Cleavage of amyloid precursor protein elicited by chronic cerebral hypoperfusion

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Abstract

In the present study, we sought to determine whether low-grade, chronic vascular insufficiency induced in a rodent model of chronic cerebrohypoperfusion is sufficient, in and of itself, to trigger cleavage of the amyloid precursor protein (APP) into βA-sized fragments. We report that chronic two vessel occlusion (2VO) results in progressive accumulation of βA peptides detected by Western analysis in aged rats correlating with a shift in the immunohistochemical localization of APP from neurons to extracellular deposits in brain parenchyma. These data indicate that the 2VO paradigm reproduces features of βA biogenesis characteristic of sporadic Alzheimer's disease. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Amyloid precursor protein; β-amyloid; Chronic vascular insufficiency; Alzheimer's disease; Chronic ischemia

1. Introduction

It is well established that the integrity of the cerebral vasculature is essential to maintaining cognitive function during aging (for a review see [8]). Chronic disruption of cerebral blood flow resulting from carotid insufficiency and other ischemic states associated with aging can induce neurological deficits and dementia [2,7,17,18]. These conditions are exacerbated during arteriosclerosis-induced dementia and Alzheimer's disease by cerebral angiopathy and morphological aberrations in brain microvasculature [11]. Although circulation-dependent behavioral deficits can be modelled in experimental paradigms of chronic cerebrovascular insufficiency, the impact of protracted low-grade blood flow reduction on amyloid precursor protein (APP) metabolism in the absence of other disease factors is still ambiguous [5,6,15,18].

In Alzheimer's disease, membrane-bound APP is proteolytically processed into βA-sized fragments (8–15kDa) by the sequential actions of β- and γ-secretases. These aberrant fragments can accumulate both intraneuronally and as extracellular deposits in brain parenchyma, exerting cytotoxic effects on neurons and increasing vascular permeability [13,21,23,24]. The cerebrovascular actions of βA peptides render transgenic animals overexpressing APP more susceptible to ischemic injury presumably by increasing vascular reactivity thereby eliciting more severe ischemia in infarcted regions [24]. Conversely, ischemic attack has also been implicated in the etiology of βA deposition. After transient ischemic attack, post-translational modification of APP into βA-sized fragments and subsequent extracellular βA deposition in brain parenchyma have been observed in ischemic tissue [1,12,20,22]. The increase in APP, however, may not always lead to neuropathological βA-associated cleavage. After 3–4 weeks of bilateral carotid artery occlusion, hippocampal APP immunoreactivity is only marginally increased [10]. Cleavage of the parent protein to βA-sized fragments after chronic ischemia was not addressed in this study [10]. In experimental models of repeated ischemic attacks, APP protein expression and βA accumulation have been demonstrated not only in damaged tissue but also in regions spared ischemic insult, thereby implicating APP synthesis in both neuropathology and neural protection [9,10]. The only report, to our knowledge, of APP processing in a chronic model of ischemia has demonstrated enhanced APP cleavage in animals treated prophylactically

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with propentofylline (and thus spared severe ischemic injury) relative to untreated ischemic animals [16]. Thus, it is unclear whether changes in APP post-translation modification after cerebral ischemia are, in fact, neuroprotective or whether they contribute to progressive neurodegeneration elicited by chronic cerebral hypoperfusion. Furthermore, it is unknown whether chronic low-grade cerebral ischemia leads to βA-deposition or whether βA-associated cleavage is only elicited by severe ischemia/reperfusion injury.

In the present study, we sought to characterize APP processing in an experimental model of sustained low-grade ischemia. In rodents, permanent ligation of the common carotid arteries (2VO) induces morphological abnormalities in neurons and quantifiable cell loss within 7 months of blood flow reduction [5,15,18]. Using the 2VO paradigm, we have demonstrated that a moderate but chronic reduction of cerebral blood flow elicits protracted gliosis, incremental apoptotic death in neurons in rat hippocampus, and progressive behavioral impairment [3,4,15]. We now report that the low grade chronic vascular insufficiency induced by 2VO is, in and of itself, sufficient to trigger cleavage of APP into βA-sized fragments and induce extracellular accumulation of APP proteolytic products.

2. Materials and methods

2.1. Induction of chronic cerebral hypoperfusion by two vessel occlusion (2VO) surgery

Permanent bilateral carotid occlusion resulting in sustained low-grade ischemia was induced in male Sprague Dawley retired breeders (Charles River Laboratories, PQ), approximately 10 months of age ($n = 20$), as described in [3,15]. Sham surgeries were performed on age-matched control rats ($n = 17$). Rats were anesthetized with ketamine hydrochloride (100 mg/kg IM) and sodium methohexital (40 mg/kg IP). A ventral midline incision was made to expose the carotid arteries and the blood vessels were gently isolated from the carotid sheath and vagus nerve. In 2VO surgery, each carotid artery was double-ligated with 5–0 silk suture just below the vascular bifurcation. In sham operations, arteries were exposed but not ligated. Rats were closely monitored during postoperative recovery and body temperature maintained with a servo-controlled heating pad. Animals were sacrificed at 2 ($n = 3$ 2VO; $n = 2$ sham), 10 ($n = 4$ 2VO; $n = 3$ sham), 20–27 ($n = 5$ 2VO; $n = 5$ sham), and 40 ($n = 8$ 2VO; $n = 7$ sham) weeks after surgery.

For Western analyses, rats sacrificed at 2, 10, and 20–27 weeks were deeply anesthetized with sodium pentobarbitol and euthanized by decapitation. Brains were rapidly removed and blocked coronally at approximately bregma −1.8 and bregma −6.04. The dorsal and ventral hippocampi were removed. Cortices and remaining medial structures were blocked horizontally at the rhinal fissure. The dorsolateral cortex encompassing the somatosensory,

parietal, and cingulate cortices was separated from thalamus and corpus callosum. The ventrolateral cortex encompassing the entorhinal and piriform cortices and amygdaloid nuclei was separated from optic tract, hypothalamus, and ventral thalamic nuclei. Hippocampal and cortical protein was isolated using Triazol (Life Sciences Research Technologies, Mississauga, ON, Canada) according to the protocol provided by the manufacturer. For $n = 5$ animals/condition sacrificed at 20–27 weeks post surgery, protein extracts were prepared separately from each animal for hippocampus and combined dorsolateral and ventrolateral cortices. For all other animals, tissue was pooled at each time point prior to protein extraction.

For immunohistochemical analysis, animals sacrificed at 40 weeks postsurgery ($n = 5$ per condition) were deeply anesthetized with sodium pentobarbitol and euthanized by cardiac perfusion with heparinized saline followed by 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4). Brains were postfixed in phosphate-buffered 4% paraformaldehyde for 24 h at 4°C and tissue was paraffin-embedded according to standard histological procedure. Coronal sections were cut on a rotary microtome at 8 μm. Sections at approximately bregma −3.8 were analyzed. All manipulations were performed in compliance with approved institutional protocols and according to the strict ethical guidelines for animal experimentation established by the Medical Research Council (Canada).

2.2. Western analyses

Protein (40 μg) was separated by SDS-PAGE under reducing conditions on 12.5% and 7.5% polyacrylamide gels containing 0.1% (w/v) SDS and electroblotted to Immobillon membrane. Membranes were incubated overnight with either polyclonal antibody anti-βA (1:5, Roche, Laval, PQ) or monoclonal antibody anti-APP (1:10; Roche). Anti-βA was raised against the synthetic peptide DAEFRHDSGYEVHHQKLLVFFAEDVGSNKGAIIGLMVGGVIA recognizing C-terminal epitopes located between amino acids 597–638 of human APP. Anti-βA detects amyloidgenic βA in both human and rat. Anti-APP was raised against the final C-terminus amino acids 642–695 of human APP and detects APP, intermediate-sized fragments, and short carboxyl fragments released during final amyloidgenic βA processing in both human and rat. Secondary antibodies were a horse radish peroxidase-conjugated anti-rabbit IgG (1:400; Roche) or a horseradish peroxidase-conjugated anti-mouse IgG (1:1000; Jackson Immunolabs, West Grove, PA, USA). Immunoreactive fragments were visualized by chemiluminescence according to the protocol established by the manufacturer (Pierce, Rockford, IL, USA). In control reactions, membranes were incubated with secondary reagent in the absence of primary antibody. In all reactions, membranes were blocked in 10 mM PBS (10 mM sodium phosphate buffer, pH 7.5, 154 mM NaCl) containing 1% heat-denatured casein and washed repeatedly between reac-
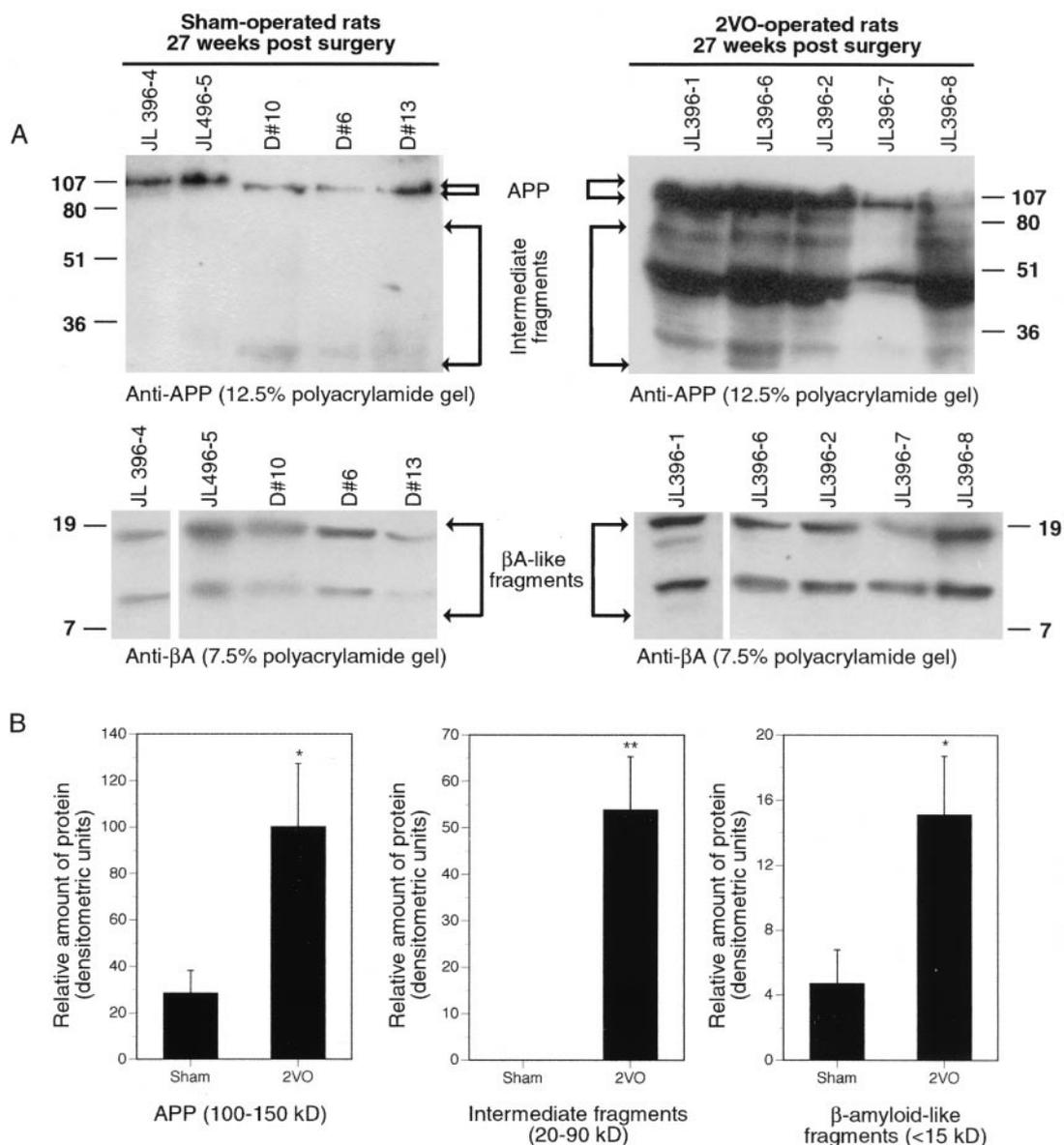
Fig. 1. Increased APP protein expression and APP cleavage into intermediate- and βA-sized fragments are detected in rat parietal-temporal cortex 20–27 weeks after 2VO surgery. Protein (40 μg) was subjected to Western analysis using anti-APP to detect APP and intermediate cleavage fragments (A, upper blots) or anti-βA to detect βA-sized fragments (A, lower blots) as described in Materials and methods. Blots were reacted with antibodies simultaneously and exposed for identical periods of time. Densitometry was performed on exposures that fell within linear range for analysis as described in Materials and methods. Figures depict longer exposure times than those used for densitometry. A significant increase in the amounts of APP detected and in the production of βA-sized fragments was detected in 2VO-operated rats relative to sham-operated rats (Panel B). A significant increase in the cleavage of intermediate sized fragments was detected in 2VO-operated rats relative to sham-operated rats (Panel B). *$P < 0.05$. **$P < 0.01$.

tions in blocking solution containing 0.35% Tween-20 (anti-βA) or 0.1% Tween-20 (anti-APP). Antibodies were diluted in blocking solution lacking Tween-20.

Densitometry was performed on a LKB Ultrascan Enhanced Laser Densitometer running GelScan XL software (Pharmacia, Baie d'Urfé, PQ). Data are presented as relative amount of protein as defined by the "Area(%)" value (the relative to total integrated area of the absorbance peak) multiplied by 100. The "Area(%)"x100 value is expressed as densitometric units. Values under 200 fall within in linear range for densitometric analysis. To obtain Western blots

within linear range, multiple exposures were developed. Statistical analysis were only performed on blots for which maximal signal intensity was below 200. Only blots that were developed simultaneously were compared statistically. Densitometric data were analyzed by Student's t test comparing 2VO versus sham with α set at $P < 0.05$.

2.3. Immunohistochemistry

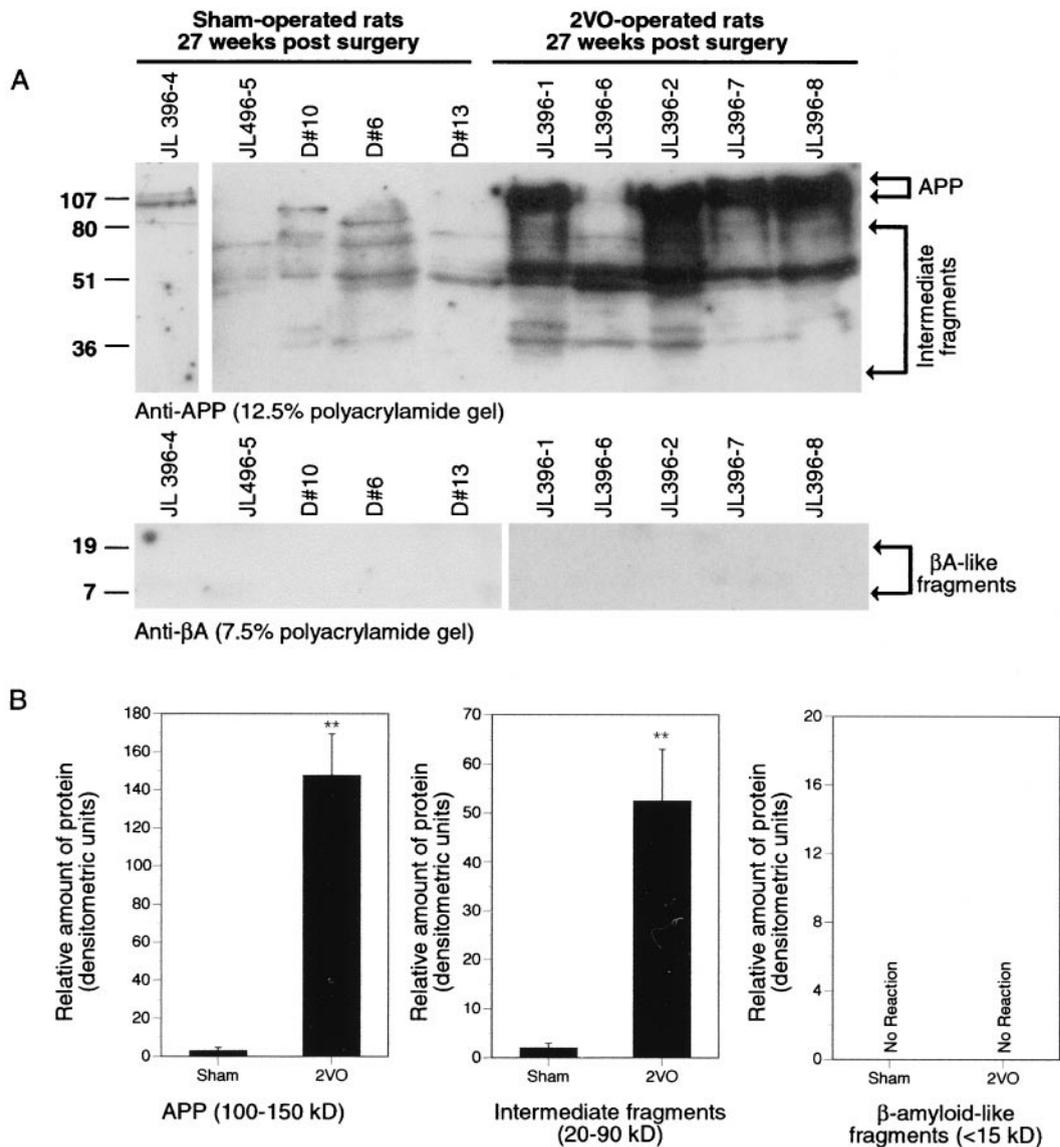Paraffin-embedded sections were deparaffinized in clearane, rehydrated through a series of graded alcohols,

Fig. 2. Increased APP protein expression and APP cleavage into intermediate-sized fragments but not βA is detected in rat hippocampus after 2VO surgery. Protein (40 μg) was subjected to Western analysis using anti-APP to detect APP and intermediate cleavage fragments (A, top) or anti-βA to detect βA-sized fragments (A, bottom) as described in Section 2. Blots were reacted with antibodies simultaneously and exposed for identical periods of time. Densitometry was performed on exposures that fell within linear range for analysis as described in Materials and methods. Figures depict longer exposure times than those used for densitometry. A highly statistically significant increase in the amounts of APP detected and in the cleavage of intermediate sized fragments was detected in 2VO-operated rats relative to sham-operated rats (B). βA-sized fragments were not detected in either condition.

and equilibrated in 10 mM PBS (10 mM sodium phosphate buffer, pH 7.2, 154 mM NaCl). Sections were reacted overnight with primary antibody (APP, 1:100) at 4°C, labeled for 1 h at room temperature with biotinylated goat anti-mouse IgG (1:300; Sigma Chemical Co., St. Louis, MO, USA), incubated for 1 h in extravidin-peroxidase (1:40, Sigma), and reacted with 1 mg/ml diaminobenzidine in 50 mM Tris-HCl, pH 8.0 containing 0.003% H_2O_2. Antibodies and tertiary reagents were diluted in Ab buffer (10 mM PBS, 0.3% Triton-X, 3% BSA, pH 7.2).

3. Results

3.1. Changes in APP processing elicited by 2VO

Retired male Sprague Dawley breeders were 10 months old at the time of sham ($n = 5$) or 2VO surgery ($n = 5$). Rats were sacrificed between 20 and 27 weeks after surgery. Brains were blocked coronally at approximately bregma −1.8 and bregma −6.04. Western immunoblots of protein extracts from the parietal-temporal cortex and the hippocampal formation were analyzed. In
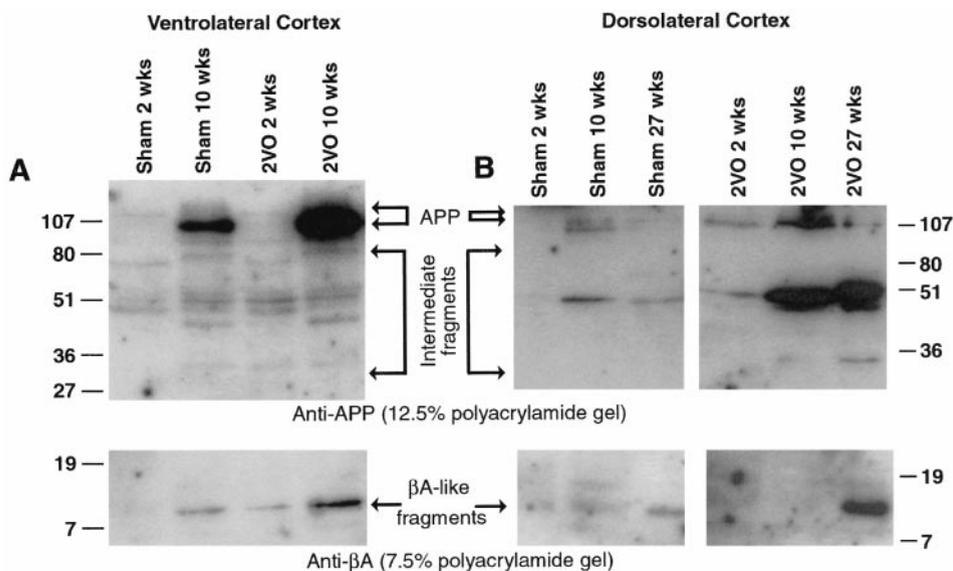
Fig. 3. A time-dependent increase in APP cleavage is detected in rat ventrolateral and dorsolateral cortex following 2VO surgery. Protein was isolated from rat ventrolateral (A) and dorsolateral (B) cortices. Protein was pooled from tissue extracted from 2-week 2VO animals ($n = 3$), 2-week sham animals ($n = 2$), 10-week 2VO animals ($n = 4$), 10-week sham animals ($n = 3$), 27-week 2VO animals ($n = 3$, dorsolateral cortex only), and 27-week sham animals ($n = 2$, dorsolateral cortex only). Western analysis was performed using anti-APP (upper blots) to detect APP and intermediate cleavage fragments or anti-βA (lower blots) to detect βA-sized fragments as described in Materials and methods.

parietal-temporal cortex, a significant increase in the amounts of APP proprotein ($t = 2.49$, df $= 8$, $P < 0.05$), intermediate sized degradative products ($t = 4.72$, df $= 8$, $P < 0.01$), and βA-like fragments ($t = 2.51$, df $= 8$, $P < 0.05$) were observed in 2VO-operated rats relative to sham-operated rats (Fig. 1A and B). In the hippocampus, a significant increase in the amounts of APP proprotein ($t = 6.58$, df $= 8$, $P < 0.01$) and intermediate-sized degradative products ($t = 4.73$, df $= 8$, $P < 0.01$) were observed in 2VO- compared to sham-operated rats (Fig. 2A and B). Cleavage of APP into βA-sized fragments was not detected in rat hippocampus 20–27 weeks after 2VO surgery.

To qualitatively examine the kinetics of this cleavage, replicate Western blots were performed on pooled protein samples extracted from animals sacrificed at 2 ($n = 3$ 2VO; $n = 2$ sham), 10 ($n = 4$ 2VO; $n = 3$ sham), or 27 ($n = 3$ 2VO; $n = 2$ sham) weeks after surgery. Densitometry was not performed, given that protein samples were pooled across subjects and immunoblots were only conducted in replicate. To more precisely isolate changes in parietal-temporal cortex, tissue was dissected into dorsolateral cortex encompassing the somatosensory, parietal, and cingulate cortices and ventrolateral cortex encompassing the entorhinal and piriform cortices and amygdaloid nuclei prior to extraction. In ventrolateral cortex of sham-operated rats, bands corresponding to full-length APP, proteolytic intermediate products, and potentially amyloidgenic βA were identified (Fig. 3A). An increase in APP proprotein levels was consistently observed over time after both sham and 2VO surgery with a more pronounced increase noted in

2VO rats (Fig. 3A, top; compare 2- and 10-week lanes in both sham and 2VO conditions). βA cleavage was detected at 10 weeks postsurgery in sham-operated animals, but not at earlier time-points (Fig. 3A, bottom; compare sham 2 and 10 week lanes). Synthesis of this potentially amyloidgenic isoform was accelerated by 2VO surgery. Animals exhibited a 12kDa anti-βA immunoreactive band 2 weeks after 2VO surgery but not sham surgery, with a marked increase in synthesis observed at 10 weeks (Fig. 3A, bottom; compare 2VO 2- versus 10-week lanes). In dorsolateral cortex, full-length APP, intermediate proteolytic products, and βA sized fragments were detected (Fig. 3B). Maximal levels of APP proprotein were observed 10 weeks after sham or 2VO surgery (Fig. 3B, top). Consistent with data presented for $n = 5$ animals in Fig. 1, 2VO rats synthesized more APP and intermediate sized fragments than sham-operated animals 27 week after surgery (Fig. 3B, top; compare sham and 2VO 27 week lanes). Although a progressive accumulation of βA fragments was observed between 2 and 27 weeks postsurgery in sham-operated animals, this increase was markedly augmented following 2VO surgery (Fig. 3B, bottom).

In hippocampus, full length APP isoforms were detected in sham-operated animals with an increase in synthesis over time after surgery (Fig. 4A; compare sham 2- and 10-week lanes). 2VO surgery was associated with an even greater increase in the levels of intermediate proteolytic fragments (Fig. 4A; compare sham and 2VO lanes). As with later time points (Fig. 2), βA proteolytic products were not detected in hippocampus following sham or 2VO surgery (Fig. 4B).
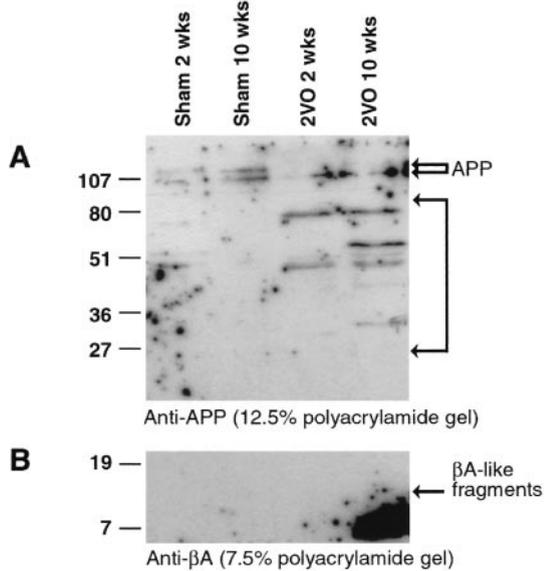
Fig. 4. APP cleavage into intermediate proteolytic fragments increases over time in rat hippocampus following 2VO surgery. Protein was isolated from 2-week 2VO animals ($n = 3$), 2-week sham animals ($n = 2$), 10-week 2VO animals ($n = 4$), or 10-week sham animals ($n = 3$). Western analysis was performed using anti-APP to detect APP and intermediate cleavage fragments (A) or anti-βA to detect βA-sized fragments (B) as described in Section 2.

3.2. Extracellular deposition of APP biosynthetic products

Immunohistochemistry for APP was performed using anti-APP at 40 weeks post 2VO or sham surgery. Consistent with the reduction in full length APP synthesis observed by Western analysis after 20–27 weeks of 2VO, a qualitative decrease in the extent of neuronal reactivity was observed 40 weeks after surgery in 2VO rats (Fig. 5B) compared to sham-operated animals (Fig. 5A). Notably, de novo extracellular deposition of APP immunoreactive deposits was evident in brain parenchyma of 2VO animals in ventrolateral cortex (Fig. 5B); an area that exhibited APP cleavage consistent with production of βA by Western analysis. Depositions were not observed in areas that failed to exhibit βA biosynthesis (i.e. hippocampus; data not shown).

4. Conclusions

In the present study, we characterize APP cleavage in aging rodent hippocampus and parietal-temporal cortex after induction of low-grade chronic vascular insufficiency. Our results indicate that chronic cerebrohypoperfusion elicited by 2VO is sufficient to stimulate aberrant APP process-
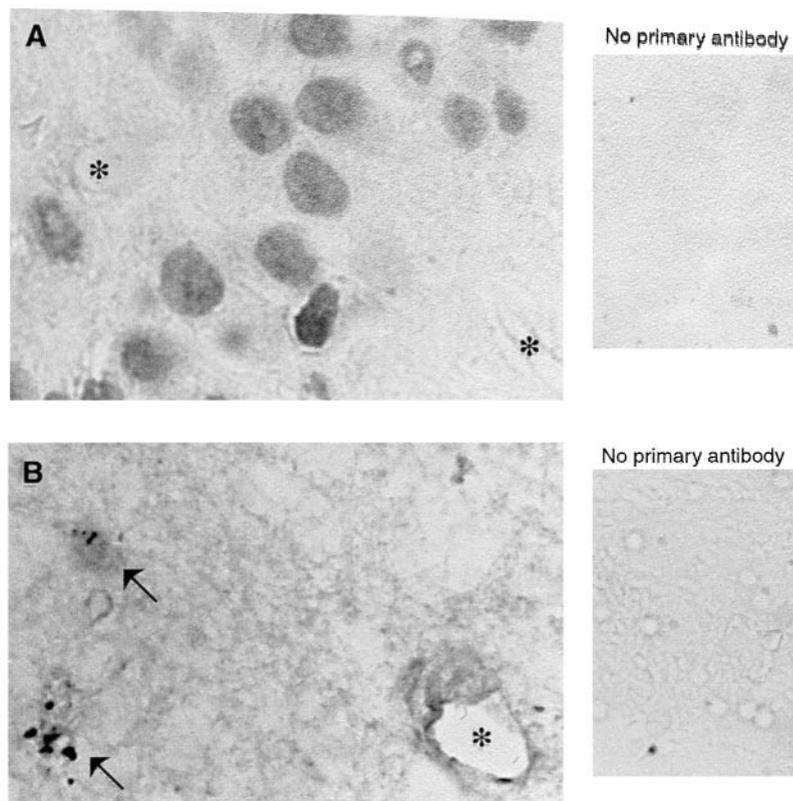

Fig. 5. Immunohistochemical localization of APP in ventrolateral cortex of sham- and 2VO-operated rats 40 weeks after surgery. Immunohistochemistry was performed using anti-APP as described in Materials and methods. (A) Sham-operated ventrolateral cortex. Note neuron-associated labeling of APP. (B) 2VO-operated ventrolateral cortex. Note the reduction of APP immunoreactivity in neurons and extracellular deposition of material in brain parenchyma (B). Anti-APP immunoreactive extracellular deposits were not detected in brain regions that failed to exhibit βA-sized fragments by Western analysis (i.e., hippocampus). Right-hand panel depicts staining of adjacent sections in the absence of primary antibody.

ing including production of potentially amyloidgenic βA and accumulation of extracellular βA deposits in rat cortex in the absence of any other disease factors. These data suggest that chronic cerebral hypoperfusion may contribute to the alterations in APP biosynthesis associated with Alzheimer's disease.

4.1. Biogenesis of βA-elicited by 2VO

APP proteolysis to βA-sized fragments was observed in rodents after 2VO in parietal-temporal cortex but not hippocampus. In cortical tissue, full-length partially glycosylated APP (100 kDa), intermediate proteolytic fragments (20–90 kDa), and smaller βA-like products (<15 kDa) were detected by Western analysis. βA biogenesis was observed at low levels in sham-operated rats in these brain regions. However, accumulation of βA-sized fragments was exacerbated over time in cortical tissue after 2VO with synthesis more pronounced in ventrolateral parietal-temporal cortex. This sustained accumulation of βA associated with 2VO surgery correlated with a subsequent shift in immunohistochemical localization of APP from neuron-associated staining to extracellular deposits in brain parenchyma. βA-associated cleavage was not detected in hippocampus, although an increase in intermediate proteolytic APP fragments was noted over time after 2VO-surgery. To our knowledge, this finding represents the first time that aberrant APP processing characteristic of normal aging and late-onset Alzheimer's disease has been demonstrated in a rodent model of low grade chronic vascular insufficiency in the absence of any other disease factors.

4.2. Comparison with APP processing in Alzheimer's brain

The primary difference between APP processing observed in the 2VO rodent model of chronic vascular insufficiency and APP proteolysis detected in Alzheimer's tissue is the regional localization of proteolytic events. In Alzheimer's brain, βA-associated cleavage is frequently detected in both hippocampus and parietal-temporal cortex. The lack of βA accumulation in rodent hippocampus may be the result of regional differences in cerebral hypoperfusion elicited. Previous work has demonstrated that the chronic reductions in blood flow after 2VO are more pronounced in cortical structures than in hippocampus. 2VO in rat elicits sustained low-grade ischemia characterized by an immediate 50% reduction in cerebral blood flow to hippocampal and a 75% reduction in cerebral blood flow to cortical structures within hours of 2VO surgery [14,19]. A chronic 25% reduction in cerebral blood flow to hippocampal and a 40% reduction in cerebral blood flow to cortical structure is observed within days of carotid ligation [14,19]. No further improvements in cerebral blood flow are detected after several months of 2VO [5,14,19]. Thus, it is consistent that βA-associated proteolytic events elicited by sustained hypoperfusion would be more pronounced in cortical than hippocampal structures.

5. Conclusion

In the present study, we sought to characterize APP processing in an experimental model of sustained low-grade ischemia. We have previously demonstrated that a moderate but chronic reduction of cerebral blood flow elicits neuropathology associated with progressive behavioral impairment. We now demonstrate that 2VO can trigger βA biogenesis and deposition of extracellular amyloid deposits in the absence of any other disease factors. These data demonstrate that chronic low grade cerebral hypoperfusion has effects on APP proteolytic processing similar to those associated with sporadic Alzheimer's disease and suggests that chronic 2VO will be a useful model to investigate the behavioral effects of aberrant APP cleavage.

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