Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 11480–11484, December 1995
Neurobiology

Discovery of adrenomedullin in rat ischemic cortex and evidence for its role in exacerbating focal brain ischemic damage

XINKANG WANG, TIAN-LI YUE, FRANK C. BARONE, RAYMOND F. WHITE, ROBERT K. CLARK, ROBERT N. WILLETTE, ANTONY C. SULPIZIO, NAMBI V. AIYAR, ROBERT R. RUFFOLO, JR., AND GIORA Z. FEUERSTEIN

Department of Cardiovascular Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406

Communicated by Stephen J. Benkovic, The Pennsylvania State University, University Park, PA, August 7, 1995 (received for review March 3, 1995)

ABSTRACT **Focal brain ischemia is the most common event leading to stroke in humans. To understand the molecular mechanisms associated with brain ischemia, we applied the technique of mRNA differential display and isolated a gene that encodes a recently discovered peptide, adrenomedullin (AM), which is a member of the calcitonin gene-related peptide (CGRP) family. Using the rat focal stroke model of middle cerebral artery occlusion (MCAO), we determined that AM mRNA expression was significantly increased in the ischemic cortex up to 17.4-fold at 3 h post-MCAO ($P < 0.05$) and 21.7-fold at 6 h post-MCAO ($P < 0.05$) and remained elevated for up to 15 days (9.6-fold increase; $P < 0.05$). Immunohistochemical studies localized AM to ischemic neuronal processes, and radioligand (^{125}I-labeled CGRP) displacement revealed high-affinity (IC_{50} = 80.3 nmol) binding of AM to CGRP receptors in brain cortex. The cerebrovascular function of AM was studied using synthetic AM microinjected onto rat pial vessels using a cranial window or applied to canine basilar arteries *in vitro*. AM, applied abluminally, produced dose-dependent relaxation of preconstricted pial vessels ($P < 0.05$). Intracerebroventricular (but not systemic) AM administration at a high dose (8 nmol), prior to and after MCAO, increased the degree of focal ischemic injury ($P < 0.05$). The ischemia-induced expression of both AM mRNA and peptide in ischemic cortical neurons, the demonstration of the direct vasodilating effects of the peptide on cerebral vessels, and the ability of AM to exacerbate ischemic brain damage suggests that AM plays a significant role in focal ischemic brain injury.**

Adrenomedullin (AM) is a recently discovered peptide that was initially identified from human pheochromocytoma (1). Biologically active AM consists of 52 amino acids in humans and 50 amino acids in rats (1, 2), and both AMs exhibit potent vasodilator activity *in vitro* and *in vivo* (3–6). AM bears homology to a family of peptides that includes calcitonin gene-related peptide (CGRP) (7–10) and amylin (11, 12). CGRP is a widely distributed neuropeptide best known for its potent vasodilator actions (13–15) and its effect on insulin functions (16). Amylin is the major protein found in islet amyloid (11, 16) in humans with non-insulin-dependent diabetes mellitus. CGRP and amylin have been found to have a wide range of biological activities, including energy metabolism, central nervous system and cardiovascular functions, and calcium metabolism (for review see refs. 16 and 17). In contrast, little is known of the biological function of AM beyond vasodilation. In contrast to CGRP, AM mRNA and peptide have not been detected in normal brain (1, 2). Very recently, AM has been associated with congestive heart failure, since elevated levels of AM were found in cardiac tissue of the failing hearts (18).

In the present report, we used a recently developed mRNA differential display technique (19) to identify genes expressed in response to brain ischemia induced by permanent occlusion of the middle cerebral artery (MCAO) in the rat. We herein report the upregulation of AM mRNA expression and peptide production after focal cerebral ischemia. Furthermore, we have demonstrated that AM is a potent cerebral vasodilator and that intracerebroventricular (i.c.v.), but not i.v., administration of AM exacerbated focal ischemic injury. Taken together, our data suggest a significant role for AM in the evolution of stroke.

MATERIALS AND METHODS

Focal Brain Ischemia. Focal cerebral ischemia was carried out in spontaneously hypertensive rats (Taconic Farms) by permanent MCAO as described in detail previously (20, 21).

mRNA Differential Display. Total cellular RNA was isolated from ipsilateral (ischemic) cortex and contralateral (nonischemic) cortex samples as described (22, 23). RNA samples from 2 h and 12 h after MCAO were used for differential display essentially as described (19, 24) using an RNAmap kit (GenHunter). The differentially expressed bands of interest were isolated, reamplified, and subcloned into a pCRII vector (Invitrogen).

Northern Blot Analysis. Northern hybridization was carried out as described earlier (23, 25).

cDNA Library Construction, Screening, and DNA Sequence Analysis. A rat cerebral ischemia cDNA library in λ ZAP II vector (Stratagene) was constructed according to manufacturer's specifications using poly(A) RNA isolated from ischemic cortex 2 and 12 h after MCAO. This library was screened using the PMCAO-9 cDNA probe isolated from differential display as described in detail previously (25). Four individual positive clones were isolated, and the corresponding phagemids were produced by *in vivo* excision. The complete cDNA sequence* of AM was determined from both strands. DNA sequence analysis and computer data base searches were performed using the Genetics Computer Group program.

Primer Extension Analysis. Primer extension was carried out as described (24) in the presence of a ^{32}P-labeled primer complementary to bases 46–64 (5′-GATGAGAAGCCGAGAAACC-3′), and 5 μg of poly(A) RNA isolated from rat ischemic cortex at 12 h after MCAO.

Immunohistochemical Study. Six-micron-thick frozen sections were incubated with rabbit anti-AM-(1–52) (human) IgG (Peninsula Laboratories) and then with fluorescein-conjugated goat anti-rabbit IgG antiserum (Organon Teknika–Cappel). Antisera were diluted in phosphate-buffered saline (PBS) containing 3% (wt/vol) bovine serum albumin. Parallel sections were incubated with either preabsorbed antiserum [i.e., preincubating anti-AM antiserum with human AM-(1–52)

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; MCAO, middle cerebral artery occlusion; ET-1, endothelin 1; i.c.v., intracerebroventricular.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. U15419).

(Peninsula Laboratories) at 10 μg/ml] or secondary antiserum only. Double labeling was performed on sections by first treating for AM immunoreactivity as above and then applying monoclonal antibodies for glial fibrillary acidic protein (Boehringer Mannheim) or neurofilaments (ICN) followed by rhodamine-conjugated goat anti-mouse IgG secondary antiserum. Sections were analyzed using OPTIMAS image analysis (BioScan, Edmonds, WA).

***In Vitro* Canine Cerebral Vessels.** Four adult mongrel dogs were euthanized via an intravenous overdose of pentobarbital. The basilar artery with intact endothelium was removed, and segments were prepared for isometric tension recording (for details, see ref. 26). An optimum resting tension of 750 mg was applied to each segment. During the equilibration period (≈2 h), the segments included in this study generated substantial spontaneous tone. The concentration-related effects of AM on spontaneous tone were then determined. In control experiments, pretreatment of the tissues with the appropriate volume of the AM vehicle (saline) had no significant effect on the spontaneous tone. The relaxation observed with AM was expressed as the mean ± SEM of the percentage of spontaneous tone.

Rat Pial Arteries. The effects of AM on pial arteries in the rat were investigated *in situ* with video microscopic techniques in a cranial window preparation with the dura intact (27). Three male Sprague–Dawley rats were prepared and maintained as described (28). Since in preliminary experiments AM produced inconsistent relaxation, endothelin 1 (ET-1; 0.2 pmol) in saline (0.5 μl) was microinjected to elicit local vasospasm; this was followed by a second dose of ET-1 (0.2 pmol) in the control group or ET-1 (0.2 pmol) plus AM (50 pmol) in the AM group. All responses were recorded on video tape and analyzed using National Institutes of Health image analysis software.

AM Administration in MCAO. Focal ischemia was produced in spontaneously hypertensive rats as described earlier. AM (i.v. at 1 μg/kg per min; $n = 6$) or vehicle (PBS with 1% bovine serum albumin; $n = 6$) was administrated continuously for 1 h pre-MCAO and 4 h post-MCAO. i.c.v. AM at doses of 0 ($n = 13$), 0.2 ($n = 9$), 2 ($n = 10$), or 8 ($n = 13$) nmol in 5 μl was administered into the ipsilateral cerebral ventricle at 1 h pre-MCAO and 6 h post-MCAO. After 24 h of MCAO, forebrain sections were removed, stained, and analyzed as described (20, 21) for percent hemispheric swelling and percent hemispheric infarct (normalized to the contralateral control hemisphere) and the actual infarct volume in cubic millimeters.

RESULTS AND DISCUSSION

Identification of Upregulated PMCAO-9 Gene Expression in Rat Ischemic Cortex Using mRNA Differential Display. Fig. 1*A* illustrates a representative autoradiograph showing the ischemia-induced expression of a band designated as PMCAO-9 at 2 and 12 h post-MCAO using mRNA differential display. The upregulation of this gene expressed in ischemic cortex was confirmed using Northern blot analysis (Fig. 1*B*). Thereafter, PMCAO-9 was subcloned into a pCRII vector and subjected to DNA sequencing analysis. Computer data base searches of the PMCAO-9 sequence (Fig. 2) demonstrated that the sequence represented an unreported cDNA.

Isolation and Analysis of AM cDNA. A rat brain ischemia cDNA library was constructed and screened using the PMCAO-9 DNA as a probe. Four individual positive clones were isolated, which differed in length at the 5′ ends (Fig. 2). Upon completion of the full-length cDNA sequencing, we found that the clone matched a rat precursor AM cDNA in the GenBank data base (accession no. D15069). However, several sequence differences were noted: 18 bases have been extended at the 5′ end of our clone compared to the sequence reported previously (2). In addition, a single base (T) deletion after base 1085 and a base replacement of C for G at base 982 were observed

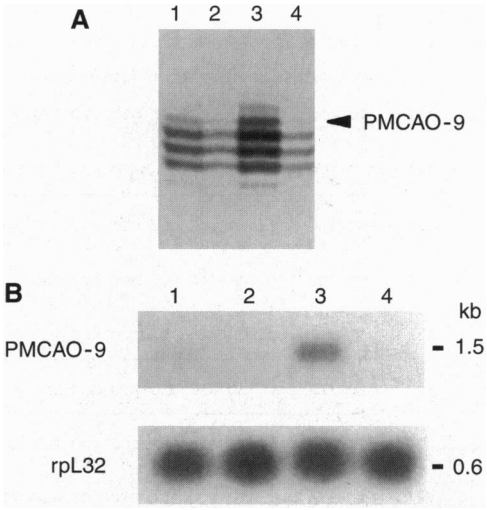

FIG. 1. Identification of altered gene expression in rat ischemic cortex after MCAO by mRNA different display. The differential display PCR was carried out using a 5′ arbitrary primer (5′-GACCGCTTGT-3′) and a 3′ $T_{12}NA$ primer and resolved by electrophoresis in the following order: lane 1, 2 h ischemic; lane 2, 2 h nonischemic; lane 3, 12 h ischemic; lane 4, 12 h nonischemic (*A*). The candidate gene indicated with an arrowhead (PMCAO-9) was confirmed by Northern analysis (*B*). Two micrograms of poly(A) RNA per lane was used with the same loading order as shown in *A*. The ribosomal protein L32 (rpL32) probe was used as a loading control (23).

in all of our four clones compared to the previously reported sequence (2). Also, a three-base deletion (CCT) after base 1227 was observed in three of our four clones, whereas the other clone has five bases (CCTGT) deleted compared to the published rat AM sequence (2). The position of this deleted sequence is tandemly followed by 23 GT repeats in all four clones. This variation is likely to be caused by *in vivo* recombination events as pointed out by a recent study (29). Furthermore, primer extension experiments revealed that the AM mRNA contains a single transcription initiation site located about 47 bases upstream of our longest cDNA clone (data not shown).

Temporal Expression of AM mRNA in Rat Ischemic Cortex After MCAO. Fig. 3*A* shows a representative Northern blot for the AM mRNA expression in the ipsilateral (ischemic) and contralateral (nonischemic) cortical samples at different time points after MCAO. Quantitative Northern blot data ($n = 4$), after normalizing to an rpL32 probe, are illustrated graphically in Fig. 3*B*. Very low levels of AM mRNA were detected in normal (data not shown) or sham-operated cortical samples. The AM mRNA expression was induced significantly in the ischemic cortex 3 h after MCAO (17.4-fold increase compared to sham; $P < 0.05$), reached its peak expression at 6 h (21.7-fold increase; $P < 0.05$), and remained elevated for at least 15 days (9.6-fold increase; $P < 0.05$) after MCAO (Fig. 3). The temporal expression profile of AM mRNA is distinctly different from that of the immediate early genes, such as c-*fos* and zif268 (30), which exhibit a more acute response profile (significant increase at 1 h and peak at 3 h), or the delayed response genes, including inflammatory cytokines, such as tumor necrosis factor α and interleukin 1β (31, 32), which are significantly elevated at 6 h and peak at 12 h after MCAO, in the same focal ischemia model. The significantly prolonged increase in AM mRNA after MCAO (Fig. 3) suggests both an early and late involvement in ischemic injury. As is the case for c-*fos* and other acute response genes, AM mRNA contains two AUUUA motifs in the 3′-untranslated region, which are believed to be associated with rapid degradation of mRNA (33, 34), suggesting that prolonged elevation of AM mRNA expression after MCAO may represent a *de novo* transcriptional event.

```
1    GACACTAGGC AGAACAACTC CAGCCTTTAC CGCTCCTGGT TTCTCGGCTT CTCATCGCAG TCAGTCTTGG ACTTTGCGGG TTTTGCCGCT

91   GTCAGAAGGA CGTCTCGGAC TTTCTGCTTC AAGTGCTTGA CAACTCACCC TTTCAGCAGG GTATCGGAGC ATCGCTACAG A

172  ATG AAG CTG GTT TCC ATC GCC CTG ATG TTA TTG GGT TCG CTC GCC GTT CTC GGC GCG GAC ACC GCA CGG CTC GAC
      M   K   L   V   S   I   A   L   M   L   L   G   S   L   A   V   L   G   A   D   T   A   R   L   D   25

247  ACT TCC TCG CAG TTC CGA AAG AAG TGG AAT AAG TGG GCG CTA AGT CGT GGG AAG AGG GAA CTA CAA GCG TCC AGC
      T   S   S   Q   F   R   K   K   W   N   K   W   A   L   S   R   G   K   R   E   L   Q   A   S   S   50

322  AGC TAC CCT ACG GGG CTC GTT GAT GAG AAG ACA GTC CCG ACC CAG ACT CTT GGG CTC CAG GAC AAG CAG AGC ACG
      S   Y   P   T   G   L   V   D   E   K   T   V   P   T   Q   T   L   G   L   Q   D   K   Q   S   T   75

397  TCT AGC ACC CCA CAA GCC AGC ACT CAG AGC ACA GCC CAC ATT CGA GTC AAA CGC TAC CGC CAG AGC ATG AAC CAG
      S   S   T   P   Q   A   S   T   Q   S   T   A   H   I   R   V   K   R   Y   R   Q   S   M   N   Q   100

472  GGG TCC CGC AGC ACT GGA TGC CGC TTT GGG ACC TGC ACA ATG CAG AAA CTG GCT CAC CAG ATC TAC CAG TTT ACA
      G   S   R   S   T   G   C   R   F   G   T   C   T   M   Q   K   L   A   H   Q   I   Y   Q   F   T   125

547  GAC AAA GAC AAG GAC GGC ATG GCC CCC AGA AAC AAG ATC AGC CCT CAA GGC TAT GGC CGC CGG CGC CGG CGT TCC
      D   K   D   K   D   G   M   A   P   R   N   K   I   S   P   Q   G   Y   G   R   R   R   R   R   S   150

622  CTG CCA GAG GTC CTC CGA GCC CGG ACT GTG GAG TCC TCC CAG GAG CAG ACA CAC TCA GCT CCA GCC TCC CCG GCG
      L   P   E   V   L   R   A   R   T   V   E   S   S   Q   E   Q   T   H   S   A   P   A   S   P   A   175

697  CAC CAA GAC ATC TCC AGA GTC TCT AGG TTA TAG GTGCGGGTGG CAGCATTGAA CAGTCGGGCG AGTATCCCAT TGGCGCCTGC
      H   Q   D   I   S   R   V   S   R   L   *                                                       186

780  GGAATCAGAG AGCTTCGCAC CCTGAGCGGA CTGAGACAAT CTTGCAGAGA TCTGCCTGGC TGCCCCTAGG GGAGGCAGAG GAACCCAAGA

870  TCAAGCCAGG CTCACGTCAG AAACCGAGAA TTACAGGCTG ATACTCTCTC CGGGCAGGGG TCTGAGCCAC TGCCTTGCCC GCTCATAAAC

960  TGGTTTTCTC ACGGGGCATA CGCCTCATTA CTTACTTGAA CTTTCCAAAA CCTAGCGAGG AAAAGTGCAA TGCTTGTTAT ACAGCCAAAG

1050 GTAACTATCA TATTTAAGTT TGTTGATGTC AAGAGGTTTT TTTTTTGTAA CTTCAAATAT ATAGAAATAT TTTTGTACGT TATATATTGT

1140 ATTAAGGGCA TTTTAAAGCG ATTATATTGT CACCTTCCCC TATTTTAAGA AGTGAATGTC TCAGCAAGGT GTAAGGTTGT TTGGTTCCCC

1230 GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTAAGG TGGAGAGCGC CTGATTACCG CCTGTGGATG AAGAAAAAAC

1320 ATTGTGTCTT CTATAATCTA TTTACATAAA ATATGTGATC TGGGAAAAAG CAAACCAATA AACTGTCTCA ATGCTG(A)n
```

FIG. 2. Nucleotide sequence of rat AM cDNA. The numbers to the left refer to the nucleotide positions and those to the right refer to the amino acid positions. The vertical arrows indicate the 5′ end of the individual cDNA clones, and the horizontal arrows are the primers for the differential display, where the unmatched bases are marked with a dot. The position of the two AUUUA motifs in the 3′-untranslated region are bold-faced and underlined, and the position of the polyadenylylation signal, AATAAA, is bold-faced. The TG repeats are underlined. The amino acid sequences predicted to be the proteolytic cleavage products are underlined. The AM peptide (amino acids 93–143) has been shown to have vasodilator properties and was used for functional analysis in Fig. 5 and Table 1.

Immunostaining of AM in the Ischemic Cortex After MCAO. Immunohistochemical analysis was carried out using brain tissues after sham surgery and from animals ($n = 3$) 6 h, 12 h, 24 h, and 5 days after MCAO. AM immunoreactivity revealed the presence of short fiber processes (highly fluorescent in a granular or vacuolar pattern within the ischemic

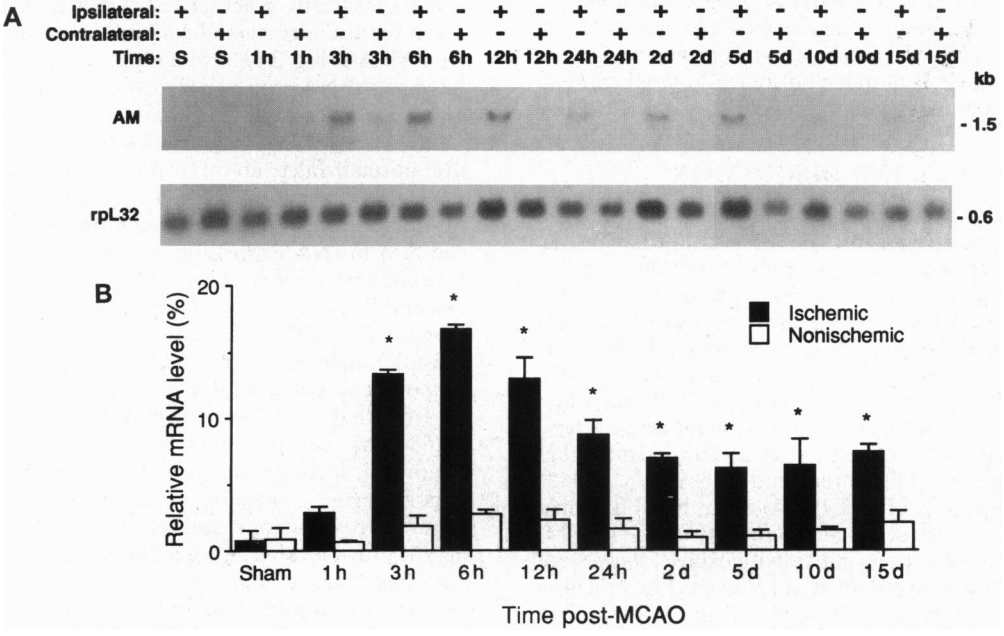

FIG. 3. Time course study of AM mRNA induction in rat ischemic cortex after MCAO. (*A*) Representive Northern blot for AM and rpL32 probes to the samples isolated at various time points and conditions from rats subjected to MCAO. Total cellular RNA (40 μg per lane) was used for this analysis. Ipsilateral and contralateral cortex samples (denoted by +) from individual rats after sham surgery (S; 12 h sacrifice) or 1, 3, 6, 12, and 24 h, and 2, 5, 10, and 15 days (d) of MCAO are depicted. (*B*) Quantitative Northern blot data for AM mRNA expression after focal brain ischemic injury. The data were analyzed using a PhosphorImager and are presented as the mean values ± SE of four animals for each time point. The data were analyzed using one-way ANOVA followed by Bonferroni-adjusted post hoc *t* test. *, $P < 0.05$ vs sham samples.

cortex). These fibers were rare and only weakly fluorescent 6 and 12 h after MCAO but were intensely immunoreactive and abundant in tissues examined 24 h and 5 days after MCAO (Fig. 4 *A* and *C*). No immunoreactivity was observed in sham-operated rats or outside of the ischemic zone after MCAO. When AM antiserum was preincubated with AM prior to incubation of tissue sections, immunofluorescence was completely eliminated (Fig. 4, *E* compared with *C*). Also, no immunoreactivity was observed in sections incubated with the second antibody only (data not shown). When double labeled with intermediate filament markers for neurons (neurofilaments) or astroglia (glial fibrillary acidic protein), the immunofluorescent data indicated colocalization of AM with neurofilaments (Fig. 4 *B, D,* and *F*), which is indicative of nerve fiber processes. In contrast, no colocalization was seen between AM and glial fibrillary acidic protein (data not shown).

AM Effects on Rat Pial Arteries *in Situ*. The effects of rat AM on rat pial microvessels (43 ± 7 μm) were determined using videomicroscopic techniques (Fig. 5*A*). The local application of ET-1 (0.2 pmol) via subarachnoid microinjection rapidly elicited a prolonged submaximal constriction of adjacent pial arteries (Fig. 5 *A* and *B*). There was little or no change in the pial artery diameter when the microinjection of ET-1 was repeated within 10 min of the first microinjection. In contrast, when AM (50 pmol) was added to the second ET-1 microinjection, a significant vasodilation of the adjacent pial artery was observed. AM (up to 10 μmol) did not interfere with ET_A receptor binding (data not shown). The percent of the control diameter was 44% ± 8% after ET-1 alone compared to 98% ± 18% after ET-1 plus AM (Fig. 5*B*). There were no changes in systemic arterial pressure after ET-1 or AM microinjection. In addition, AM had no effect on cortical perfusion when administered systemically at the same doses (data not shown).

AM Effects on Cerebral Vessels *in Vitro*. The effects of rat AM on segments of the canine basilar artery were determined *in vitro*. The canine basilar arteries used in this study slowly generated spontaneous tone, which reached a steady-state

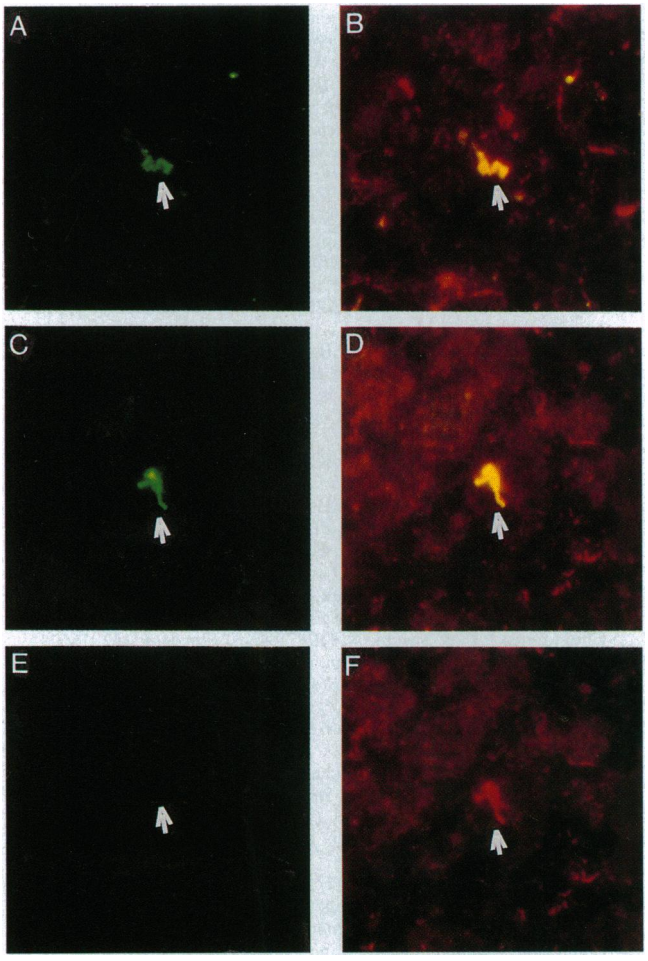

FIG. 4. Immunohistochemical detection of AM expression in ischemic cortex after MCAO in the rats. Matched computer-captured images of ischemic rat cerebral cortex immunolabeled with antiserum against AM (*A, C,* and *E*) and neurofilaments (*B, D,* and *F*). *A* and *B* are from the same matched field, as are *C–F*. Ischemic cortex at 24 h or 5 d after MCAO demonstrated intense AM immunoreactivity in a granular or vacuolar pattern (*A* and *C*, 24 h after MCAO). This immunoflourescence was restricted to the ischemic cortex. When the anti-AM antiserum was preincubated with AM, the immunoreactivity was eliminated (*E* compared with *C*). Combining computer images of brain tissue immunofluorescence for AM (green in *A* and *C*) and neurofilaments (red in *F*) allow demonstration of double-labeling in neuronal processes (yellow in double labeled *B* and *D*). Arrows indicate location of neuronal processes.

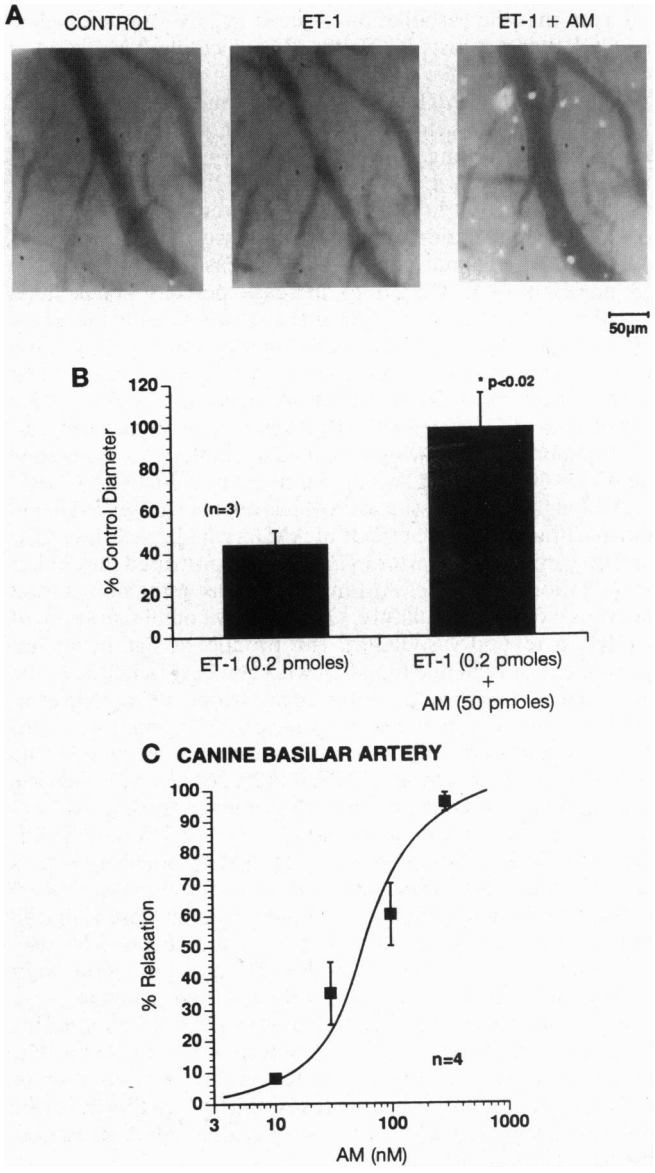

FIG. 5. The cerebrovascular effects of AM were assessed in rat pial arteries *in situ* (*A* and *B*) and canine basilar artery segments *in vitro* (*C*). Microinjection of ET-1 (0.2 pmol) produced significant vasoconstriction of rat pial arteries (*A Middle* and *B*). AM (50 pmol) administration reversed ET-1-induced vasoconstriction (*A Right* and *B*). (*C*) AM also produced a concentration-dependent relaxation of spontaneous tone in the canine basilar artery. *$P < 0.02$, compared to ET-1 treatment alone as determined by Student's *t* test.

Table 1. Effects of AM in focal ischemic injury

	i.v., ng/kg per min		i.c.v., nmol			
Measure	0	1	0	0.2	2	8
Swelling, %	1 ± 1	2 ± 2	2 ± 1	0 ± 1	0 ± 1	5 ± 1
Infarct, %	18 ± 2	17 ± 2	16 ± 1	13 ± 1	13 ± 1	20 ± 2*
Infarct volume, mm^3	110 ± 13	105 ± 14	92 ± 8	67 ± 8	72 ± 9	120 ± 9*

*$P < 0.05$ compared to vehicle, analyzed by a one-way ANOVA followed by Fisher's protected least-significant difference post hoc t test.

maximum (841 ± 115 mg) prior to the addition of AM to the tissue bath. AM produced a prolonged, concentration-dependent relaxation of spontaneous tone. The concentration required to produce half-maximal relaxation (EC_{50}) was 56 nmol, and relaxation was complete at 300 nmol (Fig. 5*C*). AM produced similar concentration-dependent vasodilation of the canine basilar artery segments when precontracted with 0.1 nmol ET-1 (data not shown). Furthermore, additional studies indicate that the vasodilation induced by AM can be blocked by CGRP antagonists (CGRP8–37), indicating AM action at CGRP or CGRP-like receptors.

AM Effects on MCAO Injury. Continuous i.v. infusion of AM beginning 1 h before and 4 h after MCAO did not produce any significant changes in ischemic injury produced by focal ischemia (Table 1). Low doses (0.2 and 2 nmol) of i.c.v. AM administration at 1 h prior to and 6 h after MCAO tended to decrease hemispheric swelling, hemispheric infarct, and infarct volume ($P > 0.05$; not significant), whereas the high i.c.v. dose (8 nmol) of AM tended to increase percent hemispheric swelling ($P > 0.05$; not significant) and significantly increased ($P < 0.05$; Table 1) percent hemispheric infarct (25.0%) and infarct volume (30.4%). Furthermore, we have established that AM may act on CGRP or CGRP-like receptors by performing radioligand (^{125}I-labeled CGRP) displacement studies (rat cortical membranes) with AM; these binding studies ($n = 3$) revealed an IC_{50} = 80.3 nmol for AM displacement of ^{125}I-labeled CGRP.

Although the expression of AM has not been observed in the normal brain (1, 2), the effects of AM on rat pial arteries *in situ* and on canine basilar arteries *in vitro* demonstrated the similar vasodilation function of AM in the brain as in the non-central nervous system vasculature (1, 3–6). i.v. administration of CGRP, a related vasodilator, can produce a significant improvement in ischemic blood flow to cerebral ischemic tissue and reduce brain injury after focal stroke (35). However, dilation of the microcirculation at the site of injury may also facilitate inflammatory cell infiltration that occurs in the ischemic cortex shortly after MCAO (20, 36, 37). Although the role of AM in ischemic brain injury requires further exploration, unlike the intraluminal vasodilatory and protective effects of CGRP in focal ischemia (35), AM vasodilatory effects are abluminal. AM administration, i.c.v., but not i.v., exacerbates focal ischemic injury at a relatively high dose range (8 nmol; Table 1), suggesting that ischemic tissue AM may contribute to permeability and disruption of the blood brain barrier and thereby to increase ischemic brain damage.

In conclusion, the upregulation of AM in the ischemic cortex suggests its involvement in the acute response to ischemia. The potential action of AM to dilate cerebral vessels and to increase permeability and infarct size call for a pathogenic role of locally expressed AM in focal stroke, although that requires further exploration.

We thank G. Sathe, J. Mao, and R. Morris for oligonucleotide synthesis and DNA sequencing.

1. Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Sakakibara, S., Matsuo, H. & Eto, T. (1993) *Biochem. Biophys. Res. Commun.* **192,** 553–560.
2. Sakata, J., Shimokubo, T., Kitamura, K., Nakamura, S., Kangawa, K., Matsuo, H. & Eto, T. (1993) *Biochem. Biophys. Res. Commun.* **195,** 921–927.
3. Ishiyama, Y., Kitamura, K., Ichiki, Y., Nakamura, S., Kida, O., Kanagawa, K. & Eto, T. (1993) *Eur. J. Pharmacol.* **241,** 271–273.
4. Nuki, C., Kawasaki, H., Kitamura, K., Takenaga, M., Kangawa, K., Eto, T. & Wada, A. (1993) *Biochem. Biophys. Res. Commun.* **196,** 245–251.
5. Perret, M., Broussard, H., LeGros T., Burns, A., Chang, J. K., Summer, W., Hyman, A. & Lippton, H. (1993) *Life Sci.* **53,** 377–379.
6. DeWitt, C., Cheng, D. Y., Caminiti, G. N., Nossaman, B. D., Coy, D. H., Murphy, W. A. & Kadowitz, P. J. (1994) *Eur. J. Pharmacol.* **257,** 303–306.
7. Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. & Evans, R. M. (1982) *Nature (London)* **298,** 240–244.
8. Morris, H. R., Panico, M., Etienne, T., Tippins, J., Girgis, S. I. & McIntyre, I. (1984) *Nature (London)* **308,** 746–748.
9. Steenbergh, P. H. Hoppener, J. W. M., Zandberg, J., Lips, C. J. M. & Jansz, H. S. (1985) *FEBS Lett.* **183,** 403–407.
10. Amara, S. G., Arriza, J. L., Leff, S. E., Swanson, L. W., Evans, R. M. & Rosenfeld, M. G. (1985) *Science* **229,** 1094–1097.
11. Cooper, G. J. S., Willis, A. C., Clark, A., Turner, R. C., Sim, R. B. & Reid, K. B. M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8628–8632.
12. Leffert, J. D., Newgard, C. B., Okamoto, H., Milburn, J. L. & Luskey, K. L. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3127–3130.
13. Brain, S. D., Williams, T. J., Tippins, J. R., Morris, H. R. & MacIntyre, I. (1985) *Nature (London)* **313,** 54–56.
14. Girgis, S. I., MacDolnald, D. W. R., Stevenson, J. C., Bevis, P. J. R., Lynch, C., Wimalawansa, S. J., Self, C. H., Morris, H. R. & MacIntyre, I. (1985) *Lancet* **2,** 14–16.
15. O'Halloran, D. J. & Bloom, S. R. (1991) *Br. Med. J.* **302,** 739–740.
16. Cooper, G. J. S. (1994) *Endocr. Rev.* **15,** 163–201.
17. Zaidi, M., Moonga, B. S., Bevis, P. J. R., Towhidul, A., Legon, S., Wimalawansa, S., MacIntyre, I. & Breimer, L. H. (1991) *Vitam. and Horm. (San Diego)* **46,** 87–163.
18. Jougasaki, M., Wei, C. M., McKinley, L. J. & Burnett, J. C. (1995) *Circulation* **92,** 286–289.
19. Liang, P. & Pardee, A. B. (1992) *Science* **257,** 967–971.
20. Barone, F. C., Hillegass, L. M., Price, W. J., White, R. F., Lee, E. V., Feuerstein, G. Z., Sarau, H. M., Clark, R. K. & Griswold, D. E. (1991) *J. Neurosci. Res.* **29,** 336–345.
21. Barone, F. C., Price, W. J., White, R. F., Willette, R. N. & Feuerstein, G. Z. (1992) *Neurosci. Biobehav. Rev.* **16,** 219–233.
22. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
23. Wang, X. K., Siren, A. L., Liu, Y., Yue, T. L., Barone, F. C. & Feuerstein, G. Z. (1994) *Mol. Brain Res.* **26,** 61–68.
24. Wang, X. K. & Feuerstein, G. Z. (1995) *BioTechniques* **18,** 448–453.
25. Wang, X. K., Lee, G., Liebhaber, S. A. & Cooke, N. E. (1992) *J. Biol. Chem.* **267,** 9176–9184.
26. Willette, R. N., Zhang, H., Mitchell, M., Sauermelch, C. F., Ohlstein, E. H. & Sulpizio, A. C. (1994) *Stroke* **25,** 2450–2456.
27. Willette, R. N. & Ohlstein, E. H. (1994) *Drug News Perspect.* **7,** 75–81.
28. Willette, R. N. & Sauermelch, C. F. (1990) *Am. J. Physiol.* **259,** H1688–H1693.
29. Gaillard, C. & Strauss, F. (1994) *Science* **264,** 433–436.
30. Wang, X. K., Yue, T. L., Young, P. R., Barone, F. C. & Feuerstein, G. Z. (1995) *J. Cereb. Blood Flow Metab.* **15,** 166–171.
31. Liu, T., McDonnell, P. C., Young, P. R., White, R. F., Siren, A. L., Hallenbeck, J. M., Barone, F. C. & Feuerstein, G. Z. (1993) *Stroke* **24,** 1746–1751.
32. Liu, T., Clark, R. K., McDonnell, P. C., Young, P. R., White, R. F., Barone, F. C. & Feuerstein, G. Z. (1994) *Stroke* **25,** 1481–1488.
33. Shaw, G. & Kamen, R. (1986) *Cell* **46,** 659–667.
34. Wilson, T. & Treisman, R. (1988) *Nature (London)* **336,** 396–399.
35. Holland, J. P., Sydserff, S. G. C., Taylor, W. A. S. & Bell, B. A. (1994) *Stroke* **25,** 2055–2059.
36. Hallenbeck, J. M., Dutka, A. J., Tanishima, T., Kochaniek, P. M., Kumaroo, K. K., Thompson, C. B., Obrenovitch, T. P. & Contreras, T. J. (1986) *Stroke* **17,** 246–253.
37. Clark, R. K., Lee, E. V., Fish, C. J., White, R. F., Price, W. J., Jonak, Z. L., Feuerstein, G. Z. & Barone, F. C. (1993) *Brain Res. Bull.* **31,** 565–572.