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Amyloid β-(1–40) stimulates cyclic GMP production via release of kinins in primary cultured endothelial cells

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Abstract

Increased β-amyloid production is believed to play a central role in the pathogenesis of Alzheimer's disease. Amyloid is deposited not only in the brain of Alzheimer patients as senile plaques but also in the cerebral vessel wall leading to cerebral amyloid angiopathy. Freshly solubilised amyloid β-(1–40) was previously reported to exert a vasoconstrictor effect. We investigated whether amyloid β-(1–40) affects the nitric oxide (NO)/cyclic GMP pathway in primary cultured endothelial cells from bovine aorta and rat coronary microvessels. Surprisingly, a significant increase in cyclic GMP production after incubation with freshly dissolved amyloid β-(1–40) was found. The stimulation of cyclic GMP production could be inhibited by the bradykinin B_2 receptor antagonist icatibant, the NO synthase inhibitor *N*-ω-nitro-L-arginine, the serine protease inhibitor 3,4-dichloroisocoumarin and the selective plasma kallikrein inhibitor Pefabloc PK, suggesting activation of the plasma kallikrein–kinin system. This is supported by a three- to four-fold increase in kinins in the supernatant of both types of endothelial cells after incubation with amyloid β-(1–40) at concentrations of 10^{-7} and 10^{-6} mol/l.

Keywords: Bradykinin; Alzheimer's disease; Amyloid β-(1–40); Vasoactivity; Endothelial cells cultured; cGMP

1. Introduction

Amyloid is deposited not only in the brain of Alzheimer patients as senile plaques but also in the cerebral vessel wall leading to cerebral amyloid angiopathy (Kalaria, 1996). Several reports have shown vasoactivity of amyloid β. Freshly solubilised, non-aggregated amyloid β-(1–40) constricted blood vessels, enhanced the vasoconstriction induced by endothelin-1 and increased resistance to relaxation triggered by nitric oxide (NO) (Crawford et al., 1998; Suo et al., 1998; Paris et al., 1999). Moreover, a vasoconstrictor and toxic effect of aggregated amyloid β-(1–42) was found in rat aorta (Thomas et al., 1996). It has been suggested by those authors that the observed vasoactivity contributes to the pathophysiology of Alzheimer's disease.

On the other hand, it has been observed that Alzheimer patients have a low blood pressure (Landin et al., 1993; Burke et al., 1994; Morrison et al., 1996) that would be difficult to reconcile with a general vasoconstrictor effect of amyloid with relevance for systemic blood pressure.

In rat aortic rings, a decrease in cyclic GMP levels after incubation with amyloid β-(1–40) was found when stimulated by endothelin-1 and was related to an enhanced cyclic GMP degradation (Paris et al., 1999). So far, it has not been investigated whether amyloid β-(1–40) affects NO production of cultured endothelial cells. A possible decrease of endothelial NO production could account for the observed vasoconstrictor effects. Therefore, the aim of our study was to find out whether amyloid β-(1–40) influences cyclic GMP in primary cultured endothelial cells from bovine aorta and rat coronary microvessels. These cells are functionally intact as we can measure basal cyclic GMP production, stimulation by the physiological agonists bradykinin and acetylcholine and by angiotensin-converting enzyme inhibitors. Endothelial angiotensin-con-

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Table 1
Stimulation of cyclic GMP production by amyloid β peptides in comparison with bradykinin and inhibitory effect of the bradykinin antagonist icatibant (10^{-7} mol/l) in primary cultured bovine aortic endothelial cells (BAEC) and rat microvascular coronary endothelial cells (RMCEC)

Endothelial cell type	Cyclic GMP (pmol/mg protein)	
	BAEC	RMCEC
Basal production	2.2 ± 0.35	0.2 ± 0.07
Bradykinin 10^{-8} mol/l	8.8 ± 0.34[a]	1.13 ± 0.2[a]
Bradykinin 10^{-8} mol/l + icatibant	2.2 ± 0.2[b]	0.22 ± 0.02[b]
Amyloid β-(1–40) 10^{-7} mol/l	5.9 ± 0.23[a]	0.59 ± 0.07[a]
Amyloid β-(1–40) 10^{-7} mol/l + icatibant	2.2 ± 0.28[b]	0.34 ± 0.02[b]
Amyloid β-(1–40) 10^{-6} mol/l	4.6 ± 0.13[a]	0.74 ± 0.09[a]
Amyloid β-(1–40) 10^{-6} mol/l + icatibant	2.2 ± 0.2[b]	0.32 ± 0.05[b]
Amyloid β-(1–42) 10^{-7} mol/l	1.9 ± 0.15	
Amyloid β-(1–42) 10^{-6} mol/l	2.4 ± 0.35	
Amyloid β-(40–1) 10^{-7} mol/l	2.49 ± 0.22	
Amyloid β-(40–1) 10^{-6} mol/l	4.68 ± 0.39[a]	
Amyloid β-(40–1) 10^{-6} mol/l + icatibant	1.8 ± 0.2[b]	

[a] $P < 0.05$ compared with basal cyclic GMP production.
[b] $P < 0.05$ effect of icatibant compared with bradykinin or amlyoid β-(1–40).

verting enzyme inhibition reduces the breakdown of endogenous kinins leading to a stimulation of NO/cyclic GMP production. This effect could be abolished by blockade of bradykinin B_2 receptors with icatibant and inhibitors of NO-synthase and serine proteases (Wiemer et al., 1994b, 1996).

Surprisingly, our preliminary data showed an increase in endothelial cyclic GMP production after incubation with freshly prepared solutions of amyloid β-(1–40). Since bradykinin stimulates cyclic GMP in our systems, we assumed a release of kinins, which we have previously demonstrated to occur in these cells, and tested whether blockade of bradykinin B_2 receptors by the selective bradykinin B_2 receptor antagonist icatibant (HOE 140), inhibition of NO-synthase by *N*-ω-nitro-L-arginine (L-NNA) and inhibition of plasma kallikrein by Pefabloc PK abolishes the stimulatory effect of amyloid β-(1–40) on cyclic GMP. Moreover, kinin levels were directly determined in the supernatant of the endothelial cells after incubation with amyloid β-(1–40).

2. Materials and methods

2.1. Effect of amyloid β-(1–40), amyloid β-(40–1) and amyloid β-(1–42) on cyclic GMP production in primary cultured endothelial cells

Endothelial cells from bovine aorta and rat coronary microvessels were isolated and cultured as described previously (Wiemer and Wirth, 1992; Wohlfart et al., 1997). Primary cultured endothelial cells grown to confluence in six-well plates were used for the determination of the effect of amyloid β-(1–40), its inverse form amyloid β-(40–1), and amyloid β-(1–42) on cyclic GMP. After removal of the culture medium, the cells were washed twice with 2 ml of warm (37°C) HEPES/Tyrode's solution. Thereafter, the cells were incubated for 30 min at 37°C with HEPES/Tyrode's solution containing isobutyl-1-methylxanthine (IBMX, 1×10^{-5} mol/l) and superoxide dismutase (20 U/ml) in the absence (control) and presence of amyloid β-(1–40), amyloid β-(40–1) or amyloid β-(1–42) at 10^{-7} and 10^{-6} mol/l. The potent and selective peptidic bradykinin B_2 receptor antagonist icatibant, previously called Hoe 140 (Hock et al., 1991; Wirth et al., 1991), was added at a concentration of 10^{-7} mol/l together with amyloid β. Bradykinin (10^{-8} mol/l) was incubated for 1 min (maximal stimulation of cyclic GMP) (Wiemer and Wirth, 1992) after a 30-min incubation period with or without icatibant. Intracellular cyclic GMP content of endothelial cells was measured by use of a radioimmunoassay as described previously (Wiemer and Wirth, 1992). Data are expressed as means ± S.E.M. of three experiments (three different cell batches) performed in triplicate dishes for each endothelial cell type.

2.2. Effects of inhibition of NO-synthase, plasma kallikrein and serine proteases on amyloid β-(1–40) stimulated cyclic GMP production in bovine aortic endothelial cells

Cells were incubated with either the NO-synthase inhibitor *N*-ω-nitro-L-arginine L-NNA (10^{-5} mol/l) or the

Table 2
Inhibition of amyloid β-(1–40) stimulated cyclic GMP in primary cultured bovine aortic endothelial cells by the NO-synthase inhibitor *N*-ω-nitro L-arginine (L-NNA), the nonselective serine protease inhibitor 3,4-dichloroisocoumarin (DCI) and the selective plasma kallikrein inhibitor Pefabloc PK

Concentration (mol/l)	Cyclic GMP (pmol/mg protein)				
	No inhibitor	Icatibant (10^{-7} mol/l)	L-NNA (10^{-5} mol/l)	DCI (10^{-6} mol/l)	Pefabloc PK (10^{-5} mol/l)
Basal	0.9 ± 0.21	2.2 ± 0.34	1.7 ± 0.25	1.2 ± 0.18	2.0 ± 0.12
Amyloid β-(1–40) 10^{-7}	5.5 + 0.62[a]	2.2 ± 0.18	2.2 ± 0.17	1.6 ± 0.28	1.2 ± 0.23
Amyloid β-(1–40) 10^{-6}	3.5 ± 0.28[a]	2.3 ± 0.31	2.3 ± 0.10	1.4 ± 0.38	1.3 ± 0.06

[a] $P < 0.05$ compared with basal cyclic GMP production.

Table 3
Release of kinins from primary cultured bovine aortic endothelial cells (BAEC) and rat microvascular coronary endothelial cells (RMCEC) by amyloid β-(1–40) in the absence and presence of the bradykinin antagonist icatibant (10^{-7} mol/l)

	Kinins (fmol/ml per mg protein)	
	BAEC	RMCEC
Basal	4.44 ± 0.6	2.84 ± 0.34
Basal + icatibant	3.89 ± 0.3	2.86 ± 0.3
Amyloid β-(1–40) 10^{-7} mol/l	10.18 ± 0.9[a]	8.49 ± 0.89[a]
Amyloid β-(1–40) 10^{-7} mol/l + icatibant	10.11 ± 0.98[a]	8.97 ± 0.98[a]
Amyloid β-(1–40) 10^{-6} mol/l	16.75 ± 1.9[a]	12.75 ± 1.9[a]
Amyloid β-(1–40) 10^{-6} mol/l + icatibant	15.18 ± 1.5[a]	13.91 ± 1.5[a]

[a] $P < 0.05$ compared with basal release of kinins.

selective plasma kallikrein inhibitor Pefabloc PK (10^{-5} mol/l) (Stürzebecher et al., 1992) or 3,4-dichloroisocoumarin (DCI) (10^{-6} mol/l), a non-selective serine protease inhibitor (Powers et al., 1989) 5 min before addition of amyloid β-(1–40).

2.3. Measurement of kinins in cultures of endothelial cells

In the previously described experiments (Section 2.1) kinins were measured concomitantly in the supernatants of bovine aortic endothelial cells and rat microvascular coronary endothelial cells by use of a specific radioimmunoassay (Wiemer et al., 1994a). Each 800 μl of the supernatants were directly transferred into EDTA-solution (final concentration 10^{44} mol/l) and subsequently lyophylised. The antibody used in this assay (from K. Shimamoto) does not distinguish between the three mammalian kinins, bradykinin, Lys-bradykinin and Meth-Lys-bradykinin (almost 100% cross-reactivity relative to bradykinin). Kinin fragments such as des-Arg^9-bradykinin did not show a significant cross-reactivity (< 0.02%–0.1% relative to bradykinin). Kinin release into the supernatant is expressed in fmol/ml supernatant related to mg cell protein.

2.4. Transmission electron microscope examination of RMCEC after incubation with amyloid β-(1–40)

To exclude that kinin release could occur as a consequence of endothelial cell damage through amyloid

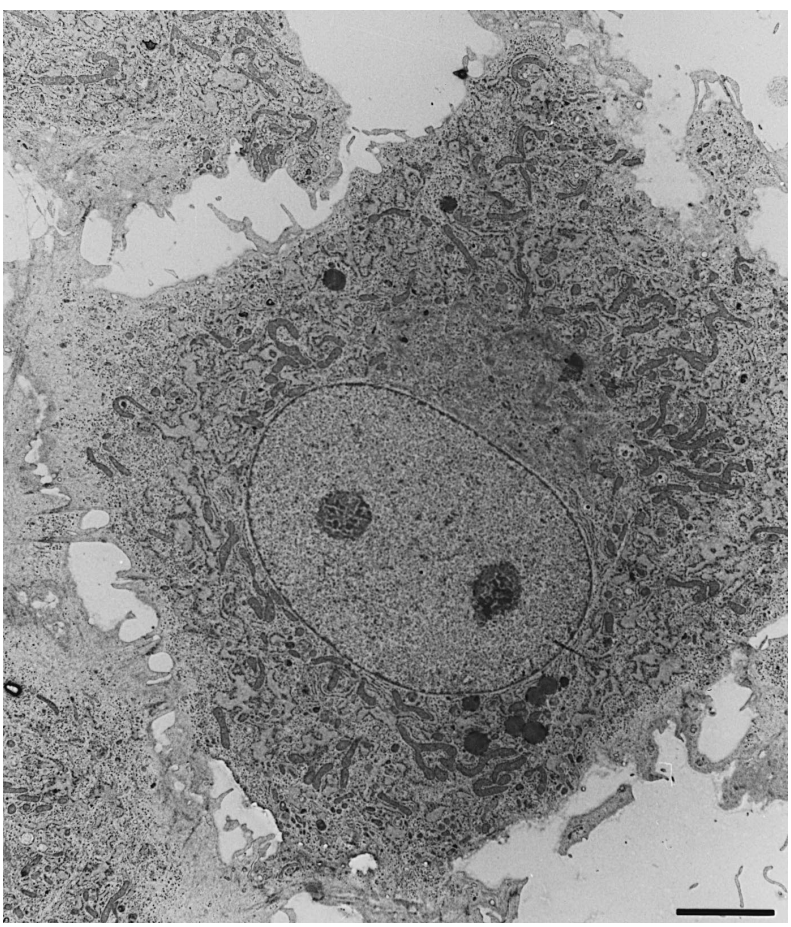

Fig. 1. Electron micrograph of primary cultured rat microvascular coronary endothelial cells (RMCEC). Untreated control with regular cellular ultrastructure. Bar represents 4 μm.

β-(1–40), transmission electron microscopical examination was performed in rat microvascular coronary endothelial cells, which were isolated and cultured as described under Section 2.1. Rat microvascular coronary endothelial cells were incubated with amyloid β-(1–40) for 30 min in concentrations up to 10^{-6} mol/l.

Preparation of specimens for electron microscopy: Cultured endothelial cells were fixed in situ by immersion with 2.5 % glutaraldehyde in phosphate buffer, postfixed with osmiumtetroxide, dehydrated with ethanol and embedded in Epon. Samples were punched out after polymerisation, glued to empty Epon blocks and cut with an ultra-microtome. The sections were stained with uranyl acetate and lead citrate, and examined in a PHILIPS EM 410 transmission electron microscope.

2.5. Materials

Amyloid β-(1–40), its inverse form amyloid β-(40–1), amyloid β-(1–42), bradykinin, L-NNA and 3,4-dichloroisocoumarin (DCI) were purchased from Sigma, St. Louis, USA, and used in freshly prepared solutions for each experiment. The bradykinin receptor antagonist icatibant (HOE 140) was synthesised at HMR. Pefabloc PK (*N*-(4-Acetamidino-phenyl)-4-phenyl-2-(toluene-4-sulfonylamino)-butyramide hydrochloride) was purchased from Pentapharm, Basel, Switzerland.

2.6. Statistics

Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test for pairwise comparisons. Differences were regarded as significant with $P < 0.05$.

3. Results

3.1. Measurement of cyclic GMP in cultured endothelial cells

Bradykinin at 10^{-8} mol/l stimulated intracellular cyclic GMP production in bovine aortic endothelial cells and rat microvascular coronary endothelial cells, and this stimulatory effect was abolished by co-incubation with the potent and selective bradykinin B_2 receptor antagonist icatibant.

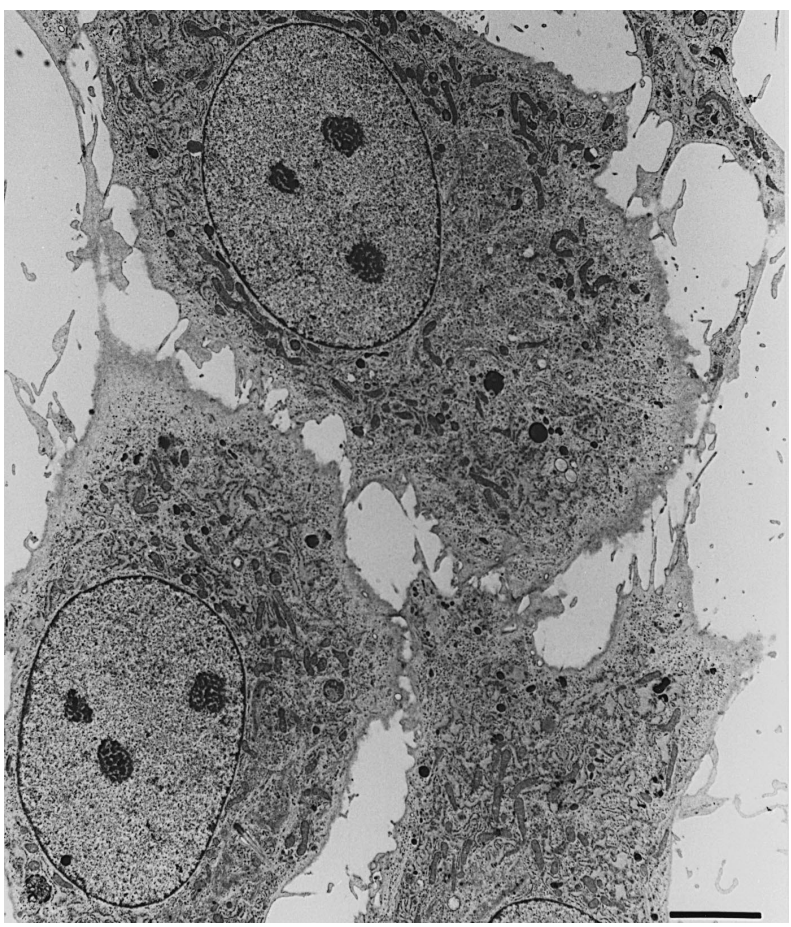

Fig. 2. Electron micrograph of primary cultured rat microvascular coronary endothelial cells (RMCEC) after 30 min of exposure with 10^{-6} mol/l amyloid β-(1–40). Note unchanged cellular ultrastructure. Bar represents 4 μm.

At concentrations of 10^{-7} and 10^{-6} mol/l amyloid β-(1–40) led to a significant increase in cyclic GMP production in both cell types. Lower concentrations of amyloid β-(1–40) showed no significant increase. The stimulation of intracellular cyclic GMP production by amyloid β-(1–40) was also abolished by co-incubation with icatibant. In contrast to amyloid β-(1–40), amyloid β-(1–42) was without effect in bovine aortic endothelial cells in a concentration up to 10^{-6} mol/l. Amyloid β-(40–1) was ineffective at 10^{-7} mol/l, whereas at 10^{-6} mol/l it significantly stimulated cyclic GMP production, which was also abolished by icatibant (Table 1).

3.2. Effects of inhibition of NO-synthase, plasma kallikrein and serine proteases on amyloid β-(1–40) stimulated cyclic GMP production in bovine aortic endothelial cells

The stimulatory effect of amyloid β-(1–40) was abolished by the bradykinin B_2 antagonist icatibant, the NO-synthase inhibitor L-NNA, the non-specific serine protease inhibitor DCI (Powers et al., 1989) and the selective plasma kallikrein inhibitor Pefabloc PK (Stürzebecher et al., 1992), which is at least 100-fold weaker on tissue kallikrein compared with plasma kallikrein. The inhibitors used did not inhibit basal cyclic GMP production (Table 2).

3.3. Measurement of the release of kinins in cultures of endothelial cells

In bovine aortic endothelial cells and rat microvascular coronary endothelial cells, 30-min incubation of amyloid β-(1–40) caused a concentration-dependent increase of kinins in the supernatants. At 10^{-6} mol/l of amyloid β-(1–40), the release of kinins was increased three- to four-fold. As expected icatibant did not affect the amyloid β-(1–40) stimulated kinin synthesis and release (Table 3).

3.4. Transmission electron microscopical examination of rat microvascular coronary endothelial cells after incubation with amyloid β-(1–40)

No signs of endothelial damage could be detected in control experiments (Fig. 1). Cellular ultrastructure was unchanged after exposure with amyloid β-(1–40), 10^{-6} mol/l, for 30 min (Fig. 2).

4. Discussion

Amyloid β-(1–40) induced an increase in cyclic GMP production in primary cultured bovine aortic endothelial cells and rat microvascular coronary endothelial cells. This increase was suppressed by the selective bradykinin B_2 receptor antagonist icatibant indicating the involvement of endogenous kinins, which could be confirmed by direct measurement of kinins. A direct action of amyloid β-(1–40) on the bradykinin B_2 receptor could also be excluded as amyloid β-(1–40) did not displace radiolabelled bradykinin in a receptor binding assay with guinea pig ileum membranes (unpublished data). The fact that the stimulatory effect of amyloid β-(1–40) could be abolished by inhibition of NO-synthase and two different serine protease inhibitors indicates an involvement of NO and the kallikrein–kinin system.

It is well documented that cultured endothelial cells can produce and release kinins (Wiemer and Wirth, 1992; Wiemer et al., 1991, 1994a). Cyclic GMP increase after bradykinin treatment is due to a stimulation of endothelial bradykinin B_2 receptors leading to an enhanced production of NO, which in turn activates the endothelial soluble guanylate cyclase to produce cyclic GMP. Since bradykinin is a potent endothelium-dependent vasodilator, amyloid β-(1–40) could be expected to cause vasorelaxation in vitro and in vivo. The opposite was found: freshly solubilised, non-aggregated amyloid β-(1–40) showed vasoconstrictor effects (Crawford et al., 1998; Suo et al., 1998; Paris et al., 1999). The possibility of superoxide radicals being responsible for the vasoconstrictor effect could be excluded (Suo et al., 1998). In rat aortic rings, however, which consists of endothelial and smooth muscle cells, a decrease in cyclic GMP production by amyloid β-(1–40) was found when stimulated by endothelin-1 (Paris et al., 1999). This is in contrast to the increase in cyclic GMP with amyloid β-(1–40) alone that occurred in our cultured endothelial cells. So far, there is no satisfactory explanation for these discrepancies.

Concerning the mechanism of kinin release the demonstration that the non-specific inhibitor of serine protease DCI completely blocked the effect of amyloid β-(1–40) indicates that kinins had to be released from their precursors, the kininogens. Since Pefabloc PK, which inhibits plasma kallikrein but not tissue kallikrein (Stürzebecher et al., 1992), abolished the effect of amyloid β-(1–40) the plasma kallikrein–kinin system must be involved. Plasma kallikrein is activated by Hageman factor (synonym Factor XII of intrinsic blood coagulation) to release bradykinin from high molecular weight kininogen, a process referred to as contact activation. Endothelial cells possess a high affinity receptor that binds either high molecular weight kininogen or Hageman Factor (Reddigari et al., 1995). Activation of this system is a process with low specificity which can typically be initiated by a number of diverse stimuli mostly non-physiological, negatively charged surfaces but also heat, cold and ischemia (Bhoola et al., 1992). The observation that kinin release could also be stimulated at least by the high concentration of amyloid β-(40–1) of 10^{-6} mol/l may be due to the fact that contact activation in general is a process with low specificity.

Evidence for contact activation by amyloid has been accumulated. Senile plaques have been shown to contain

Hageman Factor, and binding to senile plaques could be induced in vitro by different amyloid peptides (Yasuhara et al., 1994). Activation of the contact system in cerebrospinal fluid of patients with Alzheimer's disease has been reported, and amyloid β-(1–42) was found to induce the cleavage of high molecular weight kininogen (Bergamaschini et al., 1998). Zinc-dependent activation of the plasma kinin-forming cascade by aggregated amyloid β protein was reported (Shibayama et al., 1999). In both studies, activation did not occur when Hageman factor deficient plasma was used indicating contact activation. These authors used aggregated amyloid β-(1–42), however. The latter forms typical aggregates within minutes whereas amyloid β-(1–40) needs days for aggregation (Lansbury, 1995). Therefore, it is very likely that in our freshly prepared solutions amyloid β-(1–40) was still in solution and that it was able to initiate activation of plasma kallikrein as a single molecule. With this assumption, the surprising lack of effect of amyloid β-(1–42) on kinin release in our cell systems could even be explained with its rapid aggregation. Relatively immobile aggregated amyloid complexes would only slowly get in contact with Hageman factor fixed to the cell surface (Reddigari et al., 1995), and aggregation would reduce the number of possible contacts with the endothelial target. In contrast to the lack of effect of amyloid β-(1–42) in our cells, aggregated amyloid β-(1–42) stimulated contact activation in plasma, where Hageman factor is in solution (Bergamaschini et al., 1998; Shibayama et al., 1999).

Amyloid β-(1–42) and amyloid β-(1–40) were reported to be similarly neurotoxic, but they clearly differed in their toxicity to endothelial cells (Suo et al, 1997). Amyloid β-(1–40) was found to be much less toxic to human aortic endothelial cells compared with amyloid β-(1–42) and amyloid β-(25–35). This observation and the fact that amyloid β-(1–40) did not induce cell damage in our endothelial cells as shown by electron microscopic examination clearly suggests that release of kinins is not the consequence of cell damage.

In summary, our data show that amyloid β-(1–40) stimulates the production of the vasodilator NO in endothelial cells via release of kinins. The release of kinins occurs via activation of the plasma kallikrein–kinin system. The relevance of these findings for the pathophysiology of Alzheimer amyloid angiopathy remains to be determined.

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