

Cyclic exercise induces anti-inflammatory signal molecule increases in the plasma of Parkinson's patients

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Received June 2, 2003; Accepted July 11, 2003

Abstract. It has been known for many years that immune system alterations occur with Parkinson's disease (PD). Changes in lymphocyte populations in cerebrospinal fluid and blood, immunoglobulin synthesis, and cytokine and acute phase protein production have been observed in patients with PD. Hence, there is evidence for inflammation. In this report we demonstrate that cyclic exercise over months results in a significant increase in the rise of plasma anti-inflammatory signal molecules, such as interleukin-10 and adrenocorticotropin. Additionally, endogenous plasma morphine levels increase with the duration of the cyclic exercise protocol. Morphine is identified and quantified by high performance liquid chromatography coupled to electrochemical detection and nano electro-spray ionization double quadrupole orthogonal acceleration time of flight mass spectrometry. Proinflammatory cytokine, i.e., interleukin-1, interleukin-6, plasma levels did not increase. These results matched with those reported previously, demonstrating enhanced motor skills and mood elevation with this cyclic exercise protocol, suggest that this protocol induces the formation of anti-inflammatory signal molecules, which appear to be associated with alleviation of some of the clinical characteristics of PD.

Introduction

Idiopathic Parkinson's disease is a degenerative disorder of the central nervous system. Several pathogenic mechanisms have been proposed that lead to degeneration of dopaminergic neurons. These mechanisms encompass variables such as metabolic or toxic factors, oxidative stress and mitochondrial dysfunction (1). The primary anatomical features

central to PD patients include: a diminished number of myelinated dopaminergic cells in the substantia nigra (SN) and in related brain stem nuclei, a decrease in the dopamine content in nigrostriatal and mesolimbic pathways, the presence of Lewy bodies, and the deposition of neuromelanin (2,3). The perturbation of several neurotransmitters and neuropeptides has been reported in PD, indicating a more complicated and widespread pathology. The role of immune and vascular mechanisms in neurodegenerative diseases such as PD is, similarly, an important area of investigation (1,4). Death or injury to neurons in PD leads to the presence of many pro-inflammatory cytokine molecules, as well (1). This process resembles classic inflammation, but with minimal or no participation of macrophages and lymphocytes from blood (1).

In the present report, we determine the plasma level of pro-and anti-inflammatory cytokines as well as signal molecules most often associated with stress, i.e., adrenocorticotropin (ACTH) at baseline (group 1). We also measure these levels subsequent to the introduction of a 12 week cyclic exercise regime at week 4 (group 2), week 8 (group 3) and week 12 (group 4). We demonstrate that anti-inflammatory signal molecules significantly appear in the plasma months after initiating and sustaining this cyclic exercise protocol. These results are correlated with a previous study, which found that cyclic exercise in Parkinson's patients produced improvement in motor function and attitude (Rymer M, *et al*, Puijo Symposium, Kuopio, Finland, 2001).

Materials and methods

The participants were recruited from a private neurology practice in Kansas City, MO. The participants were all previously diagnosed and under treatment for Parkinson's disease. Their disease was staged at the beginning of the trial by an independent neurologist using the Hoehn and Yahr Scale (5). During their baseline testing in our program, the participants were evaluated with the standard scale, the Uniform Parkinson's Disease Rating Scale (UPDRS). They were all moderate to severely ill, average age 78 years, average time since diagnosis was around 8 years, 14 males, 5 females. The protocol described was reviewed and passed by the IRB at Saint Luke's Hospital in Kansas City, MO. Blood samples

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Key words: inflammation, neurodegeneration, immune system, nitric oxide, exercise, Parkinson's disease

(30 ml) were drawn before the exercise session at the designated time points in the 12 week protocol by a registered lab technician at the Saint Luke's Hospital out-patient laboratory on the 6th floor of Medical Plaza I building.

Experimental designs. The cyclic exercise protocol is designed to generate a series of parabolic-like waves of cardiovascular exercise and recovery and was designed by one of the authors (6) (Table I). The utility and effects of the protocol in healthy populations has been reported elsewhere (7). Cycles were tailored to the individual subjects following an initial, baseline determination of cardiovascular responsiveness during short burst of exercise followed by recovery. Five second averaged heart rates were monitored and recorded continuously using a Polar NV heart-rate monitor watch and chest strap (Polar Electro Inc. Woodbury, NY). Once the baseline determination was made, subjects began a prescribed course of cyclic exercise, 3 times weekly, monitored by a neurologist, nurse practitioner and one-on-one trainers. The cycles are designed to increase the capacity for cardiovascular acceleration and recovery, adjusted according to base heart-rate range and performance, in the context of normal circadian rhythm. This design produces a progressive set of cycles, where heart-rate targets increase (or rate of speed to targets increases) for each subsequent cycle within a set and the peak heart-rate targets increase as the cycles move from morning to afternoon, as described below (7).

The protocol included 2 stages: i) a baseline determination stage, and ii) the actual training cycle protocol. For the baseline determination, subjects were requested to refrain from caffeine and meals for 3 h prior to exercise. Prior to beginning, subjects were briefed on the 5-cycle baseline protocol, familiarized with the equipment, and instructed on the relaxation response (8). Prior to beginning of the baseline determination, subjects sat quietly for a 7-min period. Three minutes into that period, the subjects were told to initiate the relaxation response. Five minutes into that period, blood pressure was measured from the right arm of the subjects. After the 7 min elapsed, subjects were instructed to take a deep breath and begin the 1st cycle of the baseline determination.

The 1st cycle consisted of riding an exercise bicycle (Schwinn Airodyne) or climbing stairs (depending on subject preference and physical capabilities) or running on a trampoline (Body by Jake). All subjects were closely supervised. Some were physically assisted via gait belts to ensure their balance during exertion at an easy pace (3 on the 10-point modified Borg scale (9) until the heart-rate stabilized or 1 min had elapsed. A trainer used a second Polar NV watch to track the subject heart-rates and indicated to the subjects when their heart-rate had stabilized. The subjects stopped the exercise, sat down, and began the recovery part of the cycle, including the relaxation response. The trainer indicated to the subjects when their heart-rate had stabilized at a resting rate and the 2nd cycle was started. For each subsequent cycle, the sequence was the same. What differed were heart rate targets and levels of exertion. But no period of exertion exceeds 1 min. Table II summarizes the baseline set of cycles.

The trainer took the subject's blood pressure 5 min after the peak heart-rate was reached and again 10 min later. The subject was to then resume normal activity.

Table I. Cycle protocol: overview.

	Cycle set Day 1 Monday	Cycle set Day 2 Wednesday	Cycle set Day 3 Friday
Week 1 (06.00-09.00)	7 Cycles	6 Cycles	5 Cycles
Week 2 (06.00-09.00)	7 Cycles	6 Cycles	5 Cycles
Week 3 (09.00-12.00)	5 Cycles	5 Cycles	5 Cycles
Week 4 (15.00-18.00)	6 Cycles	5 Cycles	5 Cycles

Table II. Summary of baseline set of cycles.

Cycle No.	Exertion target	Level of exertion on modified Borg scale	Comment
1.	Until heart rate plateau or 1 min	3 of 10	Always recover until HR stabilizes for 15 s
2.	Cycle 1 peak HR + 10 beats	Sufficient to reach target	Trainer may ask subject to increase effort to reach target
3.	Same target as above	7-8 of 10	Reach previous target as fast as possible
4.	Cycle 2 target + 10 beats	7-8 of 10	
5.	Peak HR	9 of 10	Max effort for no longer than 1 min

The peak heart-rate achieved during baseline cycle 5 was used as the initial maximum heart-rate. Subsequent target heart-rates were set as increasing percentages of this initial maximum. For the 1st set of training cycles (performed between 06.00-09.00 h), the target heart-rate for the 1st cycle (a warm-up cycle) was 70% of the baseline peak rate. The final cycle of the 1st set is 85% of the maximum. During the 2nd week (performed between 06.00-09.00 h), the peak target heart-rate was 90% of the initial maximum, the peak heart-rate increasing 2 beats each succeeding session. The 3rd week (performed between 09.00-12.00 h), the peak heart-rate was 95% of the maximum. In addition to the increasing target heart-rates, the rate of exertion is increased as well, where effort on the particular cycles was to quickly accelerate to a 9 on the 10 point Borg scale. The 4th week of training cycles (performed between 15.00-18.00 h), include 2 such spikes in the 1st session and the latter 2 sessions consist of all spikes, save the initial, warm-up cycle. The last set of afternoon cycles may drive the heart-rate beyond the baseline maximum. If so, the new maximum heart-rate was used to determine the peak

target heart-rates for the next month of cycles. In addition, the number of cycles completed per session decreased as intensity increased within each week. The bulk of each training session consisted of recovery, as the exertion phase of each cycle never exceeded 1 min and the recovery phase was typically 3 or 4 min per cycle.

Immunocytochemical detection of signaling molecules. Blood samples (10 ml) were drawn at the Saint Luke's Hospital outpatient laboratory and duplicate plasma samples were stored in EDTA or heparin at -70°C at baseline (group 1), week 4 (group 2, week 8 (group 3) and week 12 (group 4). All plasma samples were obtained between 9 a.m. and noon. ACTH was determined in EDTA plasma. Cortisol and IL-1 β analysis required the use of heparinized plasma. For the analysis of interleukin (IL)-10, tumor necrosis factor- α (TNF α) and IL-6, the type of anti-coagulant used on the sample did not matter. Cytokines were analyzed using Endogen enzyme-linked immunosorbent assay (ELISA) kits (Woburn, MA). Samples and standards were assayed in duplicate using 50 μl of the appropriate plasma. ACTH and cortisol were analyzed by radioimmunoassay (RIA) (ICN Biomedicals, Costa Mesa, CA). For the ACTH assay, 100 μl of plasma, standards, and plasma controls were analyzed in duplicate. For the cortisol assay, two 25 μl samples, standards, and plasma controls were required. Morphine in the plasma was detected with an RIA kit purchased from Diagnostic Products Cooperation, CA, USA. This method was also used to verify the results from HPLC (see below). Four sample groups were analyzed and the data were presented as a mean of a group \pm SE. Group 1 represented 19 individuals; group 2, 18; group 3, 17; and group 4 contained 16 subjects. The detection limits for each assay are presented in Table III.

Extraction of opiate in the plasma. HCl (10 N, 20 μl) was added to 2 ml of plasma obtained from each Parkinson's patient. The samples were vigorously vortexed and extracted with 5 ml of 9:1 chloroform/isopropanol. After 5 min, homogenates were centrifuged at 3000 rpm for 15 min at room temperature. The supernatant was collected and dried with a Centrivap Console (Labconco, Kansas City, MO). The dried extract was then dissolved in 0.05% trifluoroacetic acid (TFA) water before solid phase extraction. Samples were loaded on a Sep-Pak Plus C-18 cartridge (Waters, Milford, MA) previously activated with 100% acetonitrile and washed with 0.05% TFA-water. Morphine elution was performed with a 10% acetonitrile solution (water/acetonitrile/TFA, 89.5%:10%:0.05%, v/v/v). Eluted samples were dried with in a Centrivap Console and dissolved in water prior to HPLC analysis.

HPLC and electrochemical detection of morphine in the sample. The HPLC analyses were performed with a Waters 626 pump (Waters, Milford, MA) and a C-18 Unijet microbore column (BAS). A flow splitter (BAS) was used to provide the low volumetric flow-rates required for the microbore column. The split ratio was 1/9. Operating the pump at 0.5 ml/min yielded a microbore column flow-rate of approximately 50 $\mu\text{l}/\text{min}$ of buffer. The injection volume was 5 μl . Morphine detection was performed with an ampero-metric detector

Table III. Detection limits for the plasma analyzed using RIA or ELISA.

Analyte	Detection limit
Cortisol	~ 1 $\mu\text{g}/\text{dl}$
ACTH	< 2 pg/ml
TNF α	< 1 pg/ml
IL-6	< 1 pg/ml
IL-10	< 1 pg/ml
IL-1 β	< 1 pg/ml
Morphine	1 ng/ml

LC-4C (BAS, West Lafayette, IN). The microbore column was coupled directly to the detector cell to minimize the dead volume. The electrochemical detection system was using a glassy carbon-working electrode (3 mm) and a 0.02 Hz filter (500 mV; range 10 nA). The cell volume was reduced using a 16- μm gasket. The chromatographic system was controlled by Waters Millennium³² Chromatography Manager V3.2 software and the chromatograms were integrated with Chromatograph software (Waters).

Morphine was quantified in the tissues by the method described by Zhu and Stefano (10). This method was carried out in the following manner: The mobile phases were: buffer A, 10 mM sodium chloride, 0.5 mM EDTA, 100 mM sodium acetate, pH 5; buffer B, 10 mM sodium chloride, 0.5 mM EDTA, 100 mM sodium acetate, 50% acetonitrile, pH 5. The injection volume was 5 μl . The running conditions were 100% buffer A for the first 10 min, 5% buffer B at 10 min, 50% buffer B at 25 min, and at 30 min 100% buffer B. Both buffers A and B were filtered through a Waters 0.22 μm filter and the temperature of the system was maintained at 25°C . Several HPLC purifications were performed between each sample to prevent residual morphine contamination remaining on the column. Furthermore, the fraction of blank chromatography corresponding to the elution of the morphine was checked by Q-TOF mass spectrometry analysis, confirming that no morphine remained.

Mass spectrometry determination of morphine. Identification of endogenous morphine by nano electro-spray ionization double quadrupole orthogonal acceleration time of flight mass spectrometry (Q-TOF-MS) has been documented recently (10,11). The mass spectrometry was performed using a Micromass Q-TOF system. Acetonitrile/water/formic acid, 1 μl (50:49:1, v,v,v) containing the sample was loaded in a gold coated capillary using a Micromass F-type needle. This sample was sprayed at a flow rate of 30 nl/min, giving an extended analysis time, during which we acquired an MS spectrum as well as several MS/MS spectra. During MS/MS, or tandem mass spectrometry, fragmentations are generated from a selected precursor ion by collision-induced dissociation (CID). Since not all ions fragment with the same efficiency, the collision energy typically varies between 20 and 35 V so that the parent ion is fragmented into a satisfactory number of

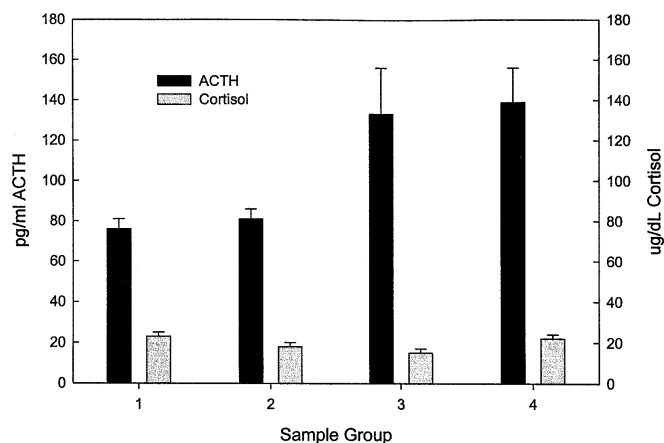


Figure 1. ACTH and cortisol levels determined in plasma by radioimmunoassay (RIA) for each group. Error bars represent the standard error of the mean of $n=19$ for group 1, 18 for group 2, 17 for group 3, and 16 for group 4. Group 3 and group 4 exhibited statistically significant increases in ACTH when compared to group 1 using the Mann-Whitney rank sum test (group 3, $p=0.02$; group 4, $p=0.002$). Plasma cortisol levels were not significantly different at any of the observation periods.

different daughter ions. Needle voltage was set at 950 V, and cone voltage at 25 V. The instrument was operated in the positive mode.

Results

Immunocytochemical assays. ACTH plasma levels in the PD patients were determined by RIA for groups 1-4 and were 76 ± 5 , 81 ± 5 , 133 ± 23 , and 139 ± 17 pg/ml, respectively (Fig. 1). Group 3 and 4 exhibited statistically significant increases when compared to group 1 using the Mann-Whitney rank sum test (group 3, $p=0.02$; group 4, $p=0.002$). Cortisol plasma concentrations were also determined by RIA for groups 1-4 and were 23 ± 2 , 18 ± 2 , 15 ± 2 , and 22 ± 2 ug/dl, respectively (Fig. 1). Plasma cortisol levels were not significantly different at any of the observation periods, suggesting that these patients were not under stress.

IL-10, an anti-proinflammatory cytokine (12), concentrations were found to be statistically different in group 3 and 4 when compared to group 1 ($p < 0.001$). The IL-10 concentration in group 1 was determined to be 1.6 ± 0.9 pg/ml, while group 2 was 3.6 ± 2.6 , group 3 was 51 ± 7 and group 4 contained 74 ± 9 pg/ml (Fig. 2), demonstrating a statistically significant increase in the last 2 observation periods. IL-6 concentrations determined for groups 1-4 were 7.5 ± 3.8 , 4.1 ± 2.0 , 4.1 ± 4.1 and 5.8 ± 3.4 pg/ml, respectively (Fig. 2). Thus, IL-6 plasma levels remained the same throughout the study. IL-1 β and TNF α were not detected in any of the plasma samples analyzed (data not shown).

Plasma morphine detected by RIA for group 1 was 0.82 ± 0.56 ng/ml; group 2, 1.48 ± 0.67 ; group 3, 1.72 ± 0.93 ; and for group 4, 1.32 ± 0.65 (Fig. 3). A two tailed t-test revealed significant differences in morphine levels when compared to group 1 (group 2, $p=0.003$; group 3, $p=0.001$; group 4, $p=0.02$). This result demonstrates that morphine levels in the plasma have changed significantly after starting the cyclic exercise protocol.

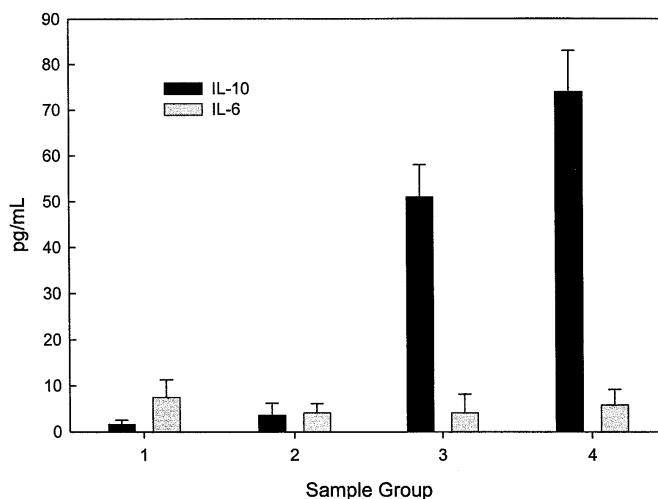


Figure 2. Cytokine (IL-10 and IL-6) levels determined in plasma by enzyme-linked immunosorbent assay (ELISA) for each group. Error bars represent the standard error of the mean of $n=19$ for group 1, 18 for group 2, 17 for group 3, and 16 for group 4. IL-10 concentrations were found to be statistically different in group 3 and 4 when compared to group 1 ($p < 0.001$). IL-6 levels did not fluctuate significantly.

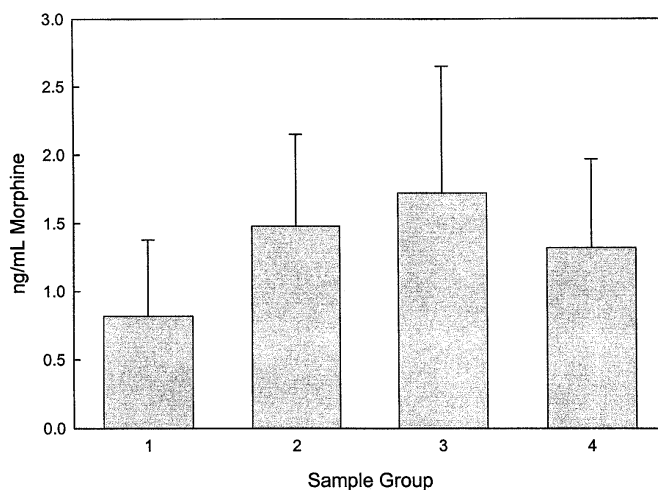


Figure 3. Morphine levels determined in plasma by radioimmunoassay for each group. Error bars represent the standard error of the mean of $n=19$ for group 1, 18 for group 2, 17 for group 3, and 16 for group 4. A two tailed t-test revealed significant differences in morphine levels when compared to group 1 (group 2, $p=0.003$; group 3, $p=0.001$; group 4, $p=0.02$).

HPLC. In order to determine the identity of this RIA identified opiate alkaloid-like material, this compound obtained from plasma was purified and quantified by HPLC. Morphine was identified in plasma by reverse phase HPLC using a gradient of acetonitrile following liquid and solid extraction and comparison to an authentic standard (Fig. 4). The morphine extracted from patient plasma (Fig. 4A) had the identical retention time when compared to an authentic morphine external standard (Fig. 4B). This finding was repeated in the samples obtained from 5 patients from group 2, all of which were never exposed to exogenous morphine. The electrochemical detection sensitivity of morphine using this method is 80 pg. The concentration of morphine was determined using the Chromatogram Manager 3.2 and extrapolated from

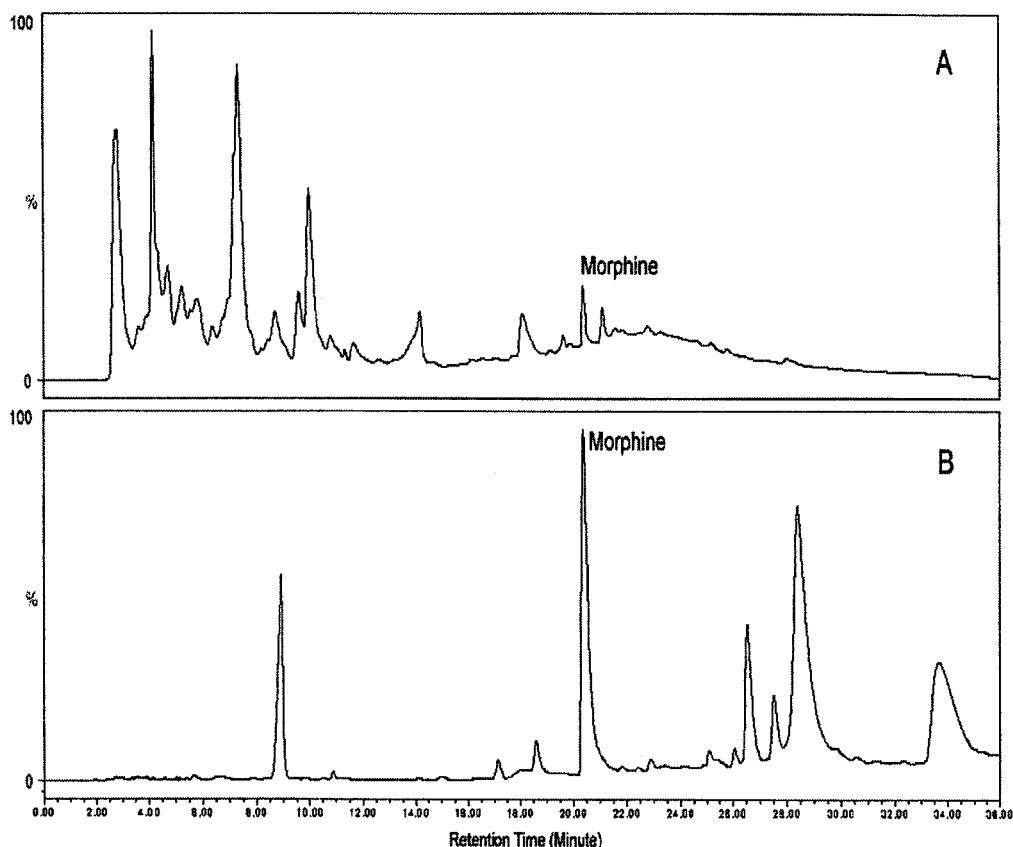


Figure 4. Purification of morphine in the plasma of Parkinson's patients. A, Chromatogram of plasma extraction (2 ml). B, Morphine external standard, 10 ng.

the peak-area calculated for the external standard. The average concentration of morphine in the 5 samples was 1.43 ± 0.58 ng/ml. This result was compatible with the RIA data. Blank runs between morphine HPLC determinations did not show a morphine residue. All the fractions corresponding to morphine blank runs were sent for mass spectrometric analysis and were negative.

Mass spectrometry. HPLC coupled to the Q-TOF system further characterized the morphine in the plasma sample. The molecular mass attributed to single charged morphine is 286.2 Da (Fig. 5A), which is consistent with the authentic standard (Fig. 5B) and theoretical value (286.14 Da). Fragmentation of both plasma morphine and the authentic standard using Collision Induced Dissociation (CID) yielded the same fragments (Figs. 5C and D), demonstrating that the original material was, indeed morphine.

Discussion

In the present report we demonstrate that anti-inflammatory molecules were significantly elevated in the plasma of Parkinson's patients for months subsequent to the initiation a cyclic exercise regimen. The level of ACTH and IL-10 in the blood of Parkinson's patients further showed a significant increase following 1 month of cyclic exercise. Endogenous plasma morphine levels, identified via Q-TOF-MS, also showed a significant increase. In addition, proinflammatory cytokine plasma levels (IL-6) did not change, or were

undetected (IL-1 and TNF α) during the entire examination period. Taken together, it would appear that the cyclic exercise protocol in Parkinson's patients induced the formation of signaling molecules associated with immune, vascular and neural down regulation (12-15).

The above findings coupled to the positive mood and physical performance measurements previously presented on these patients (Rymer M, *et al*, Puijo Symposium, Kuopio, Finland, 2001), suggests that an underlying chronic immune and/or vascular process may exist that can be alleviated by the introduction of these anti-inflammatory signal molecules via cyclic exercise. Rymer *et al* (Puijo Symposium, Kuopio, Finland, 2001) found that the motor ability, as defined by the UPDRS (Unified Parkinson's Disease Rating Scale), improved significantly. Diastolic BP was significantly reduced and immune function was significantly enhanced. In addition, participants and collaterals noted multiple improvements in the quality of life, from better and longer sleep cycles to improved affect and speech (Rymer M, *et al*, Puijo Symposium, Kuopio, Finland, 2001).

Proinflammatory cytokines are part of the inflammatory response in an injured brain (1). Increased expression of pro-inflammatory cytokines, such as IL-1 and IL-6, and TNF α have been found in cerebrospinal fluid as well as the brains of patients with PD (16). In the mouse model of MPTP-induced PD, increased levels of mRNA for IL-1 β , TNF α , IL-6, IL-10, IFN- γ in striatum have been reported (17). In addition, polymorphism of TNF α genes correlates with early onset of sporadic PD in Japanese patients (18).

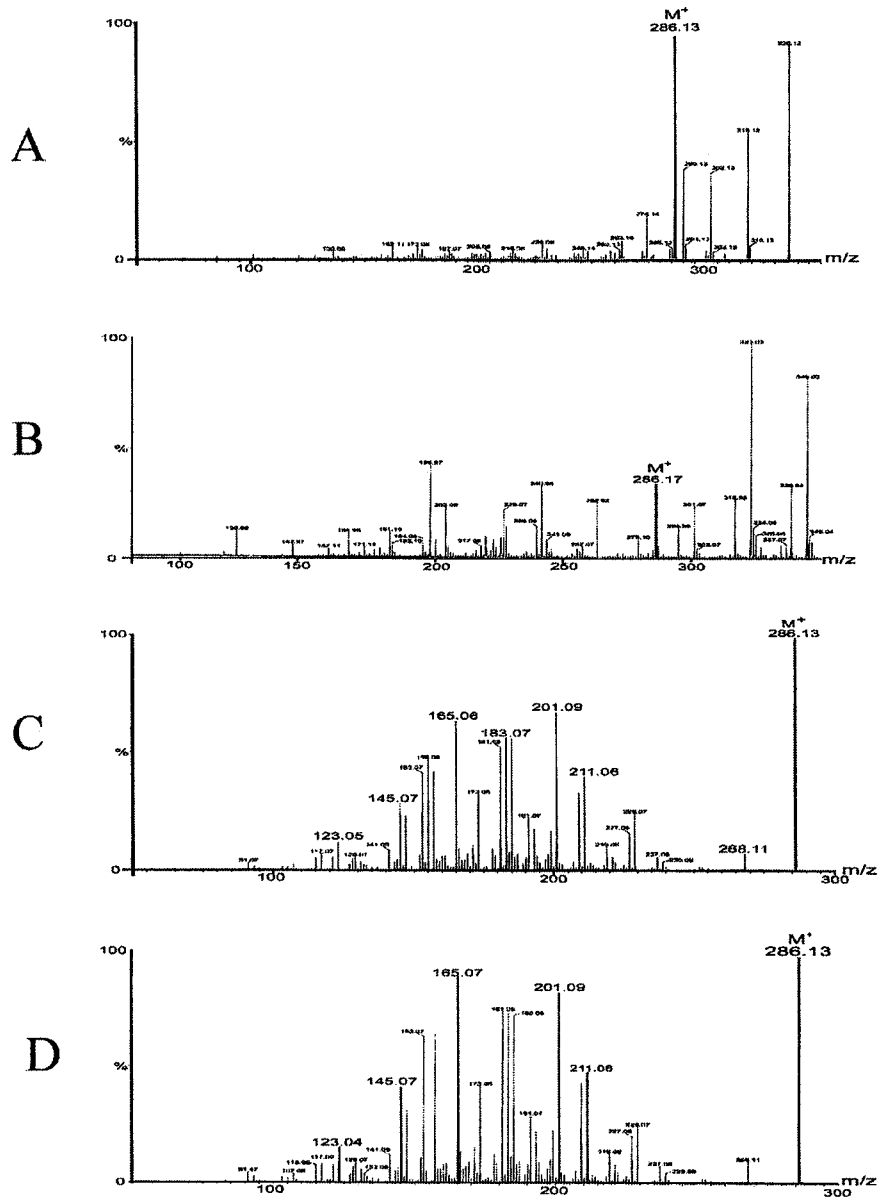


Figure 5. Mass spectrometric analysis of morphine following HPLC purification. A, External morphine standard, indicating its mass (286.13 Da); B, HPLC fraction of plasma containing morphine (286.13 Da); C, fragmentation analysis of authentic morphine molecule; D, fragmentation analysis of morphine molecule purified from patient plasma.

In regard to immune function, patients with PD exhibit changes in their cellular and humoral immune responses. Total lymphocyte count is diminished in patients with PD, and there are phenotypic alterations in circulating peripheral blood lymphocytes. The number of T-cells (CD3⁺) and B-cells (CD19⁺) are decreased in PD, especially the CD4⁺ subset (19,20). The number of memory helper T-cells is also decreased, but to a lesser extent, and the percentage of activated helper T-cells rises (19). Lymphocytes from PD patients have reduced proliferative response to mitogens such as phytohemagglutinin and concanavalin A, demonstrating that cellular immunity is compromised (21).

Alterations in immune function and cytokine levels are important in PD since proinflammatory cytokines can influence catecholamine signaling in the CNS. Interleukin-2 (IL-2) increases ³H-dopamine release *in vitro* from striatal rat slices (22). In rat hypothalamus *in vivo*, microglial-derived IL-1

was found to stimulate the release of dopamine and dihydroxyphenyl acetic acid (23). Palazzolo and Quadri demonstrated that *in vitro*, IL-1 β stimulates the release of both dopamine and norepinephrine from hypothalamus of male rats (24). IL-1 in rat hypothalamus decreased the levels of epinephrine and nor epinephrine, and their major metabolite 3-methoxy 4-hydroxyphenylglycol was elevated (25). In this study, homovanillic acid, a dopamine metabolite was elevated in the rat striatum, hypothalamus and medulla following cytokine application. Brown and colleagues (26) also noted a stimulatory effect on norepinephrine metabolism by IL-1 β in rat CNS.

Interestingly, the level of morphine in the plasma of Parkinson's patients also increased significantly 1 month after starting the cyclic exercise protocol. This result supports the data obtained by Matsubara and colleagues (27). Matsubara and colleagues (27) found an increased level of morphine in the urine of Parkinson's patients after L-dopa injection. Our

previous research has demonstrated the presence of morphine in various human tissues (13,28). Furthermore, morphine levels increased significantly after surgery in order to down regulate the immune response after trauma (29,30). This study indicates that morphine may play a role in down regulation of neurological stress/damage (30,31).

Furthermore, given the performance of the relaxation response in the cyclic exercise protocol we surmise that in future studies its contributions to the present outcome will be evaluated. This is important since the relaxation response is characterized by a decreased metabolism, heart rate, blood pressure, and rate of breathing as well as an increase in skin temperature (8,32-34). Thus, many of these outcomes can be associated with opiate signalling.

In summary, we find that a cyclic exercise protocol that elderly patients with severe PD were able to complete induces the formation of anti-inflammatory signaling molecules thereby, enhancing the very signaling processes that they mediate, i.e., down regulating inflammation. Recently, we published a report speculating on the proinflammatory nature of many disorders, including PD (35,36). However, we note that our present study did not find evidence of proinflammatory signaling. We conclude that this is due to the lack of specificity of our plasma sampling, which was necessary prior to death. Thus we were unable to sample specific brain sites where degeneration occurs or the cerebrospinal fluid. The emergence of the anti-proinflammatory signal molecules over time can be interpreted as originating from the cyclic exercise protocol since it has been found that strenuous exercise stimulates proinflammatory cytokine expression (37). The improvement in physical performance and mental outlook following this cyclic exercise protocol supports the hypothesis that the emergence of these signaling molecules is correlated with positive physical and behavioral outcomes. Thus, counter-intuitively, given the chronic and progressive nature of PD, this cyclic exercise protocol may offer a potential therapy for these patients in that, through our findings, it stimulates mild cardiovascular changes associated with nitric oxide liberation, limiting proinflammatory processes (37-40).

Acknowledgments

This work was in part supported by the following grants: NIDA 09010, VHA, Inc and LifeWaves International, LLC and The Sidney Kimmel Foundation.

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