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Reduced nitric oxide bioavailability mediates cerebroarterial dysfunction independent of cerebral amyloid angiopathy in a mouse model of Alzheimer’s disease

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Merlini M, Shi Y, Keller S, Savarese G, Akhmedov A, Derungs R, Spescha RD, Kulic L, Nitsch RM, Lüscher TF, Camici GG. Reduced nitric oxide bioavailability mediates cerebroarterial dysfunction independent of cerebral amyloid angiopathy in a mouse model of Alzheimer’s disease. Am J Physiol Heart Circ Physiol 312: H232–H238, 2017. First published November 11, 2016; doi:10.1152/ajpheart.00607.2016.—In Alzheimer’s disease (AD), cerebral arteries, in contrast to cerebral microvessels, show both cerebral amyloid angiopathy (CAA) -dependent and -independent vessel wall pathology. However, it remains unclear whether CAA-independent vessel wall pathology affects arterial function, thereby chronically reducing cerebral perfusion, and, if so, which mechanisms mediate this effect. To this end, we assessed the ex vivo vascular function of the basilar artery and a similar-sized peripheral artery (femoral artery) in the Swedish-Arctic (SweArc) transgenic AD mouse model at different disease stages. Furthermore, we used quantitative immunohistochemistry to analyze CAA, endothelial morphology, and molecular pathways pertinent to vascular relaxation. We found that endothelium-dependent, but not smooth muscle-dependent, vasorelaxation was significantly impaired in basilar and femoral arteries of 15-mo-old SweArc mice compared with that of age-matched wild-type and 6-mo-old SweArc mice. This impairment was accompanied by significantly reduced levels of cyclic GMP, indicating a reduced nitric oxide (NO) bioavailability. However, no age- and genotype-related differences in oxidative stress as measured by lipid peroxidation were observed. Although parenchymal capillaries, arterioles, and arteries showed abundant CAA in the 15-mo-old SweArc mice, no CAA or changes in endothelial morphology were detected histologically in the basilar and femoral artery. Thus our results suggest that, in this AD mouse model, dysfunction of large intracranial, extracerebral arteries important for brain perfusion is mediated by reduced NO bioavailability rather than by CAA. This finding supports the growing body of evidence highlighting the therapeutic importance of targeting the cerebrovasculature in AD.

NEW & NOTEWORTHY We show that vasorelaxation of the basilar artery, a large intracranial, extracerebral artery important for cerebral perfusion, is impaired independent of cerebral amyloid angiopathy in a transgenic mouse model of Alzheimer’s disease. Interestingly, this dysfunction is specifically endothelium related and is mediated by impaired nitric oxide–cyclic GMP bioavailability.

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Alzheimer’s disease; cerebral amyloid angiopathy; cerebrovascular pathology; endothelial dysfunction; nitric oxide; cyclic GMP

Cerebrovascular pathology is inherent to Alzheimer’s disease (AD), with several lines of evidence indicating that it also strongly contributes to the onset of the disease (1, 2, 4). However, the molecular mechanisms underlying cerebrovascular pathogenesis at the different AD stages remain unknown. Vascular amyloid-β (Aβ) deposition, known as cerebral amyloid angiopathy (CAA), severely affects cerebrovascular functionality and instigates a plethora of vascular abnormalities that play critical roles in the onset and progression of AD. These include vasculitis, vascular smooth muscle cell (VSMC) loss, and blood-brain barrier leakage (10, 12, 17, 29, 30). However, pathological remodeling of cerebral arteries is also observed before onset of AD in the absence of CAA, indicating that CAA certainly exacerbates cerebrovascular pathology, but is not required to initiate it (18, 21). Thus investigating CAA-independent changes in the functionality of cerebral vessels in AD is important for the early diagnosis of the disease.

Soluble oligomeric Aβ species decrease the vasodynamic capacity of cerebral penetrating arterioles, independent of the presence of CAA (6). Whether VSMC and/or endothelial cell functionality in the upstream, major intracranial cerebral arteries may be impaired in the absence of CAA as well is, however, not clearly delineated. Therefore, herein, we recorded endothelium-dependent and -independent ex vivo vasodilatation and constriction of the basilar artery in a transgenic mouse model of amyloidosis harboring the human Swedish and Arctic AD double mutation (SweArc mice) (20). This mouse model is characterized by progressive age-dependent cerebrovascular pathology, soluble oligomeric Aβ species generation (13, 14, 16, 17), and cerebral hypoperfusion, both before and after CAA onset (14, 17, 22). Additionally, we performed quantitative histological assessments of endothelial morphology, lipid peroxidation as a marker of oxidative stress, CAA, and cyclic...
GMP (cGMP) as a readout of nitric oxide (NO) bioavailability. We used 6- and 15-mo-old SweArc and wild-type (WT) mice to discern age- from CAA-dependent mechanisms that affect cerebral arterial function. So as to have a systemic artery control, we compared basilar artery relaxation to that of the similarly sized femoral artery. Using this experimental approach, we tested the hypothesis that dysfunction of large cerebral arteries in AD can also occur in the absence of CAA.

MATERIALS AND METHODS

Animals. The basilar artery and distal portion of the femoral artery were isolated from 6- and 15-mo-old male SweArc mice (n = 8/age group) and their WT littermates (n = 7/age group) (C57Bl6/J background). Mice were housed under optimal hygienic barrier conditions at the Laboratory Animal Services Center (LASC) of the University of Zurich (Zurich, Switzerland), with access to chow and water ad libitum. On the day of the experiments, mice were euthanized with carbon dioxide. All procedures and protocols were approved by the Veterinary Department [Bundesamt für Veterinärwesen (BVET); Swiss Animal Welfare Act, 2008, no. 455] of the Canton of Zurich and were performed according to the BVET guidelines.

Organ chamber recordings. Organ chamber recordings were performed nonblinded, as previously described (26). Briefly, the femoral and basilar artery of the mice were dissected, excised, and placed in ice-cold modified Krebs-Ringer solution (in mmol/l: NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 25.0, KH2PO4 1.18, calcium disodium EDTA 0.026, and glucose 11.1). Arterial rings were cut (0.8–1.4 mm in length, n ≥ 3/recording) and mounted in organ chambers containing Krebs-Ringer solution (37°C) aerated with 95% O2 and 5% of CO2, which were connected to a force transducer (M610 DMT). The rings were stretched progressively to their optimal resting tension according to the manufacturer’s protocol. Internal circumference of the arteries was determined corresponding to a transmural pressure of 100 mmHg. The diameter of the artery was calculated according to its internal circumference. Changes in tension were expressed as a percentage of the reference contraction to U46619, a thromboxane A2 receptor agonist, obtained at the beginning of the experiment. Artery ring recordings were acquired in the presence of sodium nitroprusside (SNP) or acetylcholine (ACh) to study endothelium-independent and -dependent relaxation in a concentration-dependent manner.

Histology. Histological procedures were performed as previously described (17). Briefly, following induction of deep anesthesia, mice were transcardially perfused with phosphate-buffered saline (PBS) and 4.0% paraformaldehyde in PBS at room temperature, followed by incubation in 30% sucrose in PBS for 36 h. Cryoprotected brains were cut into 35-µm-thick free-floating sections and were pretreated with proteinase K for antigen retrieval, immune-blocked, and incubated overnight at 4°C with an antibody against Aβ (purified mouse 6E10, 1:1000; Covance/Signet, SIG-39300), the endothelial marker CD31 (rat anti-CD31/PECAM1, 1:50; BD PharMingen, 553370), the VSMC marker α-smooth muscle actin (α-SMA) (goat anti-α-SMA, 1:250; Abcam, ab21027), cGMP (sheep anti-cGMP, 1:150; BioRad, OB5055); and/or the lipid peroxidation marker 4-hydroxynonenal (4-HNE) (rabbit anti-4-HNE, 1:200; Abcam, ab46545). The brain sections were subsequently incubated with secondary antibodies [a combination of either Alexa 488-conjugated donkey anti-rat IgG
(1:750), Alexa 594-conjugated donkey anti-goat IgG (1:750), and Alexa 647-conjugated donkey anti-mouse IgG (1:500) or Alexa 488-conjugated donkey anti-rat IgG (1:750), and Alexa 546-conjugated donkey anti-sheep (1:750), and Alexa 405-conjugated donkey anti-rabbit (1:750); Jackson ImmunoResearch, Suffolk, UK) for 2 h at room temperature. Images were acquired in sequential mode and at a 1-μm step size interval using a confocal microscope (Leica SP8; Leica, Wetzlar, Germany). They were processed as maximum projection z-stacks in Image J (National Institutes of Health, Bethesda, MD) and were analyzed using an in-house generated MATLAB script based on the Imaging Toolbox of MATLAB (version 2016b; MathWorks, Natick, MA).

Statistical analyses. The data are presented as means ± SE. For the artery ring experiments, two-way ANOVA followed by Bonferroni’s post hoc comparison test was used; the histological data were analyzed using a two-tailed, nonparametric (Mann-Whitney) Student’s t-test (GraphPad Prism, version 7; GraphPad Software, La Jolla, CA). Differences were considered statistically significant at \( P < 0.05 \).

RESULTS

General physiological parameters were assessed in the SweArc and WT mice to confirm comparable conditions in the two genotypes. As shown in Fig. 1A, the diameter of the femoral and basilar artery was comparable in the SweArc and WT mice at both 6 and 15 mo of age, with an average luminal diameter of ~170 μm (6 mo old) and ~150 μm (15 mo old) for the femoral artery, and ~150 μm (6 and 15 mo old) for the basilar artery. Likewise, the resting tension of the femoral and basilar artery was similar between and among the 6- and 15-mo-old SweArc and WT mice (~1.1 mN) (Fig. 1A). Body and heart weight in the 6-mo-old mice was 29.8 ± 9.3 g and 0.13 ± 0.03 g (SweArc), and 32.8 ± 7.1 g and 0.15 ± 0.03 g (WT), respectively; in the 15-mo-old mice it was equal to 37.7 ± 11.2 g and 0.18 ± 0.04 g (SweArc), and 44.9 ± 7.9 g and 0.19 ± 0.03 g (WT), respectively.

Next, we assessed endothelium- and VSMC-dependent vasorelaxation after U46619-induced pre-contraction using ACh and SNP, respectively (26). No significant differences in endothelium-dependent and -independent relaxation were observed between the two artery types of 6-mo-old WT and SweArc mice (Fig. 1B). In contrast, femoral arteries of 15-mo-old SweArc mice showed a significant decrease in endothelium-dependent relaxation compared with that of age-matched WT mice (area under the curve, WT and SweArc: 851 ± 31.2 % 4-HNE & cGMP staining of basilar artery

Fig. 2. Histological assessment of changes in the endothelial nitric oxide production marker cyclic GMP (cGMP) and of the presence of lipid peroxidation/oxidative stress in the basilar artery. A–D: representative images of free-floating brain tissue sections at the level of the basilar artery of a 6- and 15-mo-old wild-type (WT) littermate control (A and C, respectively) and 6- and 15-mo-old Swedish-Arctic (SweArc) (B and D, respectively) mouse stained for the endothelial cell marker CD31 (green), cGMP (red), and the lipid peroxidation/oxidative stress marker 4-hydroxynonenal (4-HNE). E: quantification of the cGMP staining calculated as percentage of the CD31 staining shows a significant decrease in cGMP in the basilar artery of 15-mo-old SweArc mice (\( n = 5 \)) compared with that in the basilar artery of 15-mo-old WT littermates (\( n = 4 \)), and of 6-mo-old WT (\( n = 3 \)) and SweArc mice (\( n = 3 \)). The percentage of 4-HNE staining is equally low between the different groups. Scale bar in A and C = 50 μm. Values are means ± SE. **\( P < 0.01 \). ***\( P < 0.001 \).
and 603 ± 30.5, respectively; \( P < 0.001 \)) (Fig. 1C). This difference was exacerbated in the basilar artery of 15-mo-old SweArc mice, showing a paradoxical vasoconstriction as opposed to the expected vasodilatation observed in the age-matched WT mice (area under the curve, WT and SweArc: 778 ± 26.1 and 384 ± 69.7, respectively; \( P < 0.001 \)). Endothelium-independent relaxation, on the other hand, was similar between the aged SweArc and WT mice (Fig. 1C).

Given the specific impairment in ACh-induced, NO-mediated vasorelaxation with a paradoxical vasoconstriction observed in the 15-mo-old SweArc mice, we assessed whether this might be related to reduced levels of cGMP, a marker of NO bioavailability. Indeed, quantitative histological analysis of cGMP in free-floating brain sections containing the basilar artery showed a significant decrease in cGMP in the 15-mo-old SweArc mice compared with that in age-matched WT littermate controls and in 6-mo-old SweArc and WT mice (Fig. 2). In line with the comparable ACh-induced vasorelaxation in the 6-mo-old SweArc and WT mice, no significant differences in basilar artery cGMP levels were observed between these two groups. Furthermore, no effect of aging on cGMP levels was observed in either the WT or SweArc mice (Fig. 2E).

Aβ may increase reactive oxygen species levels (24), thereby scavenging NO and diminishing its bioavailability (7). However, staining of the basilar artery for 4-HNE, a marker of lipid peroxidation and thus of oxidative stress, revealed equally low signals in both strains, irrespective of age (Fig. 2). This suggests that the observed reduced bioavailability of NO is not related to an increased scavenging by free radicals but perhaps to decreased NO production. Similarly, quantitative analysis of hyperplastic and/or hypertrophic changes in the endothelial cells of the SweArc basilar arteries that might underlie the observed decrease in cGMP showed no significant differences in these parameters between the SweArc and WT basilar arteries (Fig. 3). These data confirmed that the differences observed were independent of morphological changes in the basilar artery endothelium.

As our laboratory has previously shown, penetrating and intraparenchymal microvessels and arteries of SweArc mice show widespread CAA, starting at around 8 mo of age, which increases in severity with advancing age and has detrimental effects on the vasculature (14, 17). In line with the above, staining for Aβ confirmed that the intraparenchymal vessels were affected by CAA in the 15-mo-old SweArc mice (Fig. 4A); however, CAA was absent in the basilar artery (Fig. 4B), similar to the complete absence of parenchymal Aβ and CAA in the 15-mo-old WT littermate controls (Fig. 4, C and D). Of note, Aβ deposition in the vessel wall of peripheral arteries and microvessels is not observed in SweArc mice.

**DISCUSSION**

We assessed systemic vs. cerebral vascular function in a mouse model Alzheimer’s disease (AD) at different disease stages. We found CAA-independent endothelial dysfunction and reduced NO bioavailability, which was particularly accentuated in the basilar artery, whereby paradoxical vasoconstrictions in response to ACh were observed. These findings may increase the understanding of the vascular pathology and related chronic cerebral hypoperfusion present at pre-AD stages, where CAA is rare (9, 15, 23). Furthermore, our findings are in line with our laboratory’s previous histological studies in human brain and those of others showing CAA-independent pathological remodeling of vessel wall constituents regulating arterial vasodynamics (18, 21).

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**Fig. 3.** Analysis of the number of endothelial cells and their morphology in the basilar artery. A: representative images of free-floating brain tissue sections at the level of the basilar artery of a 15-mo-old Swedish-Arctic (SweArc) and wild-type (WT) littermate control mouse stained for the endothelial cell marker CD31 (green). B: quantification of the number of endothelial cells in five consecutive basilar artery sections of three SweArc and three WT mice reveals the absence of significant differences herein between the SweArc and WT littermate control mice. C: similarly, no significant differences are observed in endothelial cell width between 15-mo-old SweArc and WT littermate control mice. Values are means ± SE. Scale bar in A = 50 μm.
Our finding of a histological reduction in cGMP content, along with impairment in ACh-induced vasorelaxation in the basilar artery of 15-mo-old SweArc mice, is in line with that of another study (19). Although there the authors had not assessed vasodynamic responses as we did, they showed that both soluble and fibrillar Aβ impaired NO-cGMP signaling by inhibiting the activation of guanylate cyclase. Both of these forms of Aβ are abundantly present in aged SweArc mice (14). Indeed, soluble protofibrillar Aβ species increase in an age-dependent manner in SweArc mice, specifically from 8 mo onward (16, 25), potentially explaining the unchanged basilar artery cGMP content and conserved responses to ACh in 6-mo-old SweArc mice.

Our results indicate that, at least for the endothelium of the basilar and femoral artery, CAA is not a prerequisite for inducing endothelial dysfunction. However, although we did not detect CAA in the basilar artery, pathological changes in the CAA-affected leptomeningeal and intraparenchymal ves-

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**Fig. 4. Distribution of cerebral amyloid angiopathy (CAA) in aged Swedish-Arctic (SweArc) mice and the absence of CAA and parenchymal amyloid-β (Aβ) in wild-type (WT) littermate controls.**

A: representative images showing the presence and widespread distribution of CAA-affected parenchymal arterioles (*) and CAA-affected (penetrating) arteries (arrows) in the cortex of a 15-mo-old SweArc mouse [CAA, red; CD31, green; α-smooth muscle actin (α-SMA), blue]. Positive staining for α-SMA combined with vessel size confirms that the vessels indicated by arrows are arteries. B: representative images showing the absence of CAA in the basilar artery of a 15-mo-old SweArc mouse. C and D: representative images showing the complete absence of CAA in both the intraparenchymal vessels and basilar artery of a 15-mo-old WT littermate control mouse. Similarly, no parenchymal Aβ is detected in the WT littermate controls. The intensity of the α-SMA staining in B and D was increased using the “Brightness/Contrast” tool in ImageJ to allow proper visualization of the smooth muscle cell layers within the vessel wall. Scale bar in A and C = 200 μm; in B and D = 50 μm.

Soluble Aβ species are produced by VSMCs, as well as neurons (8); the ones produced by the latter can also reach the vascular system through perivascular drainage routes (11, 12). Thus the high level of soluble Aβ species observed in old SweArc mice, combined with the vessel-directed Aβ clearance routes, causes chronic exposure of the vessel wall to high levels of toxic soluble Aβ species, which, consequently, may alter NO levels (6). Nevertheless, despite this high vessel wall exposure to soluble Aβ, the VSMC-dependent responses to SNP were comparable in WT and SweArc mice, irrespective of age. This is in contrast to what our laboratory and others have previously shown for the deleterious effect of CAA on VSMC function and morphology (17, 18, 28).

Our results indicate that, at least for the endothelium of the basilar and femoral artery, CAA is not a prerequisite for inducing endothelial dysfunction. However, although we did not detect CAA in the basilar artery, pathological changes in the CAA-affected leptomeningeal and intraparenchymal ves-
sels downstream of the basilar artery could still partially contribute to the observed vascular dysfunction, as they are known to affect vascular integrity and function over large distances (5). Moreover, histological detection of vascular-related Aβ/CAA lacks the sensitivity that biochemical assays have for detecting (soluble) Aβ species in the vessel wall, thereby providing a limited assessment of the actual total vessel wall Aβ content (27).

Aging per se impairs cerebrovascular function; therefore, cerebrovascular impairment due to aging and/or AD pathology needs careful delineation. Our laboratory previously showed age-related impairment of endothelium-dependent basilar artery relaxation in WT mice (26). Thus the similarly blunted vasorelaxation observed in the basilar artery of 6-mo-old SweArc and 6- and 15-mo-old WT mice in the present study is most likely an effect of aging. However, the exacerbated arterial dysfunction with a paradoxical vasoconstriction observed in 15-mo-old SweArc mice indicates a combined effect of aging and Aβ-mediated reduction in NO bioavailability.

SweArc mice, like other transgenic AD mice, only partially model aspects of the vast cerebrovascular pathology that is present in AD (2), thus posing limits to the extrapolation of our results to the disease in humans. Nevertheless, taken together, our data add to the growing understanding of the multifaceted vascular insults that contribute to AD (2, 3, 18). They especially shed much needed light on the functional implications of cerebrovascular pathology in AD and provide further evidence of the need for vascular-specific therapies to combat this disease.

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DISCLOSURES
R. M. Nitsch owns stock in Neurimmune Therapeutics AG, Switzerland, and is cofounder and member of the board of directors of said company.

AUTHOR CONTRIBUTIONS


