksidatif hasarı antioksidan savunmayı artırarak engellemesinden dolayı, nöroinflamatuvar hastalıklarda bir tedavi yaklaşımı olabileceğini göstermektedir.

Anahtar Kelimeler: Benziloksikarbonil-fenilalanin-alanin-fluorometilketon (Z-FA.FMK), Katepsin B, D-galaktozamin, Oksidatif hasar, Tümor nekroz faktör-α (TNF-α).

Introduction

Inflammatory processes are important factors in neurodegenerative conditions and result primarily from the activated glial cells such as astrocytes and microglia in the brain. Controlling neuroinflammation has been considered a promising approach to treat neurodegenerative disorders. The determination of inflammatory components of disorders might be key step for attenuating inflammation therapeutically (Nathan 2002).

Cathepsins are lysosomal proteolytic enzymes and they digest unnecessary intracellular or endocyted proteins (Bohley and Seglen 1992). Molecular biological investigations revealed that these enzymes could function outside lysosomes and they can degrade extracellular matrix proteins (Buck et al. 1992). Cathepsins play important roles in several biological and pathological events such as inflammatory diseases (Chapman et al. 1997). For instance, cathepsin B is markedly upregulated in activated microglia that accumulate in pathological sites of the brain. It is thought that cathepsin B plays an essential role in inflammatory response in the brain initiated by activated microglia, because it is implicated in peripheral inflammatory diseases (Cardozo et al. 1992; Hashimoto et al. 2001). Therefore, an inhibitor of cathepsin B can be a potential therapeutic utility in these diseases. Z-FA.FMK is a potent inhibitor of cathepsin B that acts through binding to the active site of the enzyme (Lawrence et al. 2006). To the best of our knowledge, there is no study researching the effects of Z-FA.FMK on brain tissue.

Tanaka et al. (2006) suggested that activated microglia released inflammatory cytokines such as tumor necrosis factor-α (TNF-α) that plays a key role in the pathogenesis of a diverse range of neurological diseases (Feuerstein et al. 1997). Microglia are the main cellular source of TNF-α in the brain (Dziewulska and Mossakowski 2003). D-GalN is a hepatotoxin that induces liver damage *in vivo* by depletion of nucleotides with subsequent inhibition of protein and ribonucleic acid (RNA) synthesis (Leist et al. 1994). Recently, it has been reported that TNF-α affected blood-brain barrier permeability in acute liver failure induced by TNF-α and D-GalN injection to experimental animals (Lv et al. 2010).

TNF-α produces oxygen and nitrogen centered free radicals that are the important factors in development of neurodegeneration. In response to TNF-α, several reactive oxygen species (ROS) are generated, establishing a status of oxidative stress (Sugino et al. 1987). Oxidative stress may induce an alteration in the antioxidant systems including antioxidant enzymes and endogenous antioxidants such as glutathione (GSH) (Halliwell 2006). GSH detoxifies intracellular hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) *via* subsequent oxidation to glutathione disulfide (GSSG) by glutathione peroxidase (GPx). It is also capable

of conjugation to α, β-unsaturated aldehydes, which are products of lipid peroxidation by the cytosolic enzyme glutathione-S-transferase (GST) (Halliwell 1994). Alteration of GSH level in the brain contributes to the oxidative stress-mediated neuronal dysfunction (Jomova et al. 2010).

The defence against oxidative damage is undertaken by three major antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Moreno et al. 2005). SOD protects cells from the toxic effects of the endogenously generated superoxide radicals (Chrobot et al. 2000). GPx and CAT are essential in the decomposition of intracellular H_2O_2. They promote the breakdown of H_2O_2 into water and oxygen (Halliwell 1994). In addition paraoxanase 1 (PON1) participates in to detoxification of organophosphates and lipid-soluble radicals through binding to high-density lipoprotein and takes its place indirectly in the antioxidant defence system (Rajaraman et al. 2008).

Due to the fact that inhibition of cathepsin B can be a potential therapeutic utility in inflammatory diseases of the brain, we examined the effects of Z-FA.FMK, on oxidative damage and antioxidant system in the brain of TNF-α/D- GalN-injected mice used in this study.

Materials and methods

Animals

Male BALB/c mice (2.5–3-months-old, weighing 25 g) were obtained from the Istanbul University Experimental Medical Research and Application Institute (DETAE). All experiments were carried out in accordance with the guidelines of the Animal Care and Use Committee of DETAE. The mice were maintained at a constant temperature of 22±1˚C with 12-h light/dark cycles and fed a standard pellet chow, *ad libitum*. The animals were fasted for 15 h prior to treatments, but they were allowed free access to water.

Experimental design

The animals were divided into four experimental groups as follows: Group 1 (n = 10) – Animals receiving intravenous (iv) injections of physiological saline; Group 2 (n = 6) – Animals administered with 8 mg/kg Z-FA.FMK in 10% dimethyl sulphoxide by iv injection; Group 3 (n = 10) – Mice treated with 700 mg/kg D-GalN and 15 µg/kg TNF-α by sequential intraperitoneal (ip) injections; Group 4 (n = 7) – Animals treated with 700 mg/kg D-GalN and 15 µg/kg TNF-α by sequential ip injection one hour after administration of 8 mg/kg Z-FA.FMK. Groups 1 and 3 were sacrificed after 4-h (to induce injury) while the mice in groups 2 and 4 were sacrificed after 5-h (one-hour for the effect of Z-FA.FMK and 4-h to induce injury). The brain tissue was immediately taken up for biochemical investigations. Recombinant TNF-α was obtained from Calbiochem (Darmstadt, Germany). D-GalN was purchased from Acros Organics (New Jersey, USA) and Z-FA.FMK were obteired from Sigma Chemical Co (Missouri, USA).

Biochemical analysis

For biochemical analysis, brain tissue was washed in saline and kept frozen until the day of the experiments. The tissue samples in cold saline (10% [w/v]) were homogenized in a glass apparatus. After centrifugation the supernatant fraction was removed for determinations. As the markers of oxidative stress and antioxidant activity levels of tissue lipid peroxidation (LPO) and GSH, as well as SOD, CAT, GPx and PON1 activities were assessed according to following methods, respectively: Ledwozyw et al. (1986), Beutler (1975), Mylroie et al. (1986), Aebi (1984), Paglia and Valentine (1967), Furlong et al (1988), respectively. Total protein levels were measured by the method of Lowry et al. (1951).

Statistical analysis

The results were evaluated by unpaired t-test and ANOVA using the NCSS statistical computer package and reported as means ± SD. Differences were considered to be significant when they were $p<0.05$.

Results

Brain LPO and GSH levels are presented in Table 1. The LPO levels in the brain significantly increased in the D-GalN/TNF-α group as compared to the other groups ($P_{ANOVA}=0.009$). Brain LPO level was found to be significantly increased in D-GalN/TNF-α treated animals as compared to the control group ($P_{t\text{-}test} = 0.036$). Pretreatment of Z-FA.FMK to D-GalN/TNF-α injected animals caused a significant decrease of LPO level ($P_{t\text{-}test}=0.0001$).

Table 1. Brain lipid peroxidation (LPO) and glutathione (GSH) levels of all groups.

Groups	LPO (nmol MDA/ mg protein)*	GSH (nmol GSH/ mg protein)*
Control	3.07 ± 1.94	27.92 ± 5.27
Control+Z-FA.FMK	2.10 ± 0.22	22.35 ± 9.88
D-GalN + TNF-α	6.84 ± 1.36[a]	16.00 ± 1.47[c]
D-GalN + TNF-α + Z-FA.FMK	1.93 ± 0.49[b]	29.02 ± 7.58[d]
P_{ANOVA}	0.009	0.058

*Mean ± SD / [a]$P_{t\text{-}test}$ = 0.036 versus control group / [b]$P_{t\text{-}test}$ = 0.0001 versus D-GalN+TNF-α group
[c]$P_{t\text{-}test}$ = 0.005 versus control group / [d]$P_{t\text{-}test}$ = 0.012 versus D-GalN +TNF-α group

The GSH levels in the brain were decreased in the D-GalN/TNF-α group as compared to the other groups ($P_{ANOVA}=0.058$). In addition, brain GSH levels decreased in D-GalN/TNF-α-administered mice compared to control animals ($P_{t\text{-}test}=0.005$). Pretreatment of Z-FA.FMK significantly increased the GSH level in D-GalN/TNF-α treated mice ($P_{t\text{-}test}= 0.012$), (Table 1).

Brain CAT and SOD activities are presented in Table 2. Brain CAT and SOD activities were significantly decreased in the D-GalN/TNF-α group as compared to the other groups. ($P_{ANOVA} = 0.046$, $P_{ANOVA} = 0.024$, respectively). The activities of CAT and SOD in the brain of D-GalN/TNF-α injected animals were significantly lower than the control ($P_{t\text{-}test} = 0.005$, $P_{t\text{-}test} = 0.007$, respectively). The activities of these enzymes were increased in mice pretreatreated with Z-FA.FMK before D-GalN/TNF-α injection ($P_{t\text{-}test} = 0.046$, $P_{t\text{-}test} = 0.015$, respectively) (Table 2).

Table 2. Brain catalase (CAT) and superoxide dismutase (SOD) activities of all groups.

Groups	CAT (U/mg protein)*	SOD (U/g protein)*
Control	3.50 ± 0.62	29.14 ± 16.43
Control+Z-FA.FMK	2.83 ± 0.79	41.87 ± 23.61
D-GalN + TNF-α	1.94 ± 1.06[a]	6.18 ± 2.85[c]
D-GalN + TNF-α + Z-FA.FMK	3.92 ± 2.20[b]	48.49 ± 37.15[d]
P_{ANOVA}	0.046	0.024

*Mean ± SD / [a]$P_{t\text{-}test}$ = 0.005 versus control group / [b]$P_{t\text{-}test}$ = 0.046 versus D-GalN+TNF- α group
[c]$P_{t\text{-}test}$ = 0.007 versus control group / [d]$P_{t\text{-}test}$ = 0.015 versus D-GalN+TNF- α group

Brain PON1 and GPx activities were significantly decreased in the D-GalN/TNF-α group as compared to the other groups (P_{ANOVA} = 0.001, P_{ANOVA} = 0.036, respectively). PON1 and GPx activities were found significantly decreased in the D-GalN/TNF-α group ($P_{t\text{-}test}$ = 0.006, $P_{t\text{-}test}$ = 0.002, respectively). Z-FA.FMK given to the D-GalN/TNF-α increased the PON1 and GPx activities in the brain ($P_{t\text{-}test}$ = 0.0001, $P_{t\text{-}test}$ = 0.023, respectively) (Table 3).

Table 3. Brain paraoxonase1 (PON1) and glutathione peroxidase (GP_x) activities of all groups.

Groups	PON1 (U/g protein)*	GPx (U/g protein)*
Control	21.70 ± 10.43	254.37 ± 60.66
Control+Z-FA.FMK	36.54 ± 9.15	202.74 ± 99.60
D-GalN + TNF-α	4.08 ± 1.23[a]	96.68 ± 37.51[c]
D-GalN + TNF-α + Z-FA.FMK	27.31 ± 4.36[b]	223.61 ± 90.79[d]
P_{ANOVA}	0.001	0.036

*Mean ± SD
[a]$P_{t\text{-}test}$ = 0.006 versus control group
[b]$P_{t\text{-}test}$ = 0.0001 versus D-GalN+TNF-α group
[c]$P_{t\text{-}test}$ = 0.002 versus control group
[d]$P_{t\text{-}test}$ = 0.023 versus D-GalN+TNF-α group

Discussion

The activation of inflammatory signalling is an important mechanism leading to increased ROS production in several cell types, including neurons (Andrews et al. 2005). The proinflammatory cytokine, TNF-α, is known as an important mediator of neuronal damage in several inflammatory neuronal diseases (Huang et al. 2005). Overproduction of ROS is an important event in TNF-α-induced neuronal damage mechanism (Trembovler et al. 1999). Because of the mitochondria are source of ROS, it has become the main issue in the studies which investigate oxidative damage mechanism (Negre-Salvayre et al. 1997). In addition, lysosomal permeabilization has been also characterized as a property of oxidative stress-induced damage (Zdolsek et al. 1993). Thus, generation of ROS can be a possible reason of lysosomal permeabilization. Many apoptotic stimuli such as TNF-α (Manna et al. 1998) induce lysosomal permeabilization and generation of intracellular oxidants during apoptosis. Furthermore, ROS participate to neuronal apoptosis related with lysosomal damage during aging (Bahr and Bendiske 2002). ROS-induced lysosomal permeabilization seems like to occur before mitochondrial dysfunction (Guicciardi et al. 2000). For instance, cathepsin B that is a well known lysosomal enzyme has been found to act on mitochondria and promote mitochondrial ROS generation, thus, there can be a feedback mechanism that leads to more lysosomal permeabilization (Zhao et al. 2003). Here, we tested for the first time the hypothesis that cathepsin B inhibiton by Z-FA.FMK can protect brain tissue from oxidative damage induced by TNF-α/D-GalN in mice.

The brain, with high oxygen demand, and relatively deficient in anti-oxidative defence, is the most susceptible organ to oxidative damage (Li et al. 2011). The major defence mechanisms which the brain uses to reduce free radical injury include antioxidant enzymes such as SOD, CAT, GPx, PON and non-enzymatic free radical scavenging systems such as GSH physiologically reduce ROS levels (Peng et al. 2009). Since the inhibition of ROS production is believed to be one of the most important mechanisms in neuronal protection against

inflammatory agents, we have evaluated the impact of cathepsin B inhibition by Z-FA.FMK on TNF-α/D-GalN-induced alteration in SOD, CAT, GPx, PON1 activities, GSH and LPO levels in the brain tissue of mice.

The extent of LPO depends on the balance between the production of oxidants and scavenging of them by antioxidants. Thiobarbituric acid reactive substances (TBARs) are major oxidation products of peroxidized polyunsaturated fatty acids and increased TBARs content is an important indicator of lipid peroxidation (El-Demerdash, 2011). In the present study, the increase in levels of lipid peroxides indicates increased free radical generation in TNF-α/D-GalN treated mice. The significant decrease in lipid peroxides level in the brain of Z-FA.FMK treated mice compared with TNF-α/D-GalN treated mice indicates attenuation of lipid peroxidation. There was a simultaneous significant decrease in reduced GSH level in TNF-α/D-GalN treated mice. GSH is an endogenous antioxidant which reacts with the free radicals and prevents the generation of hydroxyl radicals. During this defence process, GSH is converted to the oxidized form by GPx (Meister and Anderson 1983). The decreased level of reduced GSH in TNF-α/D-GalN-treated mice observed in the present study indicates that there was an increased generation of free radicals and that the reduced GSH depleted during the process of defence against oxidative stress. The increase in the GSH levels in Z-FA.FMK treated group is due to antioxidant properties of the Z-FA.FMK.

GPx provides protection to neurons against oxidative stress through GSH. It converts H_2O_2 to H_2O and O_2. The reducing electrons are provided by GSH as it converted to GSSG (Halliwell 1994). Thus, GPx is an important parameter for indicating oxidative damage and a decrease in the activity of it can be considered to be a defence against oxidative damage. The activity of GPx in the D-GalN/TNF-α treated group was significantly lower than the control group. The decreased activity of GPx in TNF-α/D-GalN̦ group was correlated to the decreased availability of its substrate GSH. On the other hand, the activity of GPx increased in TNF-α/D-GalN̦ treated mice by pretreatment of Z-FA.FMK.

CAT and SOD are the most important antioxidant enzymes that defence against the toxic effects of oxygen metabolism. SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, while CAT converts hydrogen peroxide into water (Halliwell 1994). In TNF-α/D-GalN treated group, we observed a reduction in the activities of CAT and SOD. Kono and Fridovic (1982) reported that superoxide radical ions were able to inhibit the activity of CAT. The reduction of SOD and CAT activities in the TNF-α/D-GalN -injected mice may be resulted in the increase in superoxide radical ions and the failure of antioxidant defence system to overcome the influx of ROS. On the other hand, the pretreatment with Z-FA.FMK to this group showed an increase in the activities of these enzymes.

PON1 was demonstrated to be a major factor in detoxification of organic phosphorus compounds (Rajaraman et al. 2008). Increased oxidative stress has been shown to reduce PON1 synthesis *in vivo* and *in vitro* (Navab et al. 1997). Our study showed that treatment with TNF-α/D-GalN̦ caused a decrease in PON1 activity. The decrease in PON1 activity is directly related to the degree of oxidative damage of brain. On the other hand, pretreatment of Z-FA.FMK to this group significantly increased the PON1 activity. Z.FA.FMK prevented D-GalN/TNF-α induced oxidative damage and normalized PON1 activity in brain tissue.

In conclusion, the observation that Z-FA.FMK pretreatment can block oxidative damage induced by TNF-α/D-GalN through increasing the antioxidant defence system supports the conclusion that cathepsin B is an important mediator of oxidative stress which takes place

in neuroinflammation processes. Thus, the inhibition of cathepsin B by Z-FA.FMK can be a potential therapeutic utility in inflammatory diseases of the brain.

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