Inhibition of Alzheimer β-Fibrillogensis by Melatonin*

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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-α-phenylisotryptamine (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 potential “nonspecific” antioxidant effects of melatonin in the phenomenon 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 tubs containing 250 μM Aβ1–40, incubated for 48 h, and then examined.

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† The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid β protein; NAT, 5-hydroxy-N-acetyl-tryptamine; PBN, N-t-butyl-α-phenylisotryptamine.
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For the more amyloidogenic Aβ1–42, experiments were preformed 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.

Nuclear Magnetic Resonance Spectroscopy—To further explore structural changes of Aβ1 by melatonin, we performed one-dimensional 1H NMR spectroscopic studies on Aβ1–40. The NMR approach has the distinct advantage of being able to specifically locate the amino acid side chains that bind to a particular ligand (41). The solution conditions for the NMR and CD studies were similar, except that deuterated water (D2O) was used instead of water (H2O) as a solvent.

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 precedes fibrillogenesis (32, 33). In three independent experiments, fibrils were abundant and easily identifiable in the tubes incubated for 48 h containing Aβ1–40 incubated either alone or with NAT (B), PBN (C), or melatonin (D). Bars, 200 nm. Well formed amyloid fibrils are easily recognized in A, B, and C. Fibrils were not formed in D. EM grids were extensively and carefully examined, and a negative result was only documented when fibrils were totally absent from the grids. These results were reproduced in three independent experiments.

RESULTS

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 NAT or PBN. The amount of β-sheet structures for 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.

FIG. 1. Circular dichroism studies of Aβ1–40 (A) and Aβ1–42 (B) alone or in the presence of melatonin. Spectral tracings were generated as described under “Materials and Methods” and expressed in terms of mean residue ellipticity in units of deg cm 2 dmol −1 after subtraction of buffer base-line spectra (including melatonin, NAT, or PBN when indicated) and smoothed by a computer assisted algorithm provided by Jasco Co. The curves designate the spectra of Aβ1 alone (solid line), Aβ1 plus NAT (short dashes), Aβ1 plus PBN (long dashes), or Aβ1 plus melatonin (short and long dashes). Left panels indicate results obtained at time 0; right panels show the values obtained at 24 h for Aβ1–40 and 4 h for Aβ1–42. The corresponding percentages of the different secondary structure motifs are shown in the tables below each respective tracing. An average of 40 scans/independent experimental condition was obtained. An independent experiment yielded qualitatively similar data.
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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.

Fig. 4. Downfield 1H NMR spectral region (600 MHz) of 0.25 mM Aβ1–40 peptide (A) and 5 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>
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<tbody>
<tr>
<td>Melatonin^a</td>
<td></td>
</tr>
<tr>
<td>2H</td>
<td>7.15</td>
</tr>
<tr>
<td>4H</td>
<td>7.15</td>
</tr>
<tr>
<td>6H</td>
<td>6.89</td>
</tr>
<tr>
<td>7H</td>
<td>7.38</td>
</tr>
<tr>
<td>1′-CH2</td>
<td>2.92</td>
</tr>
<tr>
<td>2′-CH2</td>
<td>3.48</td>
</tr>
<tr>
<td>CH2-N-acetyl</td>
<td>1.91</td>
</tr>
<tr>
<td>CH2-methoxyl</td>
<td>3.87</td>
</tr>
<tr>
<td>Aβ (1–40)^b</td>
<td></td>
</tr>
<tr>
<td>His-4H</td>
<td>6.92</td>
</tr>
<tr>
<td>His-2H</td>
<td>7.78</td>
</tr>
<tr>
<td>Asp-βCH2</td>
<td>2.84–2.77</td>
</tr>
</tbody>
</table>

^a 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 His^a, His^b, and His^c.

^b Assignments for 5 mM melatonin.

Because the βCH2 for Asp^a, Asp^b, and Asp^c 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-
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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 cytoprotection, 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

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