Research Report

Gemfibrozil pretreatment proved protection against acute restraint stress-induced changes in the male rats' hippocampus

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\textbf{Abstract}

Stress predisposes the brain to various neuropathological disorders. Fibrates like gemfibrozil, commonly used for hyperlipidemia, have not yet been examined for their protective/deteriorative potential against restraint stress-induced disturbances. Pretreatment of rats with a range of gemfibrozil concentrations showed significant protection against stress consequences at 90 mg/kg of gemfibrozil, as it resulted in the highest level of antioxidant defense system potentiation among other doses. It also reduced plasma corticosterone compared with the stressed animals. Administration of gemfibrozil (90 mg/kg) before stress induction was able to significantly induce the protein levels of some protective factors including hemeoxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase-1 (NQO-1) in the antioxidant nuclear factor erythroid-derived 2-like 2 (Nrf-2) pathway, as well as mitochondrial pro-survival proteins, including peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1\textalpha) and nuclear respiratory factor 1 (NRF-1). In parallel, the level of cleaved caspase-3 and apoptosis-inducing factor (AIF), two proteins involved in apoptotic cell death, and the number of damaged neurons detected in hematoxylin-eosin (H&E) stained hippocampus sections were suppressed in

\textbf{Abbreviations:} AIF, apoptosis-inducing factor; CAT, catalase; DAB, diaminobenzidine; EAE, experimental autoimmune encephalomyelitis; GPx, glutathione peroxidase; GSH, glutathione; H&E, hematoxylin-eosin; HO-1, heme oxygenase-1; HPA, hypothalamic-pituitary-adrenal; IAPs, inhibitors of apoptosis; I/R, ischemia-reperfusion; MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; MS, multiple sclerosis; NBT, nitroblue tetrazolium; Nrf-2, nuclear factor erythroid-related factor 2; NRF, nuclear respiratory factor; (NQO)-1, NAD(P)H quinone oxidoreductase-1; PBS, phosphate-buffered saline; PGC-1\textalpha, peroxisome proliferator-activated receptor coactivator 1-alpha (PGC-1\textalpha); PPAR, peroxisome proliferator-activated receptor; PRC, PGC-1 related coactivator; ROS, reactive oxygen species; SOCS, suppression of cytokine signaling; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBI, traumatic brain injury; TFFM, mitochondrial transcription factor A; UCP2, uncoupling protein 2

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1. Introduction

Stress is an unavoidable part of our everyday life. Stress-mediated effects on brain function can be beneficial or detrimental, depending on the type of stressor, and the development stage of the organism (Dong and Csernansky, 2009). Different physical and psychosocial stresses are associated with various diseases such as mental disorders, anxiety and depression (Arborelius, Owens et al., 1999), as well as neurodegenerative diseases (Swaab et al., 2005; Jeong et al., 2006). To date, a growing body of evidence has implicated psychological stress as a potential contributing factor to increases in the formation of Aβ peptide, onset and the development of Alzheimer’s disease (Ray et al., 2011). Different areas of brain contribute to the regulation of the hypothalamic–pituitary–adrenal (HPA) axis, an important component of the stress response (Sugama, 2009). Chronic stress is shown to make the hippocampus vulnerable to aging, metabolic challenges, cognitive dysfunction and neurotoxicity (Mizoguchi et al., 1992; Conrad et al., 2004; McLaughlin et al., 2007).

In response to stress, different molecular pathways become activated, including ROS production and oxidative stress (Liu et al., 1996; Madrigal et al., 2001; Kim et al., 2005), inflammatory (Madrigal et al., 2002; García-Bueno et al., 2005; Sugama, 2009), as well as apoptotic cell death pathways (Sudo et al., 2001). These pathways are also considered as major components of the pathophysiology of neurodegenerative disorders, and in fact may predispose the nervous system to the subsequent development of neurodramages during the life when encountering stressor conditions (Emerit et al., 2004; Farooqui and Farooqui, 2011).

When cells are subjected to a variety of oxidative and/or environmental stresses, they typically respond by inducing a coordinated expression of genes encoding the set of phase II detoxifying enzymes, principally mediated by activation of the transcription factor, Nrf-2. Nrf-2 then controls the orchestrated expression of phase II enzymes and genes involved in oxidative defense, including glutathione (GSH). Nrf-2 also contributes in the stimulation of mitochondrial biogenesis pathway (Piantadosi et al., 2008; Greco and Fiskum, 2010; Tufekci et al., 2011), thereby enhancing other antioxidant defense systems, such as the antioxidant enzyme catalase (CAT) (Zhu et al., 2010). Dysfunction of both Nrf-2 and mitochondrial biogenesis molecular pathways including PGC-1α, NRF-1 and mitochondrial transcription factor A (TFAM) has been reported in various neuropathological conditions (Johnson et al., 2008; Rojo et al., 2008; Chen et al., 2009).

Fibrates are approved drugs for hyperlipidemia (Rubins et al., 1999). These drugs are considered as agonists of peroxisome proliferator-activated receptor (PPAR)-α, however, not all their effects are mediated via PPAR-α receptor-dependent mechanisms (Dasgupta et al., 2007; Jana et al., 2007). There are studies demonstrating that some of the fibrates such as fenofibrate, gemfibrozil, etc., exert antioxidant and anti-inflammatory effects in various contexts (Xu et al., 2006; Ghosh and Pahan, 2012; Jana and Pahan, 2012).

Gemfibrozil has been shown to decrease atherosclerosis in experimental diabetes in association with a reduction in oxidative stress and inflammation (Calkin et al., 2006). Benzafibrate, another fibrate, has shown protective effects against inflammatory component of colitis (Tanaka et al., 2001). Fenofibrate has reduced the number of inflammatory lesions in the heart in the experimental model of autoimmune myocarditis (Maruyama et al., 2002; Brunnmair et al., 2004). Fenofibrate has also proved neuroprotective effects against experimental models of stroke (Deplanque et al., 2003; Inoue et al., 2003) and traumatic brain injury (TBI) (Chen et al., 2007). There are also several studies indicating the potential of PPAR-α agonists, such as gemfibrozil and fenofibrate, to be used clinically for the treatment of immune-mediated inflammatory diseases, like MS and EAE (Dasgupta et al., 2007; Yang et al., 2008; Gocke et al., 2009). Although contrary to these studies, there are other reports pointing to the adverse and toxic effects of some fibrates. Individual fibrates such as ciprofibrate, gemfibrozil and fenofibrate have been shown to induce mitochondrial dysfunction via different molecular mechanisms, such as inhibition of complex I of the respiratory chain by fenofibrate (Brunnmair et al., 2004). Also, it is reported by Almad et al. (2011) that patients sustaining spinal cord injury while taking gemfibrozil might be prone to exacerbated tissue damage. In the study conducted by Guo et al. (2009) pretreatment of mice with higher doses of gemfibrozil resulted in brain swelling and exacerbation of ischemic insult in a middle cerebral artery occlusion (MCAO) model of cerebral I/R injury. In addition, we previously reported that while gemfibrozil pretreatment was neuroprotective in met-estrous female rats against global cerebral I/R injury, it led to neurotoxicity in the hippocampus of male rats.

Therefore, considering the importance of psychosocial stresses in the modern human lifestyles, and the association of stress with neurodegenerative diseases in one hand, and the limited and controversial neurological studies regarding fibrates, specifically gemfibrozil in neuronal contexts on the other hand, at the present study we decided to extend the existing knowledge about the effects of gemfibrozil pretreatment against restraint-induced stress in the hippocampus of male rats, mainly focusing on some important proteins of the molecular pathways such as antioxidant Nrf-2, mitochondrial biogenesis, inflammatory and apoptotic cell death in the male rat hippocampus, due to their established roles in determining the outcome of neuropathological conditions.
2. Results

2.1. The effects of gemfibrozil pretreatment on the rat hippocampus antioxidant defense system against restraint-induced stress

In order to assess the antioxidant defense system, activities of CAT, SOD, as well as GSH levels, as antioxidant factors on the one hand, and the level of MDA as the byproduct of lipid peroxidation, on the other hand, were measured. As shown in Fig. 1, in response to 6 h of acute restraint stress activities of CAT and SOD, as well as GSH level were reduced ($p < 0.01$; $p < 0.001$; $p < 0.001$, respectively), while MDA level was induced significantly in the stress group compared with the control. Trend of changes in antioxidant defense system factors, when different doses of gemfibrozil were used to pretreat animals against restraint stress, showed a reversed U-shaped curve representing the dose of 90 mg/kg of gemfibrozil as the best dose capable of potentiating antioxidant defense system to its highest level among other doses, proved by the significant induction of CAT and SOD activities ($p < 0.01$; $p < 0.001$, respectively), and GSH level ($p < 0.001$), as well as decline of MDA level ($p < 0.01$) in this group in comparison to the stress group. Therefore, dose of 90 mg/kg of gemfibrozil was selected to be used in animals for further analyses. “Gemfibrozil only” pretreatment at doses of 10, 30, 60 and 90 mg/kg did not show a significant difference compared with the control group over all assessments; however, dose of 120 mg/kg affected the antioxidant defense system factors negatively, proved by the significant drop of GSH level and SOD activity, as well as rise of MDA level.

2.2. Plasma corticosterone levels in experimental groups

The presence of gemfibrozil at the dose of 90 mg/kg resulted in a significant decline of plasma corticosterone level ($p < 0.05$), which was significantly increased in response to 6 h of restraint-induced stress compared with the control group ($p < 0.05$) (Table 1).

2.3. Histological evaluation

For histological analysis, we mostly focused on CA3 region of the hippocampus, as it is considered a region of high susceptibility to this kind of stress (Takuma et al., 2012). We detected degenerated and apoptotic cells in the CA3 region by H&E and TUNEL techniques, respectively. In Fig. 2A and C, H&E staining presented the percentage of damaged neurons in CA3 region which increased significantly in the stress condition compared with the control ($p < 0.001$). Moreover, TUNEL assessment confirmed involvement of apoptotic cell death. We assessed apoptosis in CA3 regions by TUNEL (Fig. 2B and D). Gemfibrozil pretreatment (90 mg/kg) was able to protect against the stress-induced neurodamage, as the percentage of apoptotic neurons were declined significantly ($p < 0.001$).

Table 1 – Plasma corticosterone levels in the experimental groups.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Plasma corticosterone level (ng/ml)</th>
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<tr>
<td>Control</td>
<td>210.11 ± 38.21</td>
</tr>
<tr>
<td>Stress</td>
<td>508.21 ± 43.16*</td>
</tr>
<tr>
<td>Gem90+stress</td>
<td>326.169 ± 28.221**</td>
</tr>
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N = 6/group; one-way ANOVA; *$p < 0.05$: compared with control group; ** $p < 0.05$: compared with stress group.
Fig. 2 – Representative hippocampus sections stained with H&E and TUNEL technique. (A) Hippocampus formation of male control, stress, gemfibrozil only and gemfibrozil-pretreated groups stained with H&E. Top panel shows the total area of hippocampus; bottom panel focuses on CA3 field with higher magnification (magnification, × 400). Damaged neurons are indicated by arrows. (B) Hippocampus formation of male control, stress, gemfibrozil only and gemfibrozil-pretreated groups stained with TUNEL technique. Top panel shows the total area of hippocampus; bottom panel focuses on CA3 field with higher magnification (magnification, × 400). Apoptotic neurons are indicated by arrows. (C) Quantitative analysis of damaged neurons which stained by H&E in the CA3 region of the hippocampus after 6 h of restraint-induced stress. (D) Quantitative analysis of damaged neurons which stained by TUNEL technique in the CA3 region of the hippocampus after 6 h of restraint-induced stress. Data are expressed as the percentage of damaged neurons to the total number of neurons (***p < 0.001; Mann–Whitney U test).
2.4. Protein levels of important mitochondrial pro-survival factors: PGC-1α and NRF-1

As shown in Fig. 3(A–D), the level of PGC-1α, known as a master regulator of mitochondrial biogenesis and a potent antioxidant, and NRF-1, another key player in the mitochondrial biogenesis signaling pathway, increased in response to stress compared with the control (p<0.05; non-significant, respectively). However, gemfibrozil pretreatment (gem90) led to a significant further induction of both factors in comparison to the stress group (p<0.01). It should be considered that the results regarding all the measured proteins in the “gemfibrozil 90 only” receiving animals did not show a statistically significant difference in comparison to the control.

2.5. Protein levels of Nrf-2 antioxidant response element signaling pathway

The proteins levels of nuclear Nrf-2, HO-1 and NQO-1 in the experimental groups of the present study are illustrated in Fig. 4. Induction of acute restraint stress for 6 h resulted in a significant incline of Nrf-2 and HO-1 compared with the control (p<0.01, respectively in Fig. 4A and B and C and D). NQO-1 followed the same pattern, although the change was not statistically significant (Fig. 4E and F). While the nuclear level of Nrf-2 detected to be equal within the control and gemfibrozil-pretreated animals, HO-1 and NQO-1 levels in the presence of gem90 were significantly higher than both control and stress groups, indicating the protective potential of gemfibrozil via induction of antioxidant factors in the context of this study.

Fig. 5 illustrates the results of time-dependent analysis of nuclear Nrf-2 level in the stressed and gemfibrozil-pretreated conditions. Hippocampus tissues were collected at 30 min, 1 h, 2 h, 4 h, and 6 h time-points following induction of acute restraint stress. Nuclear Nrf-2 reached its highest at 2 h following induction of stress, then dropped at following time-points reaching to control levels at 6 h. However, gemfibrozil pretreatment against the stress insult, stabilized nuclear Nrf-2 for a longer time; as the level of nuclear Nrf-2 at 4 h following stress induction was still high and almost equal to its level at 2 h, then dropped to control level at 6 h.

2.6. Protein levels of cell death factors; cleaved caspase-3 and AIF

The level of cleaved caspase-3, as a marker of caspase-dependent apoptotic cell death (Jänicke et al., 1998), and nuclear translocation of AIF, as a sign of activated caspase-independent apoptosis (Matsumori et al., 2005), were analyzed using western blotting. As depicted in Fig. 6A–D, while exposure to the stress resulted in a significant incline of cleaved caspase-3 and nuclear AIF levels compared with the control (p<0.01), pretreatment of animals with gem90 suppressed such increases to a considerable extent (p<0.01), against restraint-induced stress.

2.7. Protein levels of NF-κB p65 and TNF-α; important factors involved in inflammation

The expression of nuclear NF-κB p65 and TNF-α followed a similar pattern of induction in response to stress compared with the control group (Fig. 7A–D; p<0.05). Interestingly, their expressions were even further increased when animals were
pretreated with gem90 against the stress insult, in comparison with the stress group (p₀.01 and p₀.001), respectively for nuclear NF-κB p65, and TNF-α.

3. Discussion

Pretreatment of male rats with gemfibrozil (10, 30, 60, 90 and 120 mg/kg, p.o., once/day, 1 week) against acute restraint-induced stress showed a reverse U-shaped curve in its antioxidant function, assessed by the activities of CAT and SOD, as well as GSH and MDA levels. Such reversed U-shaped curve obtained here by different doses of gemfibrozil was consistent with the study of Guo et al. (2009) indicating a reversed U-shaped curve in the protective efficacies of fibrates in the brain. As the highest antioxidant activity proved to be at the dose of 90 mg/kg, it was selected as the most protective dose of gemfibrozil to be used for further analyses in this study. Plasma corticosterone level was increased in stressed rats, while gemfibrozil pretreatment reduced it significantly. It also induced the protein levels of PGC-1α and NRF-1; mitochondrial pro-survival factors in the mitochondrial biogenesis pathway, as well as HO-1 and NQO-1 in the antioxidant Nrf-2 pathway. The levels of two important proteins involved in apoptotic cell death, cleaved caspase-3 and nuclear AIF, increased in response to stress,

Fig. 4 – Western blot analysis to measure the expression of the major proteins of the antioxidant Nrf-2 pathway, nuclear Nrf-2, HO-1 and NQO-1 in the hippocampus tissues derived after 6 h of restraint-induced stress in experimental groups. (A) Immunoblot bands of nuclear Nrf-2 and lamin B2. (C) Immunoblot bands of HO-1 and β-actin. (E) Immunoblot bands of NQO-1 and β-actin. (B, D and F) The densities of corresponding bands were measured and the ratio to lamin B2 were calculated and represented as arbitrary units on the graphs for each experimental group (n=6). Bars indicate the mean ± SEM; one-way ANOVA; *p<0.05; **p<0.01; ***p<0.001.
but they declined in the presence of gemfibrozil. Consistently, morphologic analysis of hippocampus CA3 region using H&E and TUNEL staining showed that the number of damaged and apoptotic neurons increased in response to stress, while decreased in the presence of gemfibrozil. All these findings firmly indicated that gemfibrozil was protective against the acute restraint stress-induced disturbances, however, our results regarding inflammatory factors interestingly showed that gemfibrozil pretreatment resulted in an even further induction of NF-κB and TNF-α, compared with the stress group, in which gemfibrozil was absent. Previous studies in our laboratory, introduced the protective effects of gemfibrozil at dose of 30 mg/kg against oxidative stress in hippocampus of female rats while it proved neurodegeneration in hippocampus of male rats (Mohagheghi et al., 2012, 2013). Consequently we decided to develop our knowledge using the same dose in a different context, acute restraint stress.

PGC-1α is a master regulator of mitochondrial biogenesis, also possessing antioxidant activity (Chen et al., 2011). PGC-1α is activated in responses to damages resulting in mitochondrial dysfunction and ROS production to limit the damage by inducing mitochondrial biogenesis and suppressing the ROS production (Zhu et al., 2010). PGC-1α is reported to be required for the induction of many ROS-detoxifying proteins, including glutathione peroxidase (GPx), CAT, uncoupling protein 2 (UCP2) and SOD2 (St-Pierre et al., 2006). NRF-1 interacts with PGC-1α and PGC-1 related coactivator (PRC) to regulate target genes involved in the mitochondrial respiratory function, and mitochondrial biogenesis (Mattingly et al., 2008).

Gemfibrozil and some other fibrates have been shown to induce mitochondrial biogenesis involved factors in peripheral tissues such as liver and muscles (Nagai, 2002; Wenz et al., 2008), as well as in the hippocampus of met-estrous female rats undergoing cerebral I/R injury (Mohagheghi et al., 2013). Mitochondrial biogenesis as a pro-survival approach is integrally connected by redox control to the cellular defense against oxidative stress (Piantadosi et al., 2008).

Nrf-2 is a transcription factor which controls the orchestrated expression of phase II enzymes and genes involved in oxidative defense. Nrf-2 is normally kept inactive in the cytoplasm by its cytosolic inhibitor keap-1. In response to oxidative stress, it becomes activated and released from Keap-1, and then translocates to the nucleus, where through binding to promoters containing antioxidant response elements, it results in the transactivation of the respective genes for the key phase II detoxifying enzymes such as GSH, HO-1 and NQO-1, consequently leading to an enhanced antioxidant defense system containing SOD and CAT activities (Zhu et al., 2010). It is worth noting that the activation of Nrf-2 follows a time-dependent pattern. It is reported that nuclear import of Nrf-2, from time of exposure to stabilization, takes roughly 2 h, then followed by the activation of a delayed mechanism that controls switching off of Nrf-2 activation (Jain et al., 2005). According to the previous studies inflammatory factors are detectable at later time-points following induction of stress full damage. So as, our aim was detecting the neuroprotective/neurodegenerative potential mechanism of gemfibrozil through modulating antioxidant and inflammatory pathways (Madrigal et al., 2001). So, we selected 6 h for acute

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**Fig. 5 – Western blot analysis to measure the expression of nuclear Nrf-2 in the hippocampus tissues derived at 30 min, 1 h, 2 h, 4 h, and 6 h following induction of acute restraint stress in the presence and absence of gemfibrozil. (A) Immunoblot bands of nuclear Nrf-2 and lamin B2. (B) The densities of corresponding bands were measured and the ratio to lamin B2 was calculated and represented as arbitrary units on the graphs for each experimental group (n=6). Bars indicate the mean ± SEM; one-way ANOVA; **p<0.01; ***p<0.001; compared with control.
restraint stress. Our time-dependent analysis of nuclear Nrf-2 consistently confirmed previous reports. We also observed that nuclear Nrf-2 reached the peak at 2 h following stress induction, and then dropped to the control level at 6 h. In the presence of gemfibrozil, Nrf-2 was stabilized in the nucleus for a longer time; as its level at 4 h was still high and almost equal to 2 h, then declined to control level at 6 h following stress induction. Such stabilizing effect of Nrf-2 in the nucleus for a longer period of time by gemfibrozil, and at

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earlier time-points, may explain why despite induction of HO-1, NQO-1, as well as CAT, SOD and GSH in the gemfibrozil-pretreated group of the present study, nuclear Nrf-2 expression was detected to be less than its level in the stressed group. The nuclear translocation of Nrf-2 at earlier time-points has probably resulted in the enhancement of the subsequent antioxidant agents which were still detectable at the time of our experimentations. Nrf-2 and mitochondrial biogenesis pathways are reported to be interrelated (Piantadosi et al., 2008; Greco and Fiskum, 2010; Zhu et al., 2010; Tufekci et al., 2011). HO-1 has been shown to regulate cardiac mitochondria biogenesis via Nrf-2-mediated transcriptional control of NRF-1. The ensuing accumulation of nuclear NRF-1 protein has led to gene activation for mitochondrial biogenesis, which opposed apoptosis and necrosis caused by cardio-toxins like doxorubicin (Piantadosi et al., 2008). Although in the current study we did not assess mitochondrial biogenesis, increased levels of both HO-1 in the Nrf-2 pathway and NRF-1 in the mitochondrial biogenesis pathway in the hippocampus of gemfibrozil-pretreated rats were detected against the stress insult.

Consistently, we found that the level of cleaved caspase-3, the executive protease of caspase-dependent apoptosis (Jänicke et al., 1998), and nuclear translocation of AIF, which is an important factor involved in the caspase-independent apoptotic cell death (Matsumori, 2005), were increased in response to stress, while they were significantly suppressed in the presence of gemfibrozil.

A considerable amount of evidence has established the involvement of inflammation in the pathophysiology of various neurodegenerative disorders (Bettcher and Kramer, 2013; Rubio-Perez and Morillas-Ruiz, 2012). Induction of stress is associated with neuroinflammation (Madrigal et al., 2002; García-Bueno et al., 2005), leading to negative outcomes such as oxidative stress (Madrigal et al., 2003), worsening of experimental stroke (Caso et al., 2008), and the onset of inflammatory diseases (cardiovascular, neurological, digestive, or immunologic) (Baum and Posluszny, 1999). Similarly, in the hippocampus of stressed animals of our study, other than disturbances in antioxidant defense system, mitochondrial biogenesis involved proteins, Nrf-2 antioxidant pathway, as well as induction of cell death factors and corticosterone level we also detected the significant induction of proinflammatory factors such as NF-κB and TNF-α in response to acute restraint stress. Considering other changes in this group, such as potentiation of antioxidant defense system, mitochondrial pro-survival factors and antioxidant Nrf-2 signaling pathways, besides suppression of apoptotic cell death factors and corticosterone level, such induction of proinflammatory factors has not been deteriorative, at least at this time-point. Further time-point analyses are necessary to clarify whether induction of NF-κB and TNF-α would lead to negative results at delayed phases of inflammatory process.

It has been proved that the neuroinflammatory response, for example after an ischemic stroke, is not entirely detrimental. Many inflammatory components have neurotoxic as well as neuroprotective effects and they should be studied at different time-points (Ceulemans et al., 2010). Although findings reported are controversial, strong evidence supports the notion that NF-κB functions as an anti-apoptotic transcription factor in various cell populations including neurons. NF-κB activity is shown to be necessary for the anti-apoptotic activity of TGF-β1 (Glanzner et al., 2000; Mattson et al., 2000; Zhu et al., 2004). Such dual role has also been reported for TNF-α. Regarding the neurotoxic or neuoprotective effects of TNF-α, it is important to know about the specific brain regions, timing and threshold of TNF-α expression. TNF-α released in the striatum is considered to cause neurodegeneration, while its release in the hippocampus could promote neuroprotection (Mattson et al., 2000; Sriram and O’Callaghan, 2007). TNF-α in neutrophils and endothelial cell stimulate injury, whereas in neurons it is neuroprotective (Sharp et al., 2000).

TNF-α is one of the potent stimuli for the activation of NF-κB in injury settings (Kolesnick and Golde, 1994; Schreck and Baeuerle, 1994). NF-κB activation in neurons subjected to oxidative stress has resulted in negative and positive outcomes. It is believed that differential modulation of NF-κB activity by various stimuli may have a major influence on whether or not a neuron lives or dies in a particular pathophysiological condition (Mattson et al., 2000). While some studies support the proapoptotic and detrimental effects of NF-κB activity (Kasibhatla et al., 1998) in ischemic neuronal injury (Schneider et al., 1999; Nurmi et al., 2004) and Alzheimer’s disease (Yan et al., 1995; Grilli et al., 1996; Kalschmidt et al., 1997), there are other reports indicating the anti-apoptotic cell survival roles of TNF-α and NF-κB activity through various mechanisms such as inducing the production of anti-apoptotic proteins, Bcl-2, inhibitors of apoptosis (IAPs) and manganese superoxide dismutase (Mn-SOD) (Ivanov et al., 1995; Mattson et al., 1997; Mattson and Meffert, 2006). It is proposed that activation of NF-κB in neurons could promote their survival, whereas activation of NF-κB in glial cells may induce production of neurotoxin (Mattson and Meffert, 2006). In fact, according to the available data, it seems that the role of NF-κB is condition-specific and complex (Mattson et al., 2000).

Such complexity and condition-specific outcomes seem also to be true for gemfibrozil used in different neuropathological contexts. In the present study, our results such as reduction of plasma corticosterone level, suppression of cell death proteins, potentiating of antioxidant defense system including SOD, as well as induction of the pro-survival molecules involved in the mitochondrial biogenesis and Nrf-2 signaling pathways, along with the induction of NF-κB and TNF-α, in gemfibrozil-pretreated group, altogether point to the protective potential of gemfibrozil at the dose of 90 mg/kg against restraint-induced stress in the male rat hippocampus, at least at the time-point of our analyses. However, in our previous investigation, we observed that pretreatment of rats of both sexes with a different dose of gemfibrozil against global cerebral I/R insult led to a sexual-dimorphic effect; being neuroprotective in met-estrous females, while neurotoxic in males (Mohagheghi et al., 2012).

Ghosh and Pahan (2012) have suggested that gemfibrozil may find therapeutic application in neuroinflammatory and neurodegenerative disorders. They found that gemfibrozil was capable of suppressing inflammation in mouse astroglia and microglia via upregulation of the expression of suppression of
4. Experimental procedures

4.1. Animals

Male Wistar rats (275–300 g) were housed in standard cages under controlled temperature (22±2 °C), humidity and a 12 h light/dark cycle (light on 07:00–19:00), with food and water provided ad libitum. Rats acclimated to the facilities for 1 week. During this 1 week rats were gavaged with 1 ml saline to be adapted. After that, they were randomly assigned to the experimental groups, with control, stressed and gemfibrozil treated rats housed in separate chambers. Experimentation was approved by the Ethics Committee of Shahid Beheshti Medical University in accordance with the international guidelines for animal experiments. All efforts were made to minimize animal suffering and to reduce the number of animals used. No rat was eliminated during experiment due to sickness or death. Besides, we did not observed any changes in weight loss and/or gain of rats in the period of gavage.

4.2. Experimental groups

At the first step and in order to assess antioxidant defense system in the presence of different doses of gemfibrozil, animals were randomly divided into 12 experimental groups: control, stress, those received gemfibrozil pretreatment against stress, and those specified to receive “gemfibrozil only” treatment (n=6/group). Gemfibrozil (Sigma) was administered at doses of 10, 30, 60, 90 and 120 mg/kg p.o., once daily for 7 days, respectively, named as gem10, gem30, gem60, gem90 and gem120, through a feeding needle. One hour after the final dose, animals were subjected to 6 h of acute restraint stress. In stress group, animals were pretreated with vehicle (5 ml/kg of 0.5% carboxymethyl cellulose), then subjected to stress. After selection of the best protective dose, more animals were again randomly divided into four experimental groups: control, stress, gem90+stress, and “gemfibrozil (90 mg/kg) only” receiving group (n=6/group), and their hippocampus were used for western blot analysis. Additional animals were utilized to assess NF-κB, Nrf-2 and AIF proteins in the nuclear fraction of each experimental group (n=6). More animals were also specified for the experimental groups used in the time-dependent western blot analysis of nuclear Nrf-2 levels, as well as histologic evaluation (n=6/group).

4.3. Histological evaluation

4.3.1. H&E staining

After 6 h of acute restraint stress, animals in the experimental groups specified for histological assessments (n=6) were perfused transcardially with phosphate-buffered saline (PBS) (pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) under chloral hydrate anesthesia (400 mg/kg i.p.). The brains were then removed and post-fixed in 4% paraformaldehyde for 24 h and subsequently embedded in paraffin. Coronal sections (4–5 μm thickness) of hippocampal formation were prepared and stained with H&E. To obtain
the mean percentage of damaged neurons within the CA3 subfield of the hippocampus, the damaged neurons from the total number of neurons were counted on three adjacent sections. CA3 region was identified using Paxinos and Watson atlas (1986). Ten fields were chosen from each group in CA3 regions and the percentage of degenerated cells was calculated accordingly: % of degenerated cells=[(degenerated cells/degenerated cells (dark)+normal neurons (bright)]) × 100 (400 × microscopic images).

4.3.2. TUNEL staining

Whole brain tissue embedded in paraffin was used for further histopathologic preparations. Coronal sections (4–5 μm thickness) of hippocampal formation were prepared and TUNEL was performed using the In Situ Cell Death Detection Kit, POD (Roche Applied Science, Germany). Tissue sections were deparaffinized in xylene, rehydrated, and immersed in 3% hydrogen peroxide to block the endogenous peroxidase activity. After rinsing with PBS, sections were treated with protease K solution at 37 °C for 30 min to enhance the staining, incubated for 60 min at 37 °C with 50 μl of TUNEL reaction mixture, and then incubated for 30 min at 37 °C with 50 μl of converter-POD. Sections were rinsed in PBS, then incubated for 10 min at 15–25 °C with 50 μl of diaminobenzidine (DAB) substrate solution and rinsed again with PBS. Counter staining was achieved with 0.5% methyl green. Tissue was incubated in DNase solution for 10 min at 15–25 °C for positive staining. Sections were then dehydrated and coverslipped for analysis under light microscopy. Negative controls were performed by omission of the enzyme solution step. To obtain the mean percentage of apoptotic cells to normal cells within the CA1 subfield of the hippocampus, the number of TUNEL positive pyramidal neurons was counted on three adjacent sections. Ten fields were chosen from each group in CA3 regions and the percentage of apoptotic cells was calculated accordingly: % of apoptotic cells=[(apoptotic cells/apoptotic cells (dark)+normal neurons (bright)]) × 100 (400 × microscopic images).

4.4. Acute restraint stress

Stress group rats were exposed to acute restraint stress between 09:00 and 15:00 in the animal room. The restraint instrument is a small plastic cylindrical restraining tube (diameter=6.5 cm and length=15 cm). The restraint was performed using tightly fitted rodent restrainers for 6 h in their home cages whose length was adjusted with cage, so that the animal was unable to move. The control group rats were kept at the same room as restrained rats and housed without food or water for the period of restraint and handled at 9:00. Animals were euthanized immediately after restraint (still in the restrainer) using sodium pentobarbital. Blood for plasma determinations was collected by cardiac puncture and anticoagulated in the presence of trisodium citrate (3.15% w-v, 1 vol citrate per 9 vol blood). After decapitations, the brain was removed from the skull and the hippocampus structure was excised from the brain and kept at −80 °C to be used for western blot and biochemical assessments.

4.5. Plasma corticosterone level

Plasma was extracted within 1 h of collecting blood samples by centrifuging the samples at 1000 g for 15 min immediately after stress. All plasma samples were kept at −80 °C before assay by using a commercially available corticosterone EIA kit (Cayman Chemical, USA).

4.6. Biochemical analyses

Frozen hippocampus samples were weighed and homogenized in 100 mmol/l phosphate buffer (pH 7.4) containing 0.05% sodium azide in an ice bath. The homogenate was sonicated for 30 s and centrifuged (5000 g for 10 min). The supernatant was frozen at −80 °C in aliquots until further use. The protein content of the supernatant was determined using the Bradford method (Bradford, 1976).

4.6.1. Measurement of lipid peroxidation

The malondialdehyde (MDA) level as an index of lipid peroxidation was measured by the double heating method (Draper and Hadley, 1990). The method is based on spectrophotometric measurement of the purple color generated by the reaction of thiobarbituric acid (TBA) with MDA. About 0.5 ml of hippocampal homogenate was mixed with 2.5 ml of trichloroacetic acid (TCA) (10% w/v) solution followed by boiling in a water bath for 15 min. After cooling to room temperature, the samples were centrifuged at 1500 g for 10 min and 2 ml of each sample supernatant was transferred to a test tube containing 1 ml of TBA solution (0.67% w/v). Each tube was then placed in boiling water for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm with respect to the blank solution. The concentration of MDA was calculated based on the absorbance coefficient of the TBA–MDA complex (ε=1.56 × 106 cm−1 M−1) and expressed in nmol/mg protein.

4.6.2. Superoxide dismutase activity assay

SOD activity was measured based on the extent of inhibition of amino blue tetrazolium formazan formation in the mixture of nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS) and nitroblue tetrazolium (NBT) (Kakkar et al., 1984). Assay mixture contained 0.1 ml of supernatant, 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml of phenazine methosulphate (186 μM), 0.3 ml of nitroblue tetrazolium (300 μM) and 0.2 ml of NADH (750 μM). The reaction was started by addition of 0.1 ml of NADH. After incubation at 30 °C for 90 s, the reaction was stopped by the addition of 0.1 ml of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0 ml of n-butanol. Color intensity of the chromogen in butanol was measured spectrophotometrically at 560 nm. One unit of enzyme activity was defined as the amount of enzyme which caused 50% inhibition of NBT reduction per mg of protein.

4.6.3. Catalase activity assay

CAT activity was measured by the method described by Abei (1984). An aliquot (5 μl) of each tissue supernatant was added to a cuvette containing 1.995 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0 ml of freshly
prepared 30 mM hydrogen peroxide (H₂O₂). The rate of decomposition of H₂O₂ was measured spectrophotometrically at 240 nm. Activity of CAT was expressed as μmol/min/mg protein.

4.6.4. Measurement of glutathione levels

The concentration of GSH was determined in whole tissue supernatant using dithionitrobenzoic acid (DTNB) method at 412 nm (Ellman, 1959).

4.7. Western blotting

Two different protocols were used to extract the total and nuclear proteins from hippocampus tissue, according to Niimura et al. (2006) and Garcia-Bueno et al. (2008), respectively.

Western blotting was used to measure the protein expression of the following proteins: TNF-α (ABCAM; 1/2000), and NF-κB p65 subunit (Cell Signaling Technology; 1/2000), Nrf-2 (ABCAM; 1 μg/ml), HO-1 (Cell Signaling Technology; 1/1000), NQO-1 (Santa Cruz; 1/200), PGC-1α (Abcam; 1 μg/ml), NFR-1 (Santa Cruz; 1/1000), cleaved caspase-3 and nuclear AIF (Cell Signaling Technology; 1/1000).

Standard plots were generated using bovine serum albumin. Lysates equivalent to 30 μg of protein were resolved on SDS–10% polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane (Porablot, Macherey-Nagel, Germany). Then blots were blocked in 2% ECL advanced kit (Amersham Biosciences) and probed with primary antibodies overnight at 4°C. After washing, membranes were incubated for 90 min at room temperature with horseradish peroxidase-conjugated secondary antibodies (many). Then blots were blocked in 2% ECL advanced kit (Amersham Biosciences). To normalize for the loading, blots were incubated for 90 min at room temperature with horseradish peroxidase-conjugated secondary antibodies (rabbit and mouse IgG-HRP-linked antibodies). Blots were then washed and probed for β-actin or lamin B2. The density of bands was quantified using NIH Image J, and the ratio of β-actin or lamin B2 was calculated.

4.8. Statistical analysis

The number of neurons within the hippocampal CA3 region was analyzed using a non-parametric method, Mann–Whitney U test. All the Western blot, biochemical and plasma corticosterone level assessment data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey HSD for multiple comparisons, using SPSS 16.0 package programs. Data are expressed as mean ± SEM and statistical significance was set at p<0.05.

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