Prion 4:1, 26-31; January/February/March 2010; © 2010 Landes Bioscience

Pyrroloquinoline quinone inhibits the fibrillation of amyloid proteins

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Key words: amyloid β, amyloid fibril, cytotoxicity, fibril formation, inhibitor, prion, pyrroloquinoline quinone

Several neurodegenerative diseases involve the selective damage of neuron cells resulting from the accumulation of amyloid fibril formation. Considering that the formation of amyloid fibrils as well as their precursor oligomers is cytotoxic, the agents that prevent the formation of oligomers and/or fibrils might allow the development of a novel therapeutic approach to neurodegenerative diseases. Here, we show pyrroloquinoline quinone (PQQ) inhibits the amyloid fibril formation of the amyloid proteins, amyloid β (1–42) and mouse prion protein. The fibril formation of mouse prion protein in the presence of PQQ was dramatically prevented. Similarly, the fibril formation of amyloid β (1–42) also decreased. With further advanced pharmacological approaches, PQQ may become a leading anti-neurodegenerative compound in the treatment of neurodegenerative diseases.

Although neurodegenerative diseases are often characterized by the type of misfolded and deposited proteins involved, the exact causes of abnormal protein folding, as well as the mechanisms through which the accumulation of such proteins becomes cytotoxic, are not fully understood. However, these neurodegenerative diseases share one salient feature: a change in the conformation of the causative proteins from the natural to the β-strand-rich form, accompanied by the acquisition of an oligomeric status and the subsequent formation of supramolecular assemblies and amyloids.[1-3] Considering that the formation of amyloid fibrils as well as their precursor oligomers is cytotoxic,[4] agents that prevent the formation of oligomers and/or fibrils might allow the development of a novel therapeutic approach to these neurodegenerative diseases.[5]

Several types of oxidoreductases found in Gram-negative bacteria were found to possess PQQ as their cofactor (**Fig. 1**).[6] Due to the finding that PQQ also exists in plants and animals, and because of its inherent free-radical scavenging properties, PQQ has been drawing attention from both the nutritional and the pharmacological viewpoint.[7,8] Recently, it has been proposed that PQQ be classified as a new B vitamin.[9] Thanks to the inherent antioxidant and redox modulator property of PQQ in a variety of systems, the possible pharmacological applications of PQQ are also being investigated. Recently, PQQ has also been reported to show neuroprotective effects.[10]

We have reported that PQQ, an antioxidant, prevents the amyloid fibril formation and aggregation of α-synuclein (α-Syn) WT in vitro in a PQQ-concentration-dependent manner. Moreover, PQQ-conjugated α-Syn is also able to prevent α-Syn amyloid fibril formation.[11] The fact that PQQ shows anti-fibril-forming

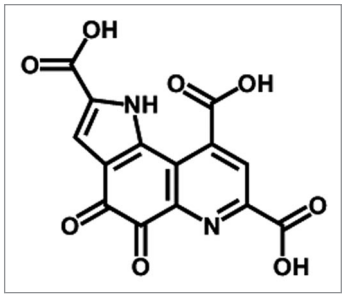

Figure 1. Structure of pyrroloquinoline quinone.

activity and that the common feature of these neurodegenerative diseases is amyloid fibril formation encouraged us to further investigate the effects of PQQ on the fibrillization of amyloid proteins, prion protein (PrP) and amyloid β (1–42) ($A\beta_{1-42}$).

The fibril formation of $A\beta_{1-42}$ was monitored by the increase in thioflavin-T (ThT) fluorescence. When monitored by ThT fluorescence in the absence of PQQ, the incubation of 50 μM $A\beta_{1-42}$ at 37°C showed a signal rise with a sigmoidal shape and a lag time of 3.8 h (**Fig. 2A**). We confirmed that there is no effect of PQQ on the intensity of ThT fluorescence in the condition of this study. Thus, we consider that the ThT fluorescence intensity means the relative amount of fibrils formed in the presence and absence of PQQ. In the presence of PQQ, fibril formation was prevented, as indicated by the reduced ThT fluorescence over the time course. The addition of 100 or 300 μM PQQ resulted in the

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Submitted: 06/16/09; Accepted: 12/04/09
Previously published online: www.landesbioscience.com/journals/prion/article/10889

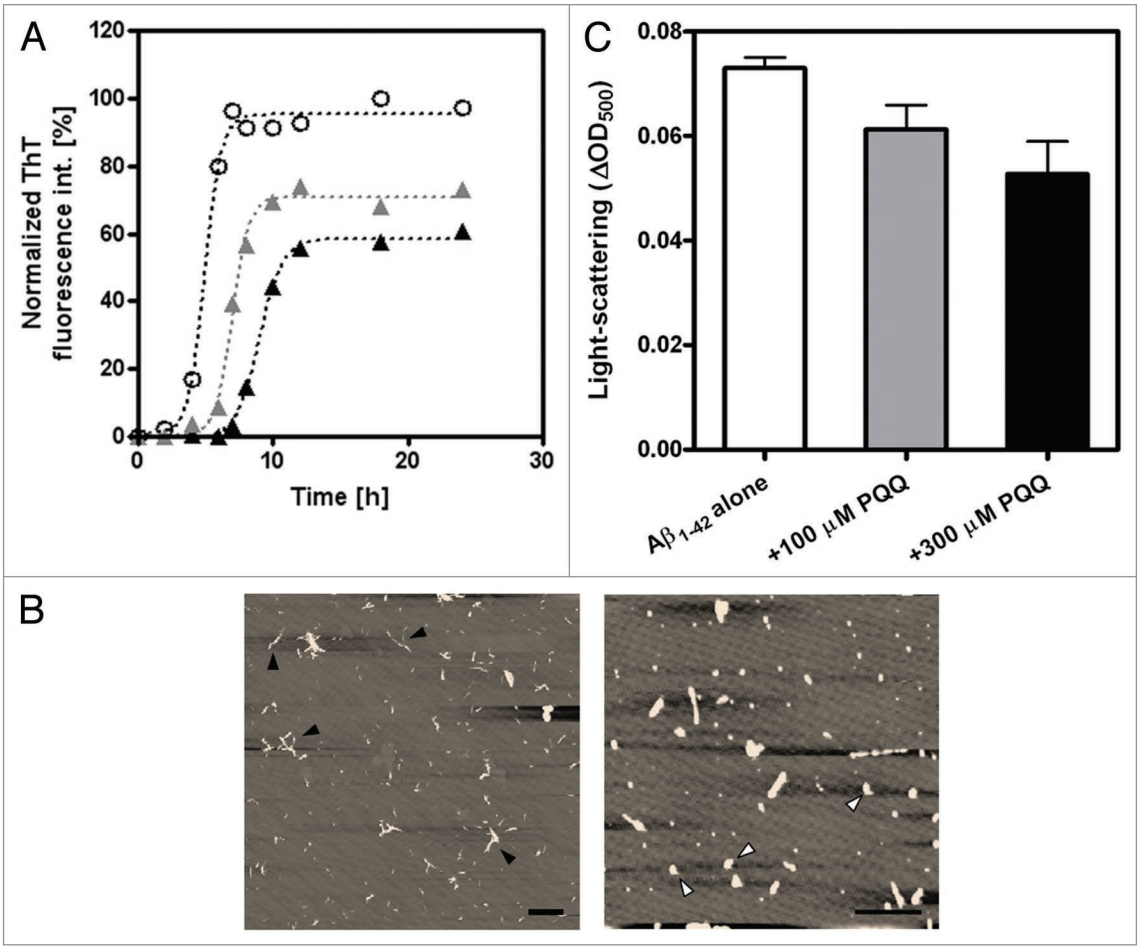

Figure 2. Inhibitory effect of PQQ on the fibril formation of amyloid β (1–42). (A) The time courses of amyloid fibril formation of $A\beta_{1-42}$ as determined by ThT fluorescence assay analysis. No additive (50 μM $A\beta_{1-42}$) (white circles), +100 μM PQQ (gray triangles), +300 μM PQQ (black triangles). The sigmoidal curve analysis was performed by PRISM (GraphPad Software). (B) AFM revealed the formation of amyloid fibrils, with short fibrillar morphology, having a diameter of 6~8 nm and a length of 0.5~1 μm (Left; 50 μM Aβ1-42). In the presence of PQQ, there are amorphous aggregates having a diameter of 4~6 nm and length of 10~500 nm were detected (Right; 50 μM $A\beta_{1-42}$ + 300 μM PQQ). Scale bars: 1 μm. All images are a height mode. (C) The effect of PQQ on the aggregation of $A\beta_{1-42}$ was monitored by light scattering at 500 nm after 20 h incubation, n = 2. 50 μM $A\beta_{1-42}$ (white bar), 50 μM $A\beta_{1-42}$ + 100 μM PQQ (gray bar), 50 μM $A\beta_{1-42}$ + 300 μM PQQ (black bar).

formation of less than 80% and 60% of the fibrils formed in the absence of PQQ and the lag time calculated from fitting-curve was increased to 5.8 h and 7.3 h, respectively.

In order to observe possible formation of amorphous aggregate, the light scattering observation was carried out with the $A\beta_{1-42}$, in the presence or absence of several concentration of PQQ (**Fig. 2C**). The formation of aggregations is monitored at 500 nm, as PQQ has a typical absorbance at around 340 nm; unlikely with the conventional light scattering observation employing 330 nm. In the absence of PQQ, the $A\beta_{1-42}$ alone increased the light scattering with time, indicating the formation of aggregates. In the presence of PQQ, however, the light scattering increased. After 20 h of incubation, the observed light scattering was 15% with 100 μM PQQ or 30% with 300 μM PQQ lower compared to those observed in the absence of PQQ. Therefore, it indicated that PQQ decreased the aggregation of $A\beta_{1-42}$ protein in dose-dependent manner, but the effect was less significant as effect of PQQ on α-synuclein.[11]

Furthermore, we performed the AFM observation to confirm the inhibitory effect of PQQ on fibril formation of $A\beta_{1-42}$ AFM observation of 25 h incubated $A\beta_{1-42}$ revealed the formation of amyloid fibrils which are shorter than other studies, having a diameter of 7~8 nm and a length of 0.3~1 μm (**Fig. 2B**, left, black head). The sample incubated in the presence of PQQ, almost amorphous aggregates (**Fig. 2B**, right, white head) like other reports[12-14] having a height of 4–6 nm and a length of 10~500 nm were detected. These results demonstrate that PQQ inhibits the fibril formation of $A\beta_{1-42}$,[15] but amorphous aggregated formed, which is consistent with the light scattering observation (**Fig. 2C**).

Previous studies have shown that the intermediate protofibril of amyloid protein is more toxic than the fibril itself.[16-18] It is therefore important to precisely evaluate the effect of PQQ on the cytotoxicity of amyloid proteins in developing a viable therapeutic strategy, because if the aggregates formed as a result of the prevention of fibril formation are more toxic than the fibrils

themselves, it is possible that PQQ will function as a risk factor in neurodegenerative diseases. We evaluated the effect of PQQ on the cytotoxicity of amyloid proteins in cultured PC12 cells (**Fig. 3**). 50 μM $A\beta_{1-42}$ samples were incubated in the absence or presence of 100 μM PQQ. In PC12 cells treated for 12 h with $A\beta_{1-42}$ incubated in the absence of PQQ, there was a ~40% loss of cell viability compared to the untreated control. By contrast, there was a ~25% loss of cell viability as a result of the addition of $A\beta_{1-42}$ incubated in the presence of PQQ. These results suggest that PQQ significantly decreased the cytotoxicity of $A\beta_{1-42}$.

We used ThT fluorescence to monitor the kinetics of fibril formation of recombinant mouse PrP by the cell-free conversion system.[19-21] PrP amyloid formation will be achievable using ScPrP, which requires strictly controlled experimental environment adopted with safety regulation. Recently, Breydo et al. reported on the cell-free conversion system of PrP the aggregation in vitro. By using this system full-length recombinant PrP with an intact disulfide bond can be folded into amyloid conformation, β-sheet rich isoform. The amyloid formation of mouse PrP (4.1 μM) was carried out under solution conditions containing the denaturant which usually required for efficient conversion to β-sheet-rich isoform in vitro with continuous shaking at 900 rpm. In the absence of PQQ, typical amyloid formation occurred, with a lag time (~13 h) followed by the rapid accumulation of formed fibrils (**Fig. 4A**). In contrast, mouse PrP fibrillization significantly decreased in the presence of PQQ. The addition of 10 μM PQQ led to a decrease in fibril formation, to less than 50% of the fibrils formed in the absence of PQQ. In the presence of 200 μM PQQ, less than 10% of the fibrils formed and the lag time was increased to 19 and 33 h by addition of 10 and 50 μM PQQ; i.e., the fibril formation of mouse PrP was dramatically inhibited.

It is also supported by AFM observation of incubated sample of PrP. Mouse PrP incubated for 84 h without PQQ showed the structure like amyloid fibril that is a height of 10 nm and a length of 2~3 μm. However the PrP incubated with 440 μM PQQ, showed only small aggregates which is a height of 5~8 nm and a length of 100 nm, there is not any formation of structure like fibril during the same time (**Fig. 4B**). These results demonstrate that PQQ inhibits fibril formation of mouse PrP. Unfortunately we can't measure the cytotoxicity of mouse PrP using this in vitro evaluation system because the sample of mouse PrP contained 1 M guanidine hydrochloride and 3 M urea.

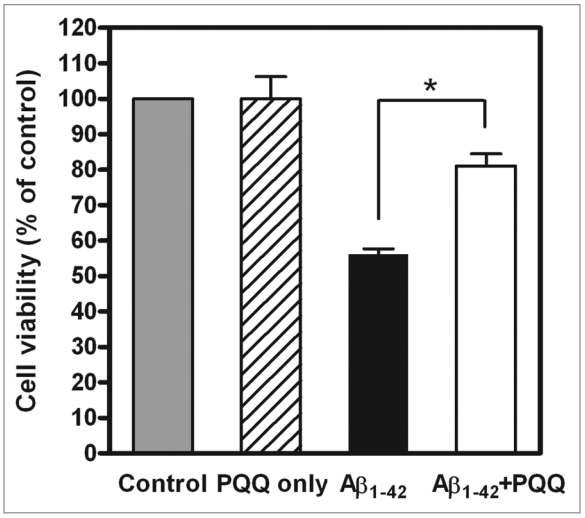

Figure 3. Effect of PQQ on cytotoxicity induced by amyloid β (1–42). Percentage of cell viability of PC12 cells added $A\beta_{1-42}$ incubated with or without PQQ from n = three independent experiments. The results are expressed as a percentage of the value of "control" (PBS) (gray bar), which is set as 100%: 10 μM PQQ only, 5 μM $A\beta_{1-42}$, 5 μM $A\beta_{1-42}$ + 10 μM PQQ. Values are expressed as mean ± SD. (t-test, *$p < 0.005$).

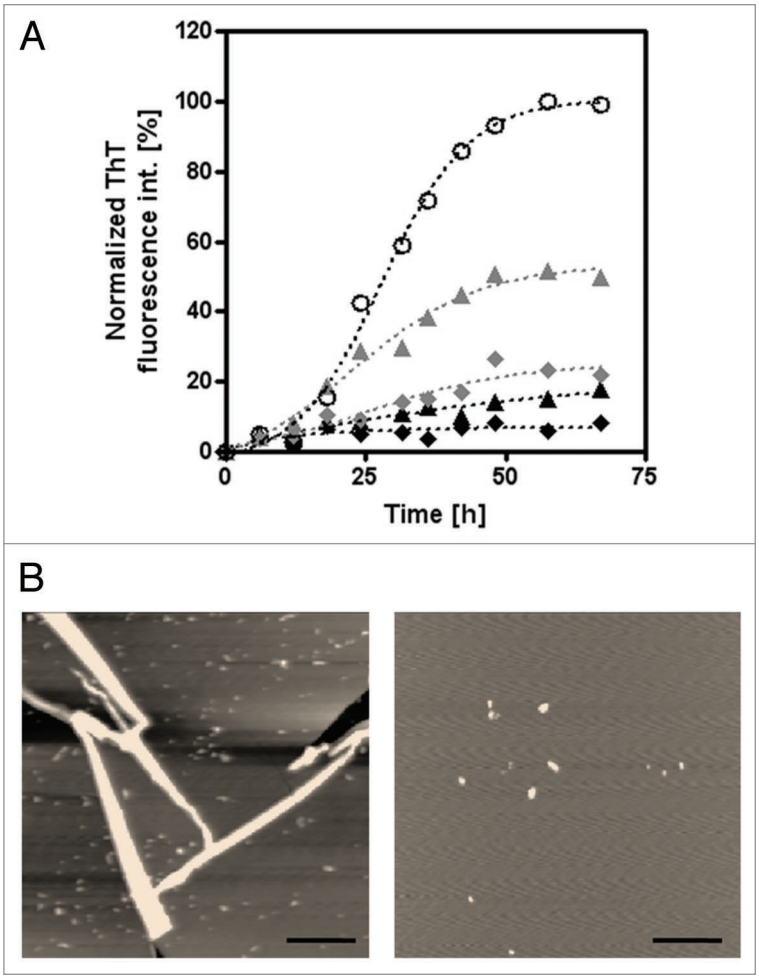

Figure 4. Inhibitory effect of PQQ on the fibril formation of mouse prion. (A) The time courses of amyloid fibril formation of mouse PrP as determined by ThT fluorescence assay analysis. No additive (4.1 μM rPrP) (white circles), +10 μM PQQ (gray triangles), +50 μM (gray diamonds), +100 μM (black triangles), +200 μM PQQ (black diamonds). The sigmoidal curve analysis was performed by PRISM (GraphPad Software). (B) In the AFM observation of PrP, it showed the typically fibrils, having a diameter of 10 nm and a length of 2~3 μm (Left; 5 μM mouse PrP). However, there are small aggregates observed in the presence of PQQ (Right; 5 μM mouse PrP + 440 μM PQQ). Scale bars: 1 μm. All images are a height mode.

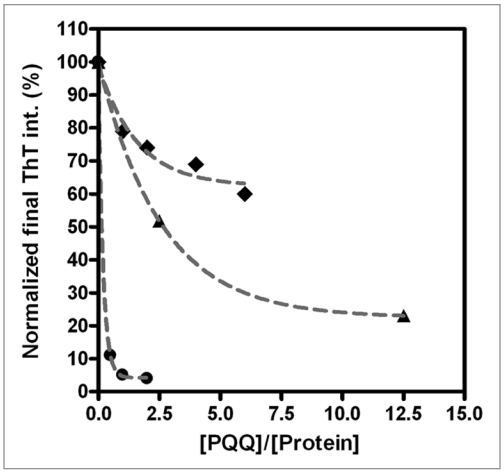

Figure 5. Efficiency of PQQ's Inhibitory activity depending on amyloid proteins. The final fluorescence intensity of ThT was normalized as a percentage of the value of control which means amyloid protein only as 100%. [PQQ]/[Protein] was the molar ratio of PQQ to each amyloid proteins; α-Syn WT (black circles), $A\beta_{1-42}$ (black diamonds) and PrP (black triangles). The data of α-Syn used here was from ref. 11.

Here we showed PQQ decreased the fibril formation of $A\beta_{1-42}$. The ThT observation suggested the presence of PQQ resulted in the decrease, but not entirely inhibited in the fluorescence intensity increase, suggesting the formation of amyloids. Similarly, the light scattering observation suggested that the presence of PQQ decreased the formation of total aggregates, but was not able to inhibit, completely. The AFM image of $A\beta_{1-42}$ incubated with PQQ clearly indicated that the absence of amyloid fibril but the formation of small amorphous aggregates. Some studies suggested that not only the amyloid fibril but rod-shaped aggregates which may be confirmed by AFM also resulted in the ThT fluorescence intensity increase.[22] Therefore, observed increase in the fluorescence intensity, during ThT observation in the $A\beta_{1-42}$ sample incubated with PQQ, was due to the formation of the amorphous aggregates. From the result of cytotoxicity assay, the $A\beta_{1-42}$ samples incubated with PQQ showed lower cytotoxicity than the $A\beta_{1-42}$ sample incubated in the absence of PQQ. Therefore, the incubation of $A\beta_{1-42}$ with PQQ may result in lower toxic intermediate status, which is no longer able to form amyloid fibril, but form the amorphous aggregates, as was observed in AFM. From these data, it is obvious that PQQ inhibits the amyloid fibril formation of amyloid $A\beta_{1-42}$, and also decreases the cytotoxicity.

We investigated not only ThT fluorescence intensity, but also the kinetics parameters like lag-time. The fibrillation of amyloid proteins is a nucleation-dependent polymerization process. The kinetics of fibrillation usually shows a sigmoidal pattern, from which the lag time can be measured to indicate the time of nucleation. This study clearly shows the increases of nucleation times and the decreases the equilibrium level of ThT fluorescence intensity in PQQ dose-dependent manner. The inhibitory concentrations affected the growth of amyloid fibrils. It suggested that PQQ may interact with the amyloid nucleus and has an effect on the nucleation phase of the aggregation of these amyloid proteins.

With previous report, we found that the dose-dependent fibrillization-inhibiting effect of PQQ was common to these amyloid proteins, but interestingly the efficacy of PQQ's inhibitory activity was different for each amyloid protein. It is obvious that PQQ has different inhibitory effect on fibrillation of amyloid proteins by comparison with the data of α-Syn (**Fig. 5**).[11] Several compounds have been reported to prevent the fibril formation of amyloid proteins, many of which are polyphenolic compounds that are drawing attention for their ability to prevent fibril formation and their potential application to therapy.[23-25] Fink et al. have reported on the function of flavonoid compounds, such as baicalein, as inhibitors of α-Syn fibril formation. They demonstrated that baicalein binds with α-Syn through the Lys residues by forming a Schiff base. Baicalein stabilizes the oligomeric state, native multimeric intermediates, of α-Syn, thereby preventing fibril formation.[26] We have reported on the UV spectrum of α-Syn incubated with PQQ, which showed remarkable differences from the typical absorbance of PQQ or α-Syn alone.[11] This suggests that PQQ forms a conjugate with α-Syn and that PQQ has reactive quinone groups, just as the oxidized form of baicalein. Therefore, it is highly probable that PQQ binds with α-Syn via a Schiff base.

Assuming that PQQ inhibits the fibrillization of amyloid proteins by binding through Lys via a Schiff base, the number and location of Lys residues will affect PQQ's inhibitory activity. The α-Syn, a natively unfolded protein, contains 15 Lys residues, 10 of which exist in the KTKEGV repeat sequences and one in the NAC region. NAC is the core region responsible for fibril formation,[27,28] and KTKEGV may play a significant role in maintaining the native unfolded state of α-Syn.[29] $A\beta_{1-42}$ has only two Lys residues, K16 and K28.[30] In this work, we used mouse PrP (207 aa),[31] which contains a folded domain (121–230) with three α-helices and two β-sheets, as well as a native unfolded region (23–120). Mouse PrP has 11 Lys residues, four of which exist in the folded region and seven in the unfolded region. Our results showed a correlation between the efficacy of PQQ's inhibition of amyloid fibril formation and the number of Lys residues in the amyloid protein. This suggests that the number of Lys residues in the region involved in fibril formation is crucial to the efficacy of PQQ's inhibitory activity.

The water solubility and charge of PQQ indicate that PQQ alone cannot be expected to easily cross the blood-brain barrier.[32] However, the recent advances in the development of drug delivery systems[33] and PQQ derivatives will enable us to transport PQQ across the blood-brain barrier. Further in vivo studies will elucidate the effect of PQQ using animal models of neurodegenerative diseases and provide us with valuable information.

The purchased $A\beta_{1-42}$ (human, 1–42) (trifluoroacetate form) was prepared as described in the previous report.[34] The reaction solution contained 50 μM $A\beta_{1-42}$ and 100 or 300 μM PQQ. These samples were incubated in PBS buffer, pH 7.3, with 0.02% NaN_3 at 37°C in a 0.5-ml tube. Aliquots of 10 μl were removed from the incubated sample and added to 1.0 ml of 25 μM ThT in PBS buffer, pH 7.3. ThT fluorescence was recorded at 486 nm with excitation at 450 nm using an ARVO MX 1420 multilabel counter (PerkinElmer). The fibril formation lag time was

calculated from the ThT fluorescence curve fitting using PRISM (GraphPad Software). Light scattering at 500 nm was used to monitor the total aggregation of these samples incubated for 20 h.

For the preparation of the amyloid fibrils of recombinant PrP (the β-sheet-rich isoform), the cell-free conversion system was used as previously reported by Bocharova et al.[19] Lyophilized mouse PrP was solubilized in 6 M guanidine hydrochloride (GdnHCl). This solution (150 µl) was adjusted to 25 µM (60 µg/ml) mouse PrP and 0, 10, 50, 100 or 200 µM PQQ in 6 M GdnHCl. The samples were preincubated for 24 h at room temperature. Then, 750 µl of conversion buffer were added and the samples containing 4.1 µM mouse PrP were incubated in 900 µl of 1 M GdnHCl, 3 M urea, 150 mM NaCl and 20 mM P.P.B. (pH 6.8) at 37°C with shaking at 900 rpm on a Thermomixer Comfort (Eppendorf). The samples withdrawn during the incubation time course were diluted into 5 mM sodium acetate buffer (pH 5.5) to a final mouse PrP concentration of 0.3 µM, and then ThT was added to a final concentration of 10 µM. The lag time was also calculated as case of $A\beta_{1\text{-}42}$.

For the AFM analysis, the sample of 50 µM $A\beta_{1\text{-}42}$ was incubated in absence or presence of 300 µM PQQ for 25 h. Also 5 µM mouse PrP was incubated in absence or presence of 440 µM PQQ for 84 h. The incubated samples were placed on freshly cleaved mica. After air drying, the mica surfaces were rinsed once with Milli-Q water. The samples were then allowed to air dry. Tapping mode imaging was performed on a Molecular Force Probe 3D (Asylum Research) using a silicon probe (OMCL-AC160TS-C2, OLYMPUS). The scanning parameters varied with the individual samples. Some typical parameters were as follows: driving amplitude, 24.24 mV; set point, 617.43 mV; resonant frequency, 307.555 kHz; scan rate, 1.00 Hz.

PC12 cells (ATCC CRL1721) were routinely cultured in RPMI-1640 medium (Sigma) containing 5% fetal calf serum, 10% horse serum and 1% penicillin-streptomycin, and maintained at 37°C in a humidified incubator with 5% CO_2 and 95% room air. For the cytotoxicity studies, the medium was removed before plating and fresh medium was gently added. In brief, cells were plated at a density of 10,000 cells per well on collagen-coated 96-well plates in 100 µl of fresh medium. After 24 h, 50 µM $A\beta_{1\text{-}42}$ incubated with or without 100 µM PQQ was added at the indicated concentration (final concentration 5 and 10 µM respectively), and the cells were incubated for an additional 1 days. ATP assay was performed using "Cellno" ATP assay reagent (TOYO, Inc.,) in accordance with the manufacturer's instructions. This assay provided a homogeneous method for determining the number of viable cells in culture based on quantitation of ATP, which indicates the presence of metabolically active cells.[35,36] After treatment of $A\beta_{1\text{-}42}$ sample, 100 µl of the ATP assay reagent was added to the culture medium, and the 96-well plate was shaken for 1 min at 400 rpm on a Thermomixer Comfort (Eppendorf). After incubation at room temperature for 10 min, the luminescence was measured with an ARVO MX 1420 multilabel counter (PerkinElmer). The results are expressed as percentages of luminescence obtained in control cells treated with PBS instead of amyloid protein.

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