Communication

Inhibition of Alzheimer β-Fibrillogenesis by Melatonin*

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Miguel Pappolla‡, Peter Bozner‡, Claudio Soto§, Haiyan Shao¶, Nickolas K. Robakis¶, Michael Zagorski¶, Blas Frangione¶, and Jorge Ghiso¶

From the ‡University of South Alabama College of Medicine, Mobile, Alabama 36617, §New York University, New York, New York 10016, ¶Case Western Reserve University, Cleveland, Ohio 44106, and ¶Mount Sinai Medical School, New York, New York 10029

It is generally postulated that the amyloid β protein (Aβ) plays a central role in the progressive neurodegeneration observed in Alzheimer’s disease. Important pathologic properties of this protein, such as neurotoxicity and resistance to proteolytic degradation, depend on the ability of Aβ to form β-sheet structures or amyloid fibrils. We report that melatonin, a hormone recently found to protect neurons against Aβ toxicity, interacts with Aβ1–40 and Aβ1–42 and inhibits the progressive formation of β-sheets and amyloid fibrils. These interactions between melatonin and the amyloid peptides were demonstrated by circular dichroism and electron microscopy for Aβ1–40 and Aβ1–42 and by nuclear magnetic resonance spectroscopy for Aβ1–40. Inhibition of β-sheets and fibrils could not be accomplished in control experiments when a free radical scavenger or a melatonin analog were substituted for melatonin under otherwise identical conditions. In sharp contrast with conventional anti-oxidants and available anti-amyloidogenic compounds, melatonin crosses the blood-brain barrier, is relatively devoid of toxicity, and constitutes a potential new therapeutic agent in Alzheimer’s disease.

Most of the recent advances in Alzheimer’s disease (AD) stem from the study of a 40–42-amino acid peptide called the amyloid β protein (Aβ) as the essential pathologic marker of this disorder (1, 2). In brains afflicted with AD, deposits of Aβ in the form amyloid fibrils are widespread within senile plaques and in cerebral and meningeal blood vessels (3, 4). Interestingly, Aβ is normally produced as a soluble peptide (5–8), and whether this form of Aβ is the immediate precursor of the amyloid deposits is still unknown. Synthetic peptides homologous to Aβ1–40 and Aβ1–42, however, undergo spontaneous rearrangements of their initial secondary structure, generating oligomeric and polymeric species with higher content of β-sheets (9–15). Such changes are either promoted or inhibited by numerous factors (9, 14, 16–22).

The secondary structure determines several important properties of Aβ that may be relevant to the pathogenesis of AD. First, it has been demonstrated that the amyloid peptide is neurotoxic (23–25) and that this characteristic is associated with formation of β-sheets (15, 26–31) or amyloid fibrils (31). Second, the ability of Aβ to form fibrils is directly correlated with the content of β-sheet structures adopted by the peptide (32). In this regard, it has been proposed that peptides with high contents of β-sheets can act as seeds for nucleation and fibril formation (33, 34). Finally, Aβ peptides with high contents of β-sheets become partially resistant to proteolytic degradation, and this may be a crucial mechanism in amyloid deposition (35). Such protease resistance and insolubility features, shared by all known forms of amyloidoses, prevent amyloid removal from tissue deposits. Thus, by preventing the formation of β-sheets one could not only reduce neurotoxicity but also facilitate clearance of Aβ via increased proteolytic degradation.

It has recently been found that melatonin has cytoprotective properties against Aβ toxicity (36). In the process of investigating the mechanisms of action of melatonin, new properties of this hormone were uncovered. As determined by CD, electron microscopy, and 1H NMR, melatonin interacted with Aβ and had a profound inhibitory effect on the formation of β-sheets and fibrils. Most interestingly, the observed changes in Aβ conformation appear to depend on specific structural characteristics of the hormone rather than on its recently established antioxidant properties (37).

MATERIALS AND METHODS

Circular Dichroism Spectroscopy—Peptides Aβ1–40 and Aβ1–42 were synthesized in the W. M. Keck Foundation (Yale University, CT), and their purity was evaluated by amino acid sequence and laser desorption mass spectrometry as described (32). Aliquots of Aβ1–40 and Aβ1–42 at a concentration of 250 μM in 5 mM Tris-HCl, pH 7.4, were incubated at room temperature alone or with 100 μM of either melatonin or the melatonin analog 5-hydroxy-N-acetyl-tryptamine (NAT) (Sigma) or N-t-butyl-a-phenylnitrone (PBN) (Sigma), a powerful free radical scavenger structurally unrelated to melatonin. Because of the antioxidant properties of melatonin (37) and because oxidative conditions may promote fibril formation (34, 38), NAT and PBN were specifically selected both as controls for the method and to discount for possible “nonspecific” antioxidant effects of melatonin in the phenomena described here.

Spectra in the far ultraviolet light (190–250 nm) were recorded at various time intervals with a Jasco-720 spectropolarimeter as described (32) using a cell path of 0.01 cm. Experiments with Aβ1–42 necessitated shorter incubation times due to the more fibrillogenic properties and faster aggregation exhibited by the longer peptide. 40 scans/experimental condition were obtained at 0.2 nm intervals over the wavelength range 190–250 nm. The data were analyzed by the Lincomb algorithm (39) to obtain the percentages of the different secondary structures motifs.

Electron Microscopy—To determine whether melatonin displayed inhibitory effects on amyloid fibril formation, transmission electron microscopy was performed following a standard method previously described (40) using a Phillips CM100 microscope and Formvar-coated nickel grids. Aβ1–40 was incubated at the same concentrations as noted for the CD and NMR experiments in the presence or absence of melatonin, and fibril formation was monitored at 0, 12, 24, 36, and 48 h in three independent experiments. Additional controls containing Aβ plus NAT and Aβ plus PBN were incubated in parallel for 48 h. To determine the minimal inhibitory concentration of melatonin on fibril formation, we performed experiments in which several melatonin concentrations (1 nM, 10 nM, 1 μM, 10 μM, 100 μM, and 200 μM) were added to tubes containing 250 μM Aβ1–40, incubated for 48 h, and then examined.

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For the more amyloidogenic Aβ1–42, experiments were performed at the same peptide concentration (250 μM) in the presence or absence of melatonin at various concentrations (100 nM, 1 μM, 10 μM, 100 μM, and 200 μM). The formation of amyloid fibrils was monitored at 0, 2, and 6 h.

Circular Dichroism Studies—As expected, the content in β-sheet conformation of Aβ1–40 incubated alone increased over time from 52% at time 0 to 66% after 24 h at 37 °C (Fig. 1A). These results are in qualitative agreement with previous work (42). The relative proportion of the structures was dramatically changed by addition of melatonin to sister tubes. At time 0, there was an immediate increase of the random conformation, whereas the original β-sheet content markedly diminished (Fig. 1A, left panel). This effect was not observed with melatonin alone, Aβ1–40 plus melatonin decreased over time, reaching 24% after 24 h of incubation (Fig. 1A, right panel). At 24 h, no structural changes were again detected in control experiments with the melatonin analog NAT, and only small effects were observed with PBN. Experiments with the more amyloidogenic Aβ1–42 showed qualitatively similar results (Fig. 1B). Melatonin caused an immediate reduction in the amount of β-sheet structures at time 0 from 89 to 65% (Fig. 1B, left panel). This percentage continued to decrease to 59% after 4 h of incubation (Fig. 1B, right panel). As observed with Aβ1–40, such striking structural changes were not elicited in parallel control preparations containing Aβ1–42 plus NAT or PBN.

Electron Microscopy Studies—Results of the ultrastructural studies reflected the conformational changes and supported the hypothesis that formation of β-sheet structures precede fibrillogenesis (32, 33). In three independent experiments, fibrils were abundant in the tubes containing Aβ1–40, incubated for 48 h, such striking structural changes were not elicited in parallel control preparations containing Aβ1–40 plus NAT or PBN.
Inhibition of Alzheimer β-Fibrillogenesis by Melatonin

FIG. 3. Aβ1–42 fibril formation in the presence or the absence of melatonin. Aβ1–42 was either incubated alone (A) or with melatonin (B) as described under “Materials and Methods.” Fibrils were only found in the tubes containing Aβ1–42 alone after 2 h of incubation (not shown) and after 6 h of incubation (A). Only amorphous material was seen in the tubes containing Aβ1–42 alone immediately after dissolution (time 0, not shown) or containing Aβ1–42 plus melatonin at the indicated time points and with a range of melatonin concentrations. In (B), amorphous material as seen at one of the concentrations of melatonin used is representatively illustrated (in this picture, melatonin concentration was 100 μM and incubation time was 6 h). Bar, 200 nm.

control sister tubes containing 250 μM Aβ1–40 plus 100 μM melatonin.

In the experiments with Aβ1–42, fibrils were identified in the tubes containing the peptide alone incubated after 2 and 6 h (no fibrils were seen at time 0). In contrast, only amorphous material was identified in the tubes containing Aβ1–42 plus melatonin at these time points (Fig. 3). All the concentrations of melatonin used in the experiments with AB1–40 (see “Materials and Methods”) were effective to inhibit fibril formation.

Nuclear Magnetic Resonance Spectroscopy—Shown in Fig. 4 are the downfield spectral regions for the Aβ1–40 peptide, melatonin (Fig. 4E) and the Aβ1–40 with 0.4, 0.8, and 1.2 molar equivalents of melatonin (Fig. 4, B–D). The three well resolved His-2H and His-4H signals are consistent with the Aβ1–40 peptide being partly folded into an ordered structure, which according to the CD studies should be β-sheet and random coil. If only random coil structure were present, then degenerate signals should be present for His6, His13, and His14 (43).

The NMR spectra of the mixtures of melatonin and Aβ1–40 show changes in chemical shifts indicative of binding and local conformational changes. The His-2H and His-4H signals shift downfield 0.05 and 0.02 ppm, respectively, whereas the aromatic peaks of melatonin also shift downfield (Fig. 4). In addition, careful analysis of the upfield spectral region (spectra not shown) revealed downfield shifts for the Asp-βCH2 groups (Table I). Control NMR experiments with NAT showed only minor chemical shift perturbations (±0.01 ppm), suggesting a specificity for the interaction of melatonin with Aβ. The lack of any line broadening or separate peaks for the bound and free states indicates that the binding is in the fast exchange limit (41). The downfield shifts can be interpreted in terms of ring-current shift contributions, with the shifted hydrogens becoming located in the planes of the melatonin and His aromatic ring (44). A remarkable feature is the identical shift seen for each of the three His residues. This result together with the observed non-linearity of the shifts with varied melatonin concentrations suggests that the binding is not localized to a particular His site on Aβ. Instead, the chemical shift changes are consistent with a residue-specific interaction between melatonin and any of the three His and Asp residues of Aβ.

FIG. 4. Downfield 1H NMR spectral region (600 MHz) of 0.25 mM Aβ1–40 peptide (A) and 5.0 mM melatonin (B), with the chemical structure of melatonin provided above the upper plot. The spectra in B, C, and D contain 0.25 mM Aβ1–40 plus 0.1, 0.2, and 0.3 mM melatonin, respectively. Assignments for the aromatic signals of melatonin and the Aβ1–40 peptide are shown, and those resonances exhibiting changes in shifts are connected by dotted lines. A duplicate independent experiment showed virtually identical results.

<table>
<thead>
<tr>
<th>Chemical shift</th>
<th>Bound chemical shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatoninb</td>
<td>cpm</td>
</tr>
<tr>
<td>2H</td>
<td>7.15</td>
</tr>
<tr>
<td>4H</td>
<td>0.09</td>
</tr>
<tr>
<td>6H</td>
<td>9</td>
</tr>
<tr>
<td>Aβ (1–40)</td>
<td>6.92</td>
</tr>
<tr>
<td>His-4H</td>
<td>0.02</td>
</tr>
<tr>
<td>His-2H</td>
<td>0.05</td>
</tr>
<tr>
<td>Asp-βCH2</td>
<td>2.84–2.77</td>
</tr>
</tbody>
</table>

* Obtained by subtracting the chemical shift of melatonin and the Aβ1–40 peptide with the present data, we are unable to assign the 2H and 4H signals specifically to His6, His13, and His14.

** Assignments for 5 mM melatonin.

Because the βCH2s for Asp1, Asp7, and Asp23 are not resolvable by 1H NMR, chemical shift ranges are presented.

DISCUSSION

Melatonin has a proposed role in the aging process (45, 46). Decreased secretion of this hormone during aging is well documented (47, 48), and more profound reductions are reported in populations with dementia (49, 50). The reported lack of toxicity of melatonin and the ease and rapidity with which this molecule crosses the blood-brain barrier following oral administration (51) makes it a prime candidate for experimental testing in humans. This hormone has been administered to human subjects at very high doses (i.e. 1 g/day) without any clinically significant toxicity (52). Our data clearly indicate that under the conditions tested, melatonin modifies the sec-
ondary structure of the Aβ peptide and inhibits the formation of amyloid fibrils. These newly found anti-amyloidogenic properties of melatonin are very rare for endogenous substances. Because of the relationship between oxidative stress and AD and the recently established antioxidant properties of this hormone, it was initially thought that the neuroprotective actions of melatonin were mostly due to its intracellular antioxidant effects (36). However, the results presented here suggest that the anti-amyloidogenic properties are dependant on structural interactions of the hormone with Aβ rather than on antioxidant properties exclusively. The His and Asp residues play important roles in β-amyloid fibril production and stability. Many physiological constituents such as transthyretin and zinc can prevent or promote aggregation by their affinities for the His residues of Aβ (53, 54). Additionally, imidazole-carboxylate salt bridges between the side chains of the His+ and the Asp− residues are critical to the formation of the amyloid β-sheet structures (12, 55–57). More significantly, disruption of these salt bridges promotes fibril dissolution (58). One possibility is that melatonin promotes the β-sheet → random coil conversion by disruption of the His+−Asp− salt bridges. Alternatively, the described effects may result from a unique combination of structural and antioxidant features of this molecule. More experiments are necessary to clarify this interpretation and dissect the relationship between cytotoxicity, changes in peptide structure, and antioxidant characteristics. The antioxidant properties of melatonin may provide additional cytoprotection at the intracellular level (36). The ratio melatonin:Aβ used in these studies is within physiological range, because the concentration of both substances in the brain are normally around 1:1 during youth (both substances are at comparable picomolar concentrations in brain tissue during the dark phase of the cycle (59, 60)). However, limitations of the methods employed required concentrations of melatonin and Aβ that deviate from actual physiological conditions. At this time, no information is available about the possible therapeutic or preventive values of melatonin or of its potential efficacy at physiologic or pharmacologic dosages. It would be premature to conclude that a subgroup of AD is caused by an age-related deficiency of this hormone, although such a possibility is nonetheless intriguing. The results reported here suggest that melatonin can provide a combination of antioxidant and anti-amyloidogenic features that can be explored either as a preventive or therapeutic treatment for AD or as a model for development of anti-amyloidogenic indole analogs.

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REFERENCES