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Interleukin-1 Beta and Tumor Necrosis Factor Alpha Upregulation and Nuclear Factor Kappa B Activation in Skeletal Muscle from a Mouse Model of Chronic/Progressive Parkinson Disease

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Skeletal muscle atrophy has been reported in patients with Parkinson disease (PD). The purpose of this study was to examine the potential implication of interleukin 1 beta (IL-1 β), tumor necrosis factor alpha (TNF- α), and nuclear factor kappa B (NF kappa B) in skeletal muscle atrophy following PD induction.

Material/Methods: Chronic Parkinsonism was induced in 10 albino mice by MPTP/probenecid treatment, while 10 other albino mice remained without treatment and were subsequently used as controls. Gastrocnemius muscles were examined for the expression of IL-1 β and TNF- α , as well as the nuclear localization of NF kappa B, indicative of its activation, using immunohistochemistry in the 2 different groups.

Results: IL-1 β and TNF- α expression and NF kappa B nuclear localization were significantly higher in the PD skeletal muscle compared with those in the controls (P value <0.01).

Conclusions: The present data are indicative of an association of PD with IL-1 β and TNF- α upregulation and NF kappa B activation in gastrocnemius muscles, potentially promoting the atrophy frequently observed in PD.

MeSH Keywords: **Interleukin-1beta • Parkinsonian Disorders • Tumor Necrosis Factor-alpha**

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Background

Parkinson disease is the second most common neurodegenerative disease in the elderly [1]. It is characterized by considerable striatal dopamine reduction and correlated motor deficits that are associated with skeletal muscle abnormalities [2]. Such skeletal muscle abnormalities are characteristic for Parkinson disease and include bradykinesia, akinesia, and at-rest tremors [3]. Parkinson disease can be induced in rodents by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which primes dopaminergic neurons to degenerate, causing significant dopamine depletion characteristic for PD [4–9].

Interleukin 1 beta and TNF- α are proinflammatory cytokines that have been reported to be upregulated in the substantia nigra in both PD patients and MPTP-treated mice [10,11]. Both IL-1 β and TNF- α have been shown to be involved in the neurodegeneration taking place in PD [12]. Studies have shown that these 2 cytokines also have been involved in the inflammation-induced muscle atrophy occurring in inflammatory diseases, such as congestive heart failure (CHF) [13,14]. Both IL-1 β and TNF- α activate NF kappa B, which is a transcription factor [15,16]. NF kappa B exists in homodimeric or heterodimeric complexes that remain inactive in the cytoplasm of cells [17]. Once activated by cytokines, translocation of free NF kappa B dimers to the nucleus occurs, upregulating the expression of several genes that encode various proteins, including cytokines and muscle atrophy-promoting proteins [17]. A noticeable rise in NF kappa B activation was described in PD tissues, including the midbrain undergoing neurodegeneration, in humans and in animal models of PD [18–21]. Furthermore, NF kappa B plays a key role in the production of inflammatory mediators implicated in the dopaminergic neurotoxicity both *in vitro* and *in vivo* [21]. Indeed, administration of NF kappa B inhibitors has been reported to significantly protect the nigro striatum from MPTP-induced neurodegeneration, leading to improved dopamine production and amelioration in the locomotor function [22,23].

Myopathies associated with mitochondrial abnormalities, inflammatory myopathies, and muscle atrophy have been described in skeletal muscle abnormalities in PD [24,25], but very little is known about the mechanisms underlying skeletal muscle atrophy reported in PD [25]. Thus, we hypothesized that IL-1 β and TNF- α , as well as NF kappa B, play pathological roles in the skeletal muscle atrophy frequently seen in PD. We used immunohistochemistry and light microscopy to examine the expression of IL-1 β and TNF- α and the nuclear localization of NF kappa B in PD gastrocnemius muscles from MPTP/p-treated mice.

Material and Methods

Animals

Twenty randomly selected, normal, albino mice were divided into 2 equal groups: a control (Cr.) group and a Parkinson diseased (PD) group. Animals were kept in separate cages, where they could freely access standard chow and water, at a temperature of 22 $\pm$ 1 $^{\circ}$ C and 12 h dark/light cycle. The Institutional Animal Care and Use Committee at Jordan University of Science and Technology approved the animal-related protocols. A previously described protocol [26] was used to induce PD in mice by the administration of 10 subcutaneous injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP-HCl) in a dose of 25 mg/kg and intraperitoneal injections of probenecid (in Tris-HCl buffer) in a dose of 250 mg/kg, over a period of 5 weeks, during which the injections were given every 3.5 days. Simultaneously, saline (25 mg/kg) was intraperitoneally injected into control mice. Cervical dislocation was performed to sacrifice mice 4 weeks following their treatment with MPTP/p, when a substantial drop in striatal dopamine levels that was concomitant with motor dysfunction and impaired performance was seen characterizing PD [26,27].

Immunohistochemistry of IL-1 β , TNF- α , and NF kappa B in the skeletal muscle

Immunohistochemistry of IL-1 β , TNF- α , and NF kappa B p65 was performed using a previously described protocol [25,28–32]. Briefly, deparaffinization and rehydration were performed in the prepared 4- μ m-thick paraffin-embedded gastrocnemius skeletal muscle sections. Then, the sections underwent antigen retrieval followed by endogenous peroxidase activity blockage using 3% hydrogen peroxide in methanol. The sections were then rinsed in phosphate-buffered saline (PBS) prior to and following incubating some of them with anti-IL-1 β antibody (Cat #: sc-7884, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and others with anti- TNF- α antibody (Cat #: ab6671, Abcam, Cambridge, MA, USA), while incubating the rest with anti-NF kappa B p65 antibody (Cat #: ab7970, Abcam, Cambridge, MA, USA), according to the dilutions recommended by the manufacturers. After that, biotinylated secondary antibody (LSAB kit, Dako, Carpinteria, CA, USA) was utilized to incubate the sections, which were subsequently rinsed with PBS. Afterwards, streptavidin horseradish peroxidase (LSAB kit, Dako, Carpinteria, CA, USA) was used to incubate the sections. Next, subsequent to rinsing sections with PBS, they were treated with 3, 3'-Diaminobenzidine (DAB) substrate and subsequently washed with tap water after the wanted color intensity was observed. Hematoxylin was used for counterstaining the sections. Primary antibody was omitted from the processing of the negative control slides. Lobular carcinoma tissue slides (ab4698, Abcam, Cambridge, MA, USA), human lymphoma slides (ab5146, Abcam,

Figure 1. Immunohistochemical staining of IL-1 β in 4- μ m-thick paraffin-embedded gastrocnemius sections. (A) From control. (B) From Parkinson-diseased (PD). Scale bar shown in (A) applies to all images in the figure. IL-1 β immunostaining is weak in the control gastrocnemius muscle. In contrast, IL-1 β immunoreactivity is very strongly observed in gastrocnemius muscles from the PD group (at the tip of the arrows). Areas of positive immunostaining of IL-1 β , shown in brown at the tips of the arrows in PD gastrocnemius muscles (B), are mapped as pixels areas at the tips of the arrows (C). (D) IL-1 β levels increased significantly in the PD gastrocnemius muscles compared to those in the control group ($P < 0.01$, *). PD – Parkinson-diseased.

Cambridge, MA, USA), and normal human liver tissue slides (ab4348, Abcam, Cambridge, MA, USA) were used as positive control slides for NF kappa B, TNF- α , and IL-1 β , respectively. Sections were viewed under light microscopy.

Data collection and analysis

Light microscopy was utilized to study 10 slides of each animal from the 2 groups. A digital camera was used to photograph 10 random regions of each section that were subsequently examined for IL-1 β expression, TNF- α expression, and nuclear localization of NF kappa B in the gastrocnemius skeletal muscle. Then, total pixels region of positive staining (Figures 1C, 2C, 3C) was computed in proportion to the entire pixels region in each photographed area of each section using Adobe Photoshop software, as described previously [25,28–32]. After that, the mean of the pixels region of positive immunostaining relative to the entire pixels region was computed for every animal in the 2 different groups.

Statistical analysis

Interleukin 1 β expression, TNF- α expression, and NF kappa B nuclear localization were evaluated in the various skeletal muscles. Subsequently, SPSS software version 19.0 (SPSS Inc., USA) was

used to perform the independent-samples *t* test and subsequently compare IL-1 β expression, TNF- α expression, and NF kappa B nuclear localization between the control and experimental groups. *P* value < 0.05 was regarded as a statistical significant level for the differences in IL-1 β expression, TNF- α expression, and NF kappa B nuclear localization.

Results

IL-1 β immunoreactivity was weak in control gastrocnemius muscle sections (Figure 1A). In contrast, PD gastrocnemius muscle sections showed very conspicuous IL-1 β immunostaining (Figure 1B). Compared with the control gastrocnemius muscle, MPTP/p-induced PD statistically significantly ($p < 0.01$) increased IL-1 β in the gastrocnemius muscle (Figure 1D).

Likewise, control gastrocnemius muscle displayed weak TNF- α immunostaining (Figure 2A). On the other hand, TNF- α immunostaining was very conspicuously observed in PD gastrocnemius skeletal muscles (Figure 2B). Hence, TNF- α expression in PD gastrocnemius muscle from MPTP/p-treated mice was significantly ($p < 0.01$) higher than that in the control gastrocnemius muscle (Figure 2D).

Figure 2. Immunohistochemical staining of TNF- α in 4- μ m-thick paraffin-embedded gastrocnemius sections. (A) From control. (B) From Parkinson-diseased (PD). Scale bar shown in (A) applies to all images in the figure. TNF- α immunostaining is weak in the control gastrocnemius muscles. However, TNF- α immunoreactivity is very conspicuously seen in gastrocnemius muscles from the PD group (at the tip of the arrows). Areas of positive immunostaining of TNF- α , shown in brown at the tips of the arrows in PD gastrocnemius muscles (B), are mapped as pixels areas at the tips of the arrows (C). (D) TNF- α levels increased significantly in the PD gastrocnemius muscles compared to those in the control group (P<0.01, *). PD – Parkinson-diseased.

Nuclear localization of NF kappa B was weak in control gastrocnemius muscles (Figure 3A). NF kappa B nuclear localization was very clearly observed in PD gastrocnemius muscles from MPTP/p-treated mice (Figure 3B). Parkinson-diseased gastrocnemius muscle from MPTP/p-treated mice illustrated significantly (p<0.01) elevated nuclear localization of NF kappa B compared with the control ones (Figure 3D).

Discussion

The present study is the first to elucidate the effect of inducing chronic PD on IL-1 β and TNF- α expression and NF kappa B activation in skeletal muscle. The resultant investigation discloses IL-1 β and TNF- α upregulation and NF kappa B activation in gastrocnemius muscle subsequent to inducing PD by MPTP/p.

We examined changes in IL-1 β and TNF- α expression and NF kappa B activation, which is indicated by its nuclear localization, in gastrocnemius muscles consequent to inducing PD in mice by treating them with MPTP/p. Hence, we investigated whether IL-1 β , TNF- α , and NF kappa B play pathological roles in the occurrence of skeletal muscle atrophy in PD that was illustrated in our previous study [25], 4 weeks after treating mice with

MPTP/p, when considerable striatal dopamine depletion as well as motor deficits that are correlated with skeletal muscle abnormalities characterizing PD have been described [24,26,27].

Consistent with our findings (Figure 1A), very low levels of the proinflammatory cytokine IL-1 β are expressed in normal skeletal muscles [33,34]. Skeletal muscle fibers are capable of producing proinflammatory cytokines, including IL-1 β [35,36]. Consistently, we observed IL-1 β overexpression consequent to PD induction (Figure 1B, 1C), which is in line with the previous report of PD skeletal muscle abnormalities, including myopathies associated with mitochondrial abnormalities [24], which enhance the generation of reactive oxygen species that stimulate the expression of proinflammatory cytokines including IL-1 β [37].

Increased iNOS expression has been demonstrated in PD gastrocnemius muscle from MPTP/p-treated mice [31]. The skeletal muscles of patients and animals with chronic heart failure (CHF) have shown elevated levels of IL-1 β and iNOS [38–42]. IL-1 β has been reported to be one of the proinflammatory cytokines that provoke iNOS expression [43]. Upregulation of IL-1 β has also been reported in the aging skeletal muscles [34,44]. Furthermore, skeletal muscles of inflammatory myopathies

Figure 3. Nuclear localization of NF kappa B p65 in 4- μ m-thick paraffin-embedded gastrocnemius sections. **(A)** From control. **(B)** From Parkinson-diseased (PD). Scale bar shown in **(A)** applies to all images in the figure. Nuclear localization of NF kappa B p65 is weak in the control gastrocnemius muscle. Dissimilarly, nuclear localization of NF kappa B p65 is strongly apparent in gastrocnemius muscles from the PD group (at the tip of the arrows). Areas of positive immunostaining of NF kappa B p65, shown in brown at the tips of the arrows in PD gastrocnemius muscles **(B)**, are mapped as pixels areas at the tips of the arrows **(C)**. **(D)** NF kappa B p65 nuclear localization increased significantly in the PD gastrocnemius muscles compared to that in the control group ($P < 0.01$, *). PD – Parkinson-diseased.

displayed raised levels of IL-1 β , which have been suggested to be associated with the muscle damage taking place in those myopathies [45]. Parkinson-diseased skeletal muscle abnormalities are reported to include inflammatory myopathies [24]. HSP90 mediates IL-1 β signaling [43]. Furthermore, inhibition of HSP90 abolished IL-1 β signaling [46]. Increased HSP90 expression has been shown in PD gastrocnemius skeletal muscles [28]. Consistently, our results illustrate increased IL-1 β expression in the skeletal muscles consequent to inducing PD.

Overexpression of IL-1 β in PD skeletal muscles, illustrated by our results (Figure 1B, 1C) is also consistent with previous studies [18–20] reporting IL-1 β upregulation in PD brains. IL-1 β was suggested to be toxic to dopaminergic neurons, causing their degeneration and the subsequent depletion of dopamine, leading to PD [47–49]. Additionally, administration of agents possessing anti-inflammatory properties has been shown to protect against MPTP neurotoxicity, preventing against degeneration of substantia nigra dopaminergic neurons through suppressing IL-1 β overexpression in substantia nigra [7,50]. IL-1 β has been shown to induce catabolism and subsequent muscle protein loss in cultured differentiated myotubes [51].

Hence, it can be proposed that IL-1 β upregulation might be a potential mechanism underlying the skeletal muscle abnormalities, particularly muscle atrophy, described in PD.

TNF- α upregulation has been shown in association with muscle atrophy in inflammatory diseases [13,14]. Consistently, we observed TNF- α overexpression (Figure 2B, 2D) in the gastrocnemius muscle, which was shown to manifest atrophy in our previous study subsequent to PD induction by MPTP/p treatment [25]. Increased TNF- α expression has already been shown in the substantia nigra of PD patients and MPTP-treated animals [10–12], indicating its involvement in the neurodegeneration causing PD.

Both IL-1 β and TNF- α induce transcription factor NF kappa B activation, which is equivalent to its nuclear localization, causing skeletal muscle atrophy [15,16,52]. Once activated, NF kappa B in turn promotes the expression of the proinflammatory cytokines IL-1 β and TNF- α , leading to a positive feed-back loop and a consequent overstimulation of NF kappa B and the development of muscular abnormalities [51,53]. Consistently, we observed that NF kappa B activation was correlated with IL-1 β

and TNF- α overexpression consequent to inducing chronic PD in mice by treating them with MPTP/p (Figure 3A–3D).

iNOS overexpression and NF kappa B activation have been shown in skeletal muscles of patients with chronic heart failure (CHF) [38–42]. NF kappa B activation has been reported to be essential for iNOS expression and the subsequent NO generation, leading to the establishment of a positive regulatory loop [54,55]. NF kappa B activation has been suggested to contribute to age-related muscle loss in the elderly [53]. Additionally, NF kappa B activation has been demonstrated in inflammatory myopathies and Duchenne muscular dystrophy, where NF kappa B activity leads to muscle wasting and atrophy recognized in those conditions [56–58]. NF kappa B activation has been reported to cause degradation of particular skeletal muscle proteins, induce inflammation, and prevent post-injury myofiber regeneration, leading to skeletal muscle loss and subsequent atrophy in various conditions such as muscular dystrophy and chronic obstructive pulmonary disease [53]. NF kappa B has been suggested as a target for blocking skeletal muscle loss [59] because its genetic or pharmacological inhibition has been shown to ameliorate muscle pathogenesis and augment skeletal muscle regeneration [59]. Consistently, our previous study illustrated atrophic PD skeletal muscle fibers that showed angular profiles and substantial decline in cross-sectional area, which was suggestive of muscle atrophy [25,60–64], after PD induction. Gastrocnemius skeletal muscle atrophy was correlated with the upregulation of IL-1 β and TNF- α and activation of NF kappa B, as illustrated by our results (Figure 1–3). Upregulated IL-1 β and TNF- α , indeed, have been suggested to promote skeletal muscle atrophy by activating NF kappa B in inflammatory diseases [65]. Once activated, NF kappa B was described to alter gene expression, causing prolonged alterations

in muscle protein levels and the subsequent atrophy promotion by stimulating muscle catabolism or disrupting muscle differentiation process [66–68]. Hence, NF kappa B activation may be a potential mechanism underlying the skeletal muscle atrophy observed in PD.

Nevertheless, a dual role has been described for NF kappa B in skeletal muscle: a pathological role and a protective role. NF kappa B is suggested to have a potential role in modulating the immune response and muscle regeneration by regulating myogenesis and muscle repair in polymyositis, dermatomyositis, and Duchenne muscular dystrophy [69,70]. Thus, future studies must be conducted to investigate whether NF kappa B can play any potential protective role in the PD skeletal muscle, because this is very important in targeting NF kappa B as a therapeutic approach.

Conclusions

In summary, our present findings show a correlation of PD with IL-1 β and TNF- α upregulation and NF kappa B activation in gastrocnemius muscles, potentially promoting the atrophy frequently observed in PD. Thus, IL-1 β and TNF- α may be therapeutic targets in an attempt to ameliorate and/or prevent skeletal muscle atrophy occurring in PD.

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