Pioglitazone suppresses neuronal and muscular degeneration caused by polyglutamine-expanded androgen receptors

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Spinal and bulbar muscular atrophy (SBMA) is a neuromuscular disease caused by the expansion of a CAG repeat in the androgen receptor (AR) gene. Mutant AR has been postulated to alter the expression of genes important for mitochondrial function and induce mitochondrial dysfunction. Here, we show that the expression levels of peroxisome proliferator-activated receptor-γ (PPARγ), a key regulator of mitochondrial biogenesis, were decreased in mouse and cellular models of SBMA. Treatment with pioglitazone (PG), an activator of PPARγ, improved the viability of the cellular model of SBMA. The oral administration of PG also improved the behavioral and histopathological phenotypes of the transgenic mice. Furthermore, immunohistochemical and biochemical analyses demonstrated that the administration of PG suppressed oxidative stress, nuclear factor-κB (NFκB) signal activation and inflammation both in the spinal cords and skeletal muscles of the SBMA mice. These findings suggest that PG is a promising candidate for the treatment of SBMA.

INTRODUCTION

Expansion of the trinucleotide CAG repeat in a coding region causes a group of neurodegenerative disorders including Huntington’s disease that share several molecular pathomechanisms such as transcriptional dysregulation and mitochondrial dysfunction (1). Spinal and bulbar muscular atrophy (SBMA) is an adult-onset motor neuron disease that exclusively affects males and is caused by the expansion of a CAG repeat in the androgen receptor (AR) gene. Spinal and bulbar muscular atrophy is characterized by proximal muscle atrophy, weakness, fasciculations and bulbar involvement (2–4). No specific treatment for this disease has been identified. Previous studies showed that polyglutamine-expanded ARs accumulate in the nuclei of motor neurons in a testosterone-dependent manner and that the pathogenic AR perturbs the transcription of diverse genes that play important roles in the maintenance of neuronal function, thereby leading to neuronal dysfunction and the impairment of retrograde axonal transport in the mouse model of SBMA (5–8).

Recent studies have shown that the pathogenesis of SBMA is a result of both neurogenic and myopathic changes. Spinal and bulbar muscular atrophy patients present with extensive motor neuron loss together with signs of muscle degeneration, including the presence of central nuclei and the degeneration of fibers (3,9). The elevated levels of serum creatine kinase (CK) also support a myopathic pathogenesis in SBMA (10,11). Histopathological analyses of muscle tissues from the SBMA mice revealed both neurogenic and myopathic features (5,12–14). In the knock-in mouse model of SBMA, muscle degeneration occurs prior to the onset of spinal cord pathology (14). Furthermore, suppression of muscle pathologyameliorates motor

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neuron degeneration in mouse models of SBMA, supporting the hypothesis that skeletal muscle is a primary target of polyglutamine-expanded-AR toxicity (13,15,16).

In the cellular model of SBMA, the accumulation of polyglutamine-expanded ARs in the presence of the relevant ligand results in mitochondrial membrane depolarization and an increase in the levels of reactive oxygen species that is blocked by treatment with the antioxidants co-enzyme Q10 and idebenone (17). Cytochrome c oxidase subunit Vb has been shown to interact with normal and mutant AR in a hormone-dependent manner, which may provide a mechanism for mitochondrial dysfunction in SBMA (18). Pathogenic ARs have also been shown to repress the transcription of subunits of peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1), a transcriptional co-activator that regulates mitochondrial biogenesis and function; this finding suggests that polyglutamine-mediated transcriptional dysregulation is associated with mitochondrial dysfunction (17). Mitochondrial dysfunction is a common pathomechanism of polyglutamine-mediated neurodegenerative disorders, and the transcriptional repression of PGC-1 caused by mutant huntingtin is also reported in Huntington’s disease (19).

Peroxisome proliferator-activated receptor-γ (PPARγ) is a nuclear receptor and a ligand-activated transcription factor that regulates the expression of genes linked to a variety of physiological processes such as mitochondrial function, cell proliferation, atherosclerosis and immunity (20). Eicosanoids and nuclear receptor and a ligand-activated transcription factor that is associated with mitochondrial dysfunction (17). Mitochondrial dysfunction is a common pathomechanism of polyglutamine-mediated neurodegenerative disorders, and the transcriptional repression of PGC-1 caused by mutant huntingtin is also reported in Huntington’s disease (19).

RESULTS

The expression level of PPARγ decreases in cellular models of SBMA

To examine the alteration in the PPARγ signaling pathway in SBMA model cells, we performed immunoblotting using neuronal cells (NSC34 cells) and muscular cells (C2C12 cells) transected with a truncated AR (tAR-24Q or tAR-97Q). Immunoblotting analyses revealed that the expression levels of PPARγ were lower in tAR-97Q-transfected neuronal and muscular cells (tAR-97Q cells) than those in tAR-24Q-transfected cells (tAR-24Q cells), suggesting that the pathogenic AR protein bearing an expanded polyglutamine tract down-regulates the expression level of PPARγ (Fig. 1A–F). The expression levels of PPARγ were up-regulated by the administration of PG in NSC34 and C2C12 cells (Fig. 1A–F). On the other hand, the expression levels of AR were not altered by PG (Fig. 1A–F). Quantitative real-time polymerase chain reaction (RT-PCR) analyses showed that mRNA levels of PPARγ were lower in tAR-97Q cells of NSC34 and C2C12 cells than those in tAR-24Q cells; however, these levels were increased in cells treated with PG (Fig. 1A–F). Human neuroblastoma SH-SY5Y cells transfected with tAR-97Q had a lower luciferase activity under control of the PPARγ promoter compared with the cells transfected with tAR-24Q, indicating that the pathogenic AR inhibits the activity of the PPARγ promoter (Supplementary Material, Fig. S1A). SH-SY5Y cells, which stably express full-length AR-97Q also attenuated the activity of PPARγ promoter compared with the cells with full-length AR-24Q, and dihydrotestosterone (DHT) treatment intensifies this effect (Supplementary Material, Fig. S1B). These findings suggest that the decrease of PPARγ is associated with the nuclear accumulation of the pathogenic AR and that the transcriptional down-regulation of PPARγ by AR is, at least partially, hormone dependent. The expression levels of PPARγ in the spinal cords and skeletal muscles of autopsied specimens of SBMA patients had lower immunoreactivities to PPARγ than samples from control patients (Supplementary Material, Fig. S2A, B). We next investigated the effect of the increased expression of PPARγ on the cellular viability of tAR-97Q cells (Fig. 1G–L). The transient overexpression of PPARγ improved cellular viability and mitochondrial activity and attenuated cellular damage according to results of the lactate dehydrogenase (LDH) assay in both the NSC34 and C2C12 cells transfected with tAR-97Q. These findings led us to believe that the PPARγ agonist PG is a possible candidate for the treatment of SBMA.

Next, we analyzed the effects of PG on polyglutamine-mediated cytotoxicity in NSC34 and C2C12 cells by measuring cellular viability, cell death, Annexin V-positive cells, and LDH release. The transient overexpression of tAR-97Q resulted in diminished cellular viability and increased cell death, apoptotic cells and LDH release in NSC34 and C2C12 cells (Fig. 1M–T). Treatment with PG at a dose of 0.1 μM improved cellular viability of both cell lines (Fig. 1M, Q). Cell death, apoptotic cells and LDH release were also reduced by treatment with 0.1 μM PG (Fig. 1N–P, R–T). Cellular viability and cytotoxicity assays using primary cortical neurons showed similar findings (Supplementary Material, Fig. S3A, B). We further confirmed the beneficial effects of PG on hormone-dependent pathogenesis in SBMA. Pioglitazone improved the cell viability and attenuates apoptosis of a cellular model of SBMA, which stably expresses full-length AR-97Q and was treated with DHT (Supplementary Material, Fig. S4A, B). In contrast, the knock-down of PPARγ via RNAi increased LDH release and decreased mitochondrial activity in the NSC34 and C2C12 cells (Supplementary Material, Fig. S5A–C). Moreover, the effect of PG treatment was not seen when PPARγ was knocked-down, indicating that the neuroprotection by PG is dependent on PPARγ. To confirm the beneficial effects of PG on neural function, we measured the length of the axons of treated and untreated NSC34 cells (Supplementary Material, Fig. S6A, B). The axons of tAR-97Q cells were shorter than those of tAR-24Q cells, and this phenotype was improved by treatment with 0.1 μM PG.

Pioglitazone improves the histopathological findings of the spinal cord and skeletal muscle of SBMA

To test the effects of PPARγ activation in vivo, PG was administered to 6-week-old SBMA (AR-97Q) transgenic mice at
concentrations of 0.01 and 0.02% in the feed until the end of analysis. No differences in feed intake were observed among the untreated, 0.01% PG-treated and 0.02% PG-treated mice at 8 weeks (data not shown). The AR-97Q mice fed the 0.02% PG-treated diet consumed 26.5 ± 1.04 mg/kg/day of PG at 8 weeks and 24.3 ± 4.44 mg/kg/day at 12 weeks. The oral administration of 0.02% PG from 6 weeks of age onward improved the body weight, performance on the rotarod task, grip power and lifespan of AR-97Q mice, although PG administered at a 0.01% dose did not improve the AR-97Q phenotype (Fig. 2A–D). Pioglitazone at 0.02% also limited the muscle atrophy and improved the stride length of the AR-97Q mice.

Figure 1. Pioglitazone (PG) improves the viability of cellular models of SBMA. (A–F) The protein and mRNA levels of PPARγ in NSC34 cells (A–C) and C2C12 cells (D–F) transfected with tAR-24Q or tAR-97Q and treated with or without PG. The ratio of PPARγ levels to monomeric and oligomeric AR levels (B, E). Quantitative analysis of PPARγ and AR was performed using densitometry. The PPARγ mRNA levels were measured using RT-PCR (C, F) (n = 3 per group). (G–L) The viability, LDH release and mitochondrial activity of NSC34 (G–I) and C2C12 cells (J–L) co-transfected with tAR-97Q and a mock or PPARγ vector (n = 3 per group). (M–P) The viability, cell death, Annexin V-positive cells and LDH release of NSC34 cells (M–P) and C2C12 cells (Q–T) transfected with tAR-24Q or tAR-97Q and treated with or without PG (n = 6 per group). Error bars indicate s.e.m. *P < 0.05 and **P < 0.01 by unpaired t-test (G–L) or ANOVA with Dunnett’s test (B, C, E, F, M–T).
On the other hand, the administration of 0.02% PG had no detectable effects on the phenotypes of wild-type mice (Supplementary Material, Fig. S7A–D). There was a tendency that wild-type mice treated with PG gained weight compared with untreated wild-type mice, although the difference was not statistically significant (Supplementary Material, Fig. S7A–D). On the other hand, PG treatment increased the amount of food intake of the wild-type mice at the beginning of the treatment, but this effect faded with aging: 132.6 ± 2.5 mg/g/day of the diet without PG, and 148.5 ± 2.3 mg/g/day of the 0.02% PG-treated diet at 8 weeks (P < 0.05 by unpaired t-test); 118.2 ± 5.0 mg/g/day of the diet without PG, and 108.8 ± 10.4 mg/g/day of the 0.02% PG-treated diet at 12 weeks (P > 0.05).

We also examined the effects of PG when the administration was initiated after the onset of neurological symptoms. The oral administration of 0.02% PG from 8 weeks of age onward improved the body weight, performance on the rotarod task, grip power and lifespan of AR-97Q mice; however, its effects on survival were weaker than those observed when the treatment was initiated at 6 weeks of age. Specifically, the lifespan of the
mice treated at 6 and 8 weeks was 52.9 and 31.5% longer, respectively, than that of the untreated AR-97Q mice (Supplementary Material, Fig. S8A–D).

Next, we examined the biological effects of PPARγ-targeted therapy on AR-97Q mice. In agreement with the results of the cellular experiments, quantitative analyses using densitometry revealed that the expression levels of PPARγ were lower in spinal cords of untreated AR-97Q mice than those of wild-type mice; however, these levels were increased by PG treatment (Fig. 2H, I). Little difference was observed in the expression levels of AR in spinal cords of untreated and PG-treated AR-97Q mice (Fig. 2J, K). Similar findings were also observed in the skeletal muscle of AR-97Q mice (Fig. 2L–O). PPARγ mRNA levels were also lower in the spinal cords and skeletal muscles of untreated AR-97Q mice than in those of wild-type mice; these levels were also up-regulated in PG-treated AR-97Q mice (Fig. 2P, Q). The mRNA levels of PGC1α in the spinal cord were lower in untreated AR-97Q mice than those in wild-type mice. Although not significant, there was a trend that PG treatment increases the mRNA level of PGC1α in the spinal cord of AR-97Q mice (Supplementary Material, Fig. S9).

To investigate the pathological changes underlying the change in phenotype induced by PG, we performed immunohistochemistry on the spinal cords and skeletal muscles of wild-type, untreated AR-97Q and PG-treated AR-97Q mice. The number of 1C2-positive cells in the spinal cords and skeletal muscles was not significantly different between untreated and PG-treated AR-97Q mice (Fig. 3A–D). Immunohistochemistry for choline acetyltransferase (ChAT) in the anterior horn of the spinal cord revealed that motor neurons were atrophied in untreated AR-97Q mice but not in wild-type mice; however, the neurons were larger in PG-treated AR-97Q mice (Fig. 3E, F). Immunoreactivity to ChAT was also restored by PG treatment. Hematoxylin and eosin staining demonstrated that skeletal muscle fibers were atrophied in untreated AR-97Q mice but not in wild-type mice; however, the neurons were larger in PG-treated AR-97Q mice (Fig. 3E). Immunohistochemistry for glial fibrillary acidic protein (GFAP—a marker of reactive astrogliosis) detected increased immunoreactivity in the anterior horn of the spinal cord of untreated AR-97Q mice; however, astrogliosis was attenuated by PG treatment (Fig. 3I, J). PPARγ immunohistochemistry in the spinal cord and skeletal muscle revealed that the signal intensity of PPARγ in the motor neurons and skeletal muscles was down-regulated in untreated AR-97Q mice and up-regulated by PG treatment (Supplementary Material, Fig. S10A–D). To evaluate the side effects of PG, blood was collected from the mice at 13 weeks to measure CK, aspartate aminotransferase, alanine aminotransferase and LDH serum levels. No abnormal values were observed in PG-treated AR-97Q mice, indicating that the oral administration of 0.02% PG did not induce systemic adverse effects (Supplementary Material, Fig. S11A–D). Fasting blood glucose levels were not significantly different among wild-type, untreated AR-97Q and PG-treated AR-97Q mice (Supplementary Material, Fig. S11E).

### Pioglitazone suppresses oxidative stress in SBMA mice

Rosiglitazone, another PPARγ agonist, has been reported to prevent mitochondrial dysfunction and oxidative stress in mutant huntingtin-expressing cells (24). We therefore investigated the effects of PG on oxidative stress to understand the molecular basis of neuronal and muscular protection conferred by PG. We measured the mitochondrial activity of NSC34 cells and C2C12 cells that were transfected with tAR-24Q or tAR-97Q and treated with or without PG (Fig. 4A, B). The mitochondrial activity of tAR-97Q cells was lower than that of tAR-24Q cells and was improved by treatment with 0.1 µM PG.

We next measured the activity of cytochrome c oxidase (CCO—a marker of mitochondrial function) in the skeletal muscles of AR-97Q mice (Fig. 4C). In total, 1.5–3.0% of fibers were negative for CCO in untreated AR-97Q mice; in contrast, almost no CCO-negative fibers were found in wild-type or PG-treated AR-97Q mice (P < 0.05 by ANOVA with Dunnett’s test for CCO-negative fibers in untreated AR-97Q mice compared with wild-type or PG-treated AR-97Q mice). To examine the expression levels of proteins related to oxidative stress, we performed immunohistochemistry using antibodies against nitrotyrosine and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the spinal cords and skeletal muscles of 13-week-old mice (Fig. 4D–G). The immunoreactivity to nitrotyrosine (a marker of oxidized protein) in the motor neurons and skeletal muscles was higher in untreated AR-97Q mice than that in wild-type mice; however, the intensity was lowered after PG treatment (Fig. 4D, E). Similar effects were also observed for 8-OHdG, a marker of oxidative stress in nucleic acids (Fig. 4F, G). Specifically, PG limited the levels of nuclear 8-OHdG, particularly in motor neurons (Fig. 4F, G). We next examined the daily urinary 8-OHdG excretion of 13-week-old mice (Fig. 4H) and found that 8-OHdG excretion was higher in untreated AR-97Q mice than that in wild-type mice and was lowered by PG treatment. In a similar manner, the urinary 8-OHdG levels of SBMA patients were reported to be significantly higher than those of control patients and strongly correlated with motor function scores (25). Furthermore, immunoblot analyses using anti-4-hydroxy-2-nonenal (HNE) antibodies in the spinal cords and skeletal muscles of 13-week-old mice revealed that the expression levels of HNE, a marker of lipid peroxidation chain reactions, in the spinal cord and skeletal muscle of untreated AR-97Q mice were up-regulated and suppressed by PG treatment (Fig. 4I–K). These results suggest that PG alleviates the oxidative stress that appears to underlie the pathogenesis of SBMA.

### Pioglitazone suppresses the activation of nuclear factor-κB (NFκB) pathway in SBMA mice

Nuclear factor-κB is a nuclear transcription factor that regulates the expression of a large number of genes that are critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation and various autoimmune disorders. Previous studies have demonstrated that PG exerts anti-inflammatory effects via the attenuation of NFκB activation in the central nervous system (26,27). The augmented expression of NFκBp50 and NFκBp65 in NSC34 cells and C2C12 cells decreased cellular viability and mitochondrial activity and increased cellular damage (Supplementary Material, Fig. S12A–D). The nuclei of spinal motor neurons and skeletal muscles in autopsied specimens of SBMA patients showed increased levels of immunoreactivity to NFκB relative to control patients (Supplementary Material, Fig. S13A, B). To evaluate the activity of the NFκB pathway in cellular and mouse models of SBMA, we performed

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immunoblotting for nuclear NFκBp65, cytoplasmic inhibitory protein-κ-Bα (IκBα) and phosphorylated IκBα (pIκBα) in NSC34 and C2C12 cells that were transfected with tAR-24Q or tAR-97Q and treated with or without PG. The expression levels of nuclear NFκBp65 and cytoplasmic pIκBα were up-regulated in untreated tAR-97Q cells compared with tAR-24Q cells but were down-regulated by PG treatment (Fig. 5A–H). The expression level of cytoplasmic IκBα was not significantly different among tAR-24Q, untreated tAR-97Q and PG-treated tAR-97Q cells. Anti-NFκBp65 and pIκBα immunohistochemistry also showed that the immunoreactivities of the spinal motor neurons and skeletal muscles were higher in untreated AR-97Q mice than those in wild-type mice and lower in the PG-treated AR-97Q mice (Fig. 6A–D). The alteration of NFκB signaling shown in cellular models was also observed in immunoblot analyses of spinal cords and skeletal muscles in 13-week-old wild-type untreated AR-97Q and PG-treated AR-97Q mice (Fig. 6E–L). These findings suggest that PG inhibits the activity of the NFκB pathway in both the spinal cord and skeletal muscle of AR-97Q mice.

Figure 3. Effect of PG on the histopathology of SBMA mice. (A–D) Immunohistochemistry with quantitative analyses for 1C2 (an anti-polyglutamine antibody) in 13-week-old mice. (E, F) Anti-ChAT immunostaining of 13-week-old mice. (G, H) Hematoxylin and eosin staining of the skeletal muscles (G) and quantitation of muscle fiber size (H) in 13-week-old mice. (I, J) Immunohistochemistry with quantitative analysis for GFAP (a marker of reactive astrogliosis) in 13-week-old mice. Quantitative analyses were performed with \( n = 3 \) per group. Error bars indicate s.e.m. Statistical analyses were performed using the unpaired t-test (B, D). *\( P < 0.01 \) by ANOVA with Dunnett’s test (F, H, J). N.S., not significant. Scale bars: 25 μm (A, C, E, G, I).
Effects of PG on microglia

PPARs also act as master regulators governing the polarization of macrophages and microglia into ‘M2’ or ‘alternative’
activation states that suppress inflammation and promote phagocytosis and tissue repair (28, 29). In contrast, microglia in ‘M1’ or ‘classical’ activation states promote the formation of the extremely toxic compound peroxynitrite, which causes damage...
to healthy tissue. Therefore, we investigated the state of microglia and the effects of PG on these cells in AR-97Q mice. Immunohistochemical analyses showed that cells in the anterior horn of the spinal cords of untreated AR-97Q mice had more microglia that were positive for CD86, an M1 glial cell surface marker, than the corresponding cells in wild-type mice; however, this phenomenon was mitigated by PG treatment (Fig. 7A, B). In contrast, PG increased immunoreactivity against Arg1, an M2 glial marker, in the microglia of the anterior horn of the spinal cords of AR-97Q mice; untreated AR-97Q mice had decreased immunoreactivities to Arg1 (Fig. 7C, D). Immunohistochemistry using anti-Iba1, a general microglial marker, demonstrated little difference in the levels of this marker among wild-type, untreated AR-97Q and PG-treated AR-97Q mice (Fig. 7E, F). Similar findings were observed in immunoblot analyses of the anterior part of the spinal cords of 13-week-old mice (Fig. 7G–J). The M1/M2 ratio was therefore markedly higher in the microglia of the anterior horn of the spinal cords of untreated AR-97Q mice than in those of wild-type mice. However, the M1/M2 ratio was restored to normal levels by PG treatment, suggesting that the release of proinflammatory molecules is associated with the pathogenesis of SBMA. In addition, PG treatment induced the phenotypic conversion of microglia from a proinflammatory M1 state to an anti-inflammatory M2 state, which is associated with neuroprotection. The results of immunohistochemical analyses revealed that there were more CD86-positive macrophages in the skeletal muscles of untreated AR-97Q mice than those of wild-type mice, and this phenomenon was suppressed by PG treatment (Supplementary Material, Fig. S14A). In contrast, PG increased the number of Arg1-positive macrophage in the skeletal muscles of AR-97Q mice; untreated AR-97Q mice had less immunoreactivities to Arg1 compared with wild-type mice (Supplementary Material, Fig. S14B).

**The expression of genes related to inflammation is significantly altered in the spinal cord and muscle of PG-treated AR-97Q mice**

To understand the global molecular changes induced by the administration of PG, we prepared total mRNA samples from the spinal cords and skeletal muscles of 13-week-old untreated AR-97Q and PG-treated AR-97Q mice and performed gene expression analyses. The microarray analyses found that the expression levels of 83 genes and 422 genes were significantly increased (>2-fold and >3-fold, respectively) in the spinal cords and skeletal muscles, respectively, of PG-treated mice (P < 0.05) (Supplementary Material, Fig. S15, S16). We then performed functional analysis using gene ontology (GO) of these genes, classifying them into several functional categories: immune response, extracellular matrix, cell adhesion, metabolism and others (Fig. 8A, B). As for the down-regulated genes, there were no GO terms, the frequency of which was found to be significantly decreased (less than one-half in the spinal cords and less than one-third in the skeletal muscles). Genes related to the immune response and extracellular matrix were predominantly altered by PG treatment, suggesting that the PPARγ agonist modulates the inflammatory response (especially along the NFκB pathway). The results also demonstrated that genes from similar functional categories are found in the spinal cord and skeletal muscle, indicating that PG attenuates the toxicity of polyglutamine-expanded AR in both neural and muscular tissues via a similar mechanism (Supplementary Material, Fig. S17A, B).

**DISCUSSION**

Recent studies have shown that mitochondrial dysfunction and oxidative stress-mediated neuronal toxicity are implicated in
various neurodegenerative diseases, including Huntington’s disease, amyotrophic lateral sclerosis (ALS) and Friedreich’s ataxia (30–35). In the present study, we demonstrated that the mRNA and protein levels of PPAR<sub>g</sub>, a regulator of mitochondrial function, were decreased in mouse and cellular models of SBMA. The results of reporter assay showed that the pathogenic AR suppressed the activity of PPAR<sub>g</sub> promoter. The mRNA levels of PGC1<sub>a</sub>, the co-activator of PPAR<sub>g</sub>, were also downregulated in the spinal cord of our mouse model of SBMA, as reported in another mouse model of this disease (17). Decreased mRNA expression of PGC1<sub>a</sub> and PGC1<sub>a</sub>-regulated factors is also reported in the ALS mouse model and in human sporadic ALS, suggesting that mitochondrial dysfunction is a common pathological feature of motor neuron diseases (36,37). We also demonstrated that the overexpression of PPAR<sub>g</sub> and the administration of PG, a PPAR<sub>g</sub> agonist, improved the viability of cellular models of SBMA. Moreover, the oral administration of PG to SBMA mice improved neurological symptoms and histopathological findings. Pioglitazone mitigated oxidative stress, mitochondrial dysfunction and the activation of the NFκB pathway in mouse and cellular models of SBMA, providing a mechanistic basis for this treatment. In addition, PG modulated microglial populations in the spinal cord of the SBMA mice. These findings suggest that the PPAR<sub>g</sub> pathway can inhibit oxidative stress, the NFκB pathway and inflammation in SBMA. PPAR<sub>g</sub> agonists have been proven effective in mouse and cellular models of neurodegenerative disorders, trauma and stroke (21,24,27,38–45). For example, PG has been shown to

Figure 6. Effect of PG on NFκB signals of SBMA mice. (A–D) Immunohistochemistry for NFκBp65 (A, B) and pIκBa (C, D) in 13-week-old mice. (E–L) ImmunobLOTS for nuclear NFκBp65, cytoplasmic pIκBa and IκBa in the spinal cords (E–H) and skeletal muscles (I–L) of 13-week-old mice. Quantitative analyses were performed using the densitometry of NFκBp65 (F, J), pIκBa (G, K) and the ratio of pIκBa to IκBa (H, L) (n = 3 per group). Error bars indicate s.e.m. * P < 0.05 by ANOVA with Dunnett’s test (F–H, J–L). Error bars indicate s.e.m. * P < 0.05 by ANOVA with Dunnett’s test (F–H, J–L). Scale bars: 25 μm (A–D).
be neuroprotective in the G93A SOD1 transgenic mouse model of ALS (38), improving motor performance and increasing survival by reducing microglial activation and gliosis. Despite the promising preclinical trials using SOD1 mouse model, PG had no beneficial effects on the survival of ALS patients as add-on therapy to riluzole, indicating the difficulty of the translation from mouse models to human (46).

Pioglitazone has also been shown to reduce iNOS, NFκB and 3-nitrotyrosine immunoreactivity, and several of these findings were replicated in the present study. Pioglitazone treatment also improved motor function and mitigated oxidative stress and mitochondrial enzyme activity in a rat model of Huntington’s disease induced by quinolinic acid (40). However, the administration of rosiglitazone to R6/2 transgenic Huntington’s disease mice did not alter the course of survival or weight loss of the animals, possibly because insufficient levels of rosiglitazone were administered to sustain effective therapeutic levels or prevent rapid neurodegeneration (47).

Interestingly, in the present study, similar molecular changes related to oxidative stress and NFκB activation were observed in the motor neurons and skeletal muscles of SBMA mice. Microarray analyses showed that PG mitigated

Figure 7. Effect of PG on the microglia of SBMA mice. (A–F) Immunohistochemistry with quantitative analyses of CD86 (A, B), Arg1 (C, D) and Iba1 (E, F) in 13-week-old mice. The arrows indicate the CD86- (A) and Arg1- (C) positive cells. Quantitative analyses of the ratio of CD86- (B), Arg1- (D) and Iba1- (F) positive cells in the anterior horn were performed with n = 3 per group. (G) Immunoblots for CD86, Arg1 and Iba1 in the anterior part of the spinal cord from 13-week-old mice. (H–J) Quantitative analysis using densitometry of CD86 (H), Arg1 (I) and Iba1 (J) (n = 3 per group). Error bars indicate s.e.m. * P < 0.05 by ANOVA with Dunnett’s test (B, D, F, H–J). N.S., not significant. Scale bars: 25 μm (A, C, E).
These cellular events in both neuronal and muscular tissues. These effects were also observed in cellular experiments using neuronal and muscular cell lines. The results of the present study suggest that PG has direct effects on both neuronal and muscular degeneration in SBMA and that skeletal muscle is an important target for therapies that alleviate neuromuscular symptoms of SBMA.

Although we tested the ability of PG to modify mitochondrial function, our results also indicate that oxidative stress, the activation of the NFκB pathway and neuroinflammation play important roles in the pathogenesis of SBMA. Mitochondrial dysfunction, NFκB activation and the M1 microglial phenotype are known to be connected in several ways. For example, mitochondrial toxins caused by primary damage to the mitochondrial respiratory chain induce microglial activation and neuroinflammatory processes. Proinflammatory cytokines (such as tumor necrosis factor-α) released by M1 microglia alter the morphology and function of mitochondria (48,49). Furthermore, NFκB was recently identified as a physiological regulator of mitochondrial respiration, and NFκB-induced oxidative stress is thought to contribute to mitochondrial dysfunction in diabetic mice (50,51). NFκB activation in microglia also regulates inflammatory processes that exacerbate diseases such as ischemia and Alzheimer’s disease (52). These findings implicate mitochondrial dysfunction, NFκB and microglial alteration in the pathogenesis of neurodegenerative diseases.

The results of the present study provide important insights into the NFκB-mediated pathogenesis of SBMA. The NFκB pathway was activated in the mouse and cellular models and in patients with SBMA. The overexpression of NFκBp50 or p65, key molecules of the signaling pathway, induced cellular damage in neuronal and muscular cells; however, PG treatment suppressed the activity of the NFκB pathway in the mouse and cellular models of SBMA. These findings suggest that NFκB signaling plays an important role in the pathogenesis of SBMA. NFκB activation has been implicated in the pathogenesis of various neurodegenerative disorders (35,53,54), and this pathway appears to be a commonly considered target of therapy for devastating neurological diseases.

Neuroinflammation is a common pathological feature of many neurodegenerative diseases and appears to play an important role in the non-cell autonomous pathogenesis (55,56) of several animal models of neurodegenerative disease. In animal models of ALS, lowering mutant superoxide dismutase 1 (SOD1) expression levels in microglia using the Cre–Lox recombination system significantly extended the survival of transgenic mice carrying a mutant human SOD1G37R gene (57–59). Moreover, microglia in the ALS mouse model appear to switch from an M2 state to an M1 state as the disease progresses (58,59). The present results suggest that PG exerts a neuroprotective effect on SBMA mice via the modulation of inflammation.

In conclusion, we demonstrated that PPARγ is down-regulated in SBMA and that the activation of this nuclear receptor by PG mitigates oxidative stress, NFκB activation and neuroinflammation and improves the symptoms and pathological findings of SBMA mice. Our results corroborate the decrease in PPARγ expression and the increases in oxidative stress and NFκB activity observed in autopsy samples from SBMA patients. Given that PG is already used as an oral-hyperglycemic drug and crosses the blood–brain barrier, this drug appears to be a safe and effective therapy for SBMA and other neurodegenerative diseases.
MATERIALS AND METHODS

Cell culture and transfection

Mouse NSC34 motor neuron-like cells (kindly provided by N.R. Cashman, University of British Columbia, Vancouver, Canada) and mouse C2C12 myoblast cells (DS Pharma Biomedical, Osaka, Japan) were cultured in a humidified atmosphere of 95% air/5% CO2 in a 37°C incubator in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). DMEM/F12 with 10% FBS was used to culture SH-SY5Y human neuroblastoma cells (ATCC No. CRL-2266) and those stably expressing the human full-length AR-97Q. The plasmids were transfected using OPTI-MEM (Gibco, Germany) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. NSC34 cells were differentiated in DMEM for 96 h. Fetal horse serum (2%) was added to the medium for 48 h to differentiate the C2C12 cells. SH-SY5Y cells were differentiated in DMEM/F12 supplemented with 2% FBS and 20 μM retinoic acid. SH-SY5Y cells stably expressing AR-97Q were differentiated in DMEM/F12 supplemented with 20 μM retinoic acid and 1 mM 5α-dihydrotestosterone as described earlier (6).

Plasmid constructs and siRNA

The pEVRF-p65 vector encoding the murine RelA protein and the pCMV4-p50 vector encoding the NFκBp50 subunit were kindly provided by Drs. Genevieve Soucy and Jean-Pierre Julien (Department of Psychiatry and Neuroscience, Laval University, Research Centre of CHUL, Canada). The pcDNA3.1-PPARγ expression vector was cloned as previously described (60). The pCR3.1–AR–24Q and pCR3.1–AR–97Q plasmids were cloned as previously described (61). This truncated protein consisted of an N-terminal fragment of human AR containing 24 or 97 CAG repeats (1–645 bp and 1–864 bp, respectively) subcloned into pcDNA3.1 (6,62,63). To knock-down PPARγ, the following oligonucleotide siRNA duplexes were synthesized by Invitrogen and used to transfect the NSC34 and C2C12 cells: sense sequence, CAGAGCAAAGAGGUGGCCAUCCGAA; antisense sequence, UUCCGAGUGCACUCCUUCGUCUG. We used Stealth RNAi negative control duplex (Invitrogen) as the control siRNA. The NSC34 and C2C12 cells were transfected with the siRNA oligonucleotide duplex using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Cell viability, toxicity and apoptosis assays

The cell viability assays were performed using WST-8 (Roche Diagnostics, Mannheim, Germany). The cells were cultured in 24-well plates. Twenty-four hours after each treatment with the indicated concentrations of PG, the cells were incubated with the WST-8 substrate for 3–4 h and spectrophotometrically assayed at 450 nm using a plate reader. For detecting apoptotic cells, we used Multi-Parameter Apoptosis Assay Kit (Cayman Chemical, MI, USA). The cells were cultured in 96-well black plates. After the treatment, plate reader fluorescence detection was performed according to the manufacturer’s instructions.

Mitochondrial activity assay

The assessment of mitochondrial membrane potential was determined using MitoTracker™ green FM (Invitrogen) dyes. The dyes passively diffuse across the plasma membrane and accumulate in active mitochondria, whose accumulation is dependent upon membrane potential. The cells were incubated with 100 nM of the dyes for 30 min at 37°C in serum-free DMEM and washed with pre-warmed serum-free DMEM. The cells were then observed using a fluorescence microplate reader (Powerscan HT) at excitation and emission wavelengths of 490 and 516 nm, respectively.

Animals

AR-97Q (Line #7–8) male mice were bred and maintained as previously described (5,64). They have backcrossed at least 15 generations to C57BL/6 before used in the present study. The mice were genotyped by PCR using DNA from their tail (5). In the experiments, PG was administered at concentrations of 0.01 or 0.02% in the feed from 6 or 8 weeks of age until the end of the analysis, unless otherwise mentioned. Only males were used in this study. The litters were randomly allocated to PG-containing or normal chow. For pathological and biochemical analyses, the feed was administered to the mice beginning at 6 weeks of age.

Behavioral analysis

All of the tests were performed on a weekly basis, and the data were analyzed prospectively. The rotarod performance was assessed weekly using an Economex Rotarod (Ugo Basile, Comerio, Italy) as previously described (64). The grip strength was measured with a Grip Strength Meter (MK-380M, Muromachi Kikai, Tokyo, Japan) as described elsewhere (65).

Immunoblotting

Mice anesthetized with ketamine–xylazine were perfused with 4% paraformaldehyde fixative in phosphate buffer (pH 7.4). The tissues (whole brain, spinal cord, brainstem and skeletal muscle) were dissected and snap-frozen with powdered CO2 in acetone. The tissues were homogenized in buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS and 1 mM 2-mercaptoethanol with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA, USA) and centrifuged at 2500 × g for 15 min. The cultured cells were also lysed in the same reagent after intervention. NE-PER Nuclear Cytoplasmic Reagents (Thermo Scientific) were used for the analysis of the NFκB pathway. Equal amounts of protein were separated by 5–20% SDS–PAGE gels and transferred to Hybond-P membranes (GE Healthcare, Piscataway, NJ, USA). Primary antibody binding
was probed with horseradish peroxidase-conjugated secondary antibodies at a dilution of 1 : 5000, and the bands were detected using an immunoreaction enhancing solution (Can Get Signal; Toyobo, Osaka, Japan) and enhanced chemiluminescence (ECL Prime; GE Healthcare). An LAS-3000 imaging system (Fujiﬁlm, Tokyo, Japan) was used to produce digital images. The signal intensities of these independent blots were quantiﬁed using IMAGE GAUGE software version 4.22 (Fuji) and expressed in arbitrary units. The membranes were reprobed with an anti-GAPDH (MAB374, 1 : 5000; Santa Cruz) antibody or Histone H1 (#05-457, 1 : 1000; Millipore, Billerica, MA, USA) for normalization.

Histology and immunohistochemistry

The mouse tissues were dissected, post-ﬁxed in 10% phosphate-buffered formalin and processed for parafﬁn embedding. The sections to be stained with the anti-polyglutamine antibody (1C2) were treated with formic acid for 5 min at room temperature. The sections to be incubated with the anti-ChAT, GFAP, nitrotyrosine, pIκBα and Iba-1 antibodies were boiled in 10 mM citrate buffer for 15 min. The speciﬁcity of the monoclonal antibody against 8-OHdG (N45.1) has been characterized in a previous study (66). The sections to be incubated with the anti-8OHdG antibody were boiled in 0.1 M glycine buffer (pH 2.2) for 15 min. The sections to be incubated with an anti-NFxBp65 antibody were autoclaved using the 2100-Retriever (Funakoshi Corporation, Tokyo, Japan). Primary antibody binding was probed with a secondary antibody labeled with a polymer as part of the Envision+ system containing horseradish peroxidase (Dako Cytomation, Go¨ttingen, Denmark). The immunohistochemical sections were photographed with an optical microscope (Axio Imager M1, Carl Zeiss AG, Göttingen, Germany). The immunoreactivity and cell sizes were analyzed with WinROOF (Mitani, Tokyo, Japan). The means ± s.e.m. were expressed in arbitrary units. For hematoxylin and eosin (H&E) staining, 6-μm-thick sections of the gastrocnemius muscles were air-dried and stained.

Quantitative analysis of immunohistochemistry

To assess 1C2-, CD86-, Arg1- and Iba1-positive cells, at least 50 consecutive 6-μm-thick axial sections of the thoracic spinal cord and skeletal muscle were prepared, and every ﬁfth section was immunostained with each antibody. The numbers of 1C2-positive cells were counted in all of the neurons of the anterior horn of the 10 axial sections from the thoracic spinal cord of each group of mice (n = 3) under a light microscope (Bx51; Olympus, Tokyo, Japan). For the purposes of counting, we deﬁned a motor neuron by its presence within the anterior horn and the obvious nucleolus in a given 6-μm-thick section. The numbers of 1C2-positive cells in skeletal muscles were calculated for >500 ﬁbers in randomly selected areas of the 10 axial sections. To quantify the expression levels of nitrotyrosine, 8OHdG, NFκBp65 and pIκBα, we performed immunohistochemistry on every ﬁfth section of the 25 consecutive sections. We measured the intensity of nuclear immunoreactivities for nitrotyrosine and pIκBα in the thoracic anterior horn of the 5 axial sections and in >500 cells in 5 randomly selected ×400 microscopic ﬁelds of the 5 sections of skeletal muscles from each group of mice (n = 3). We calculated the intensities by multiplying the staining concentration by cell sizes or nuclear sizes, which were quantiﬁed with WinROOF. To quantify the size of the motor neurons and the region of anti-GFAP immunoreactivity in the spinal anterior horn, we analyzed every ﬁfth section of the 25 consecutive 6-μm-thick axial sections from the thoracic spinal cord using an image analyzer (WinROOF). To calculate the cell size of the skeletal muscles, >500 H&E stained ﬁbers in randomly selected areas were examined using an image analyzer (WinROOF).

Cytochrome c oxidase staining of skeletal muscle

Cryostat sections (6 μm) were ﬁrst incubated in the medium containing 0.1% manganese chloride, 0.1% hydrogen peroxide and 5.5 mM of diaminobenzidine tetrahydrochloride in 0.1 M sodium acetate pH 5.6 at 37°C for 60 min. The sections were washed in distilled water, incubated in 1% copper sulfate medium for 5 min, dehydrated in a graded ethanol series (70, 80, 90 and 100%) and cleared in xylene.

Quantitative RT-PCR

Total RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen) and from mouse spinal cords and skeletal muscles using TRIzol (Invitrogen). The extracted RNA was then reverse-transcribed into ﬁrst-strand cDNA using the SuperScript III First-Strand synthesis system (Invitrogen). RT-PCR was performed in a total volume of 25 μl that contained 12.5 μl of 2× QuantiTect SYBR Green PCR Master Mix and 0.3 μl of each primer (Sigma–Aldrich, MO, USA); the ampliﬁed products were detected with the iCycler system (Bio-rad Laboratories, Hercules, CA, USA). The reaction conditions were as follows: 95°C for 1 min, 40 cycles of 15 s at 94°C, 15 s at 59°C and 30 s at 72°C. The expression level of the internal control β2 microglobulin was simultaneously quantiﬁed. The following primers were used: 5′ ctgtgacaccagacgctg 3′ and 5′ aatgaggattctcct 3′ for the cell and mouse PPARγ, 5′ acagctccaa gagcaggaaa 3′ and 5′ ctaaatgctcctcat 3′ for the mouse PGC1α, 5′ ggtagggactgataagaagc 3′ and 5′ aaggggactaactgaacgc 3′ for the cell and mouse β2 microglobulin. The weight of the gene contained in each sample was equal to the log of the starting quantity, and the standardized expression level of each cell and mouse was equal to the weight ratio of each gene to that of β2 microglobulin.

Microarray analysis of the mouse spinal cord and skeletal muscle

Gene expression in the spinal cords and skeletal muscles of untreated and PG-treated AR-97Q mice was assessed using a SurePrint G3 Mouse GE 8 × 60K Microarray (Agilent Technologies, Santa Clara, CA, USA). For each group (untreated AR-97Q and PG-treated AR-97Q), we examined the male mice at 13 weeks of age. Most of the 13-week untreated AR-97Q mice were very weak and had experienced profound muscle atrophy. The RNA from the spinal cord and skeletal muscle was isolated from three mice of each group using TRIzol (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer’s speciﬁcations. The RNA samples were puriﬁed using the PureLink™ RNA Mini Kit (Life Technologies Corporation). The cDNA
We analyzed the data using the unpaired Student’s t-test for two group comparisons and analysis of variance (ANOVA) with PASW Statistics 18 (Chicago, IL, USA). The survival rate was analyzed by Kaplan–Meier and log-rank tests using STATVIEW software version 5 (Hulinks, Tokyo, Japan). We denoted P-values of 0.05 or less as statistically significant.

Study approval

The collection of autopsied human tissues and their use for this study were approved by the Ethics Committee of Nagoya University Graduate School of Medicine (No. 902-3), and written informed consent was obtained from the patients’ next-of-kin. Experimental procedures involving human subjects were conducted in conformance with the principles expressed in the Declaration of Helsinki. All of the animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under the approval of the Nagoya University Animal Experiment Committee (No. 25087).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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REFERENCES

14. Yu, Z., Dadgar, N., Albertelli, M., Grus, K., Jordan, C., Robins, D.M. and Lieberman, A.P. (2006) Androgen-dependent pathology demonstrates preparation, hybridization process and microarray data analysis were performed by TAKARA BIO (Otsu, Japan). The quality of total RNA was assessed with the Agilent 2100 Bioanalyzer, and the RNA integrity number values showed sufficiently high quality (ranging from 8.7 to 9.2). Agilent one-color microarray-based gene expression profiling was used to detect differential gene expression in the spinal cords and skeletal muscles of untreated and PG-treated AR-97Q mice. Array image analysis and the calculation of spot intensity measurements were performed with Agilent’s Feature Extraction Software. The criteria used to detect the differences in gene expression were a 2-fold and 3-fold change in the spinal cords and muscles, respectively, of PG-treated AR-97Q mice compared with untreated AR-97Q mice. The expression profiles were analyzed using GeneSpring 12.5 software (Silicon Genetics, CA, USA). The microarray profiling data were deposited in the Gene Expression Omnibus data base with the accession number GSE51807.

Urinary analyses of the AR-97Q mice

The mice were placed in metabolic cages, and urine was collected over a 24-h period. The concentration of 8-OHdG was measured with an ELISA kit for 8-hydroxy-2′-deoxyguanosine (Japan Institute for the Control of Aging (JaICA), Shizuoka, Japan) and that of creatinine was measured with an ELISA kit (Japan Institute for the Control of Aging (JaICA), Shizuoka, Japan) and that of creatinine was measured with an ELISA kit for creatinine (Cayman) according to the manufacturer’s instructions. The urinary levels of 8-OHdG were normalized in relation to creatinine levels to adjust for the urine volume.

Statistical analysis

We analyzed the data using the unpaired Student’s t-test for two group comparisons and analysis of variance (ANOVA) with Dunnett’s test for multiple comparisons using PASW Statistics 18 (Chicago, IL, USA). The survival rate was analyzed by Kaplan–Meier and log-rank tests using STATVIEW software version 5 (Hulinks, Tokyo, Japan). We denoted P-values of 0.05 or less as statistically significant.

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The collection of autopsied human tissues and their use for this study were approved by the Ethics Committee of Nagoya University Graduate School of Medicine (No. 902-3), and written informed consent was obtained from the patients’ next-of-kin. Experimental procedures involving human subjects were conducted in conformance with the principles expressed in the Declaration of Helsinki. All of the animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under the approval of the Nagoya University Animal Experiment Committee (No. 25087).


