Role of cocaine- and amphetamine-regulated transcript in estradiol-mediated neuroprotection

Yun Xu*†, Wenri Zhang‡, Judith Klaus*, Jennifer Young‡, Ines Koerner‡, Laird C. Sheldahl§, Patricia D. Hurn*‡§, Francisco Martínez-Murillo¶, and Nabil J. Alkayed*‡§‖

Departments of *Anesthesiology and Critical Care Medicine, and ¶Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21287; Departments of ‡Anesthesiology and Perioperative Medicine, and §Physiology and Pharmacology, Oregon Health and Science University, Portland, OR 97239; and †Department of Neurology, Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing 210008, China

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Estrogen reduces brain injury after experimental cerebral ischemia in part through a genomic mechanism of action. Using DNA microarrays, we analyzed the genomic response of the brain to estradiol, and we identified a transcript, cocaine- and amphetamine-regulated transcript (CART), that is highly induced in the cerebral cortex by estradiol under ischemic conditions. Using *in vitro* and *in vivo* models of neural injury, we confirmed and characterized CART mRNA and protein up-regulation by estradiol in surviving neurons, and we demonstrated that i.v. administration of a rat CART peptide is protective against ischemic brain injury *in vivo*. We further demonstrated binding of cAMP response element (CRE)-binding protein to a CART promoter CRE site in ischemic brain and rapid activation by CART of ERK in primary cultured cortical neurons. The findings suggest that CART is an important player in estrogen-mediated neuroprotection and a potential therapeutic agent for stroke and other neurodegenerative diseases.

ischemia | stroke | estrogen

Postmenopausal women are at high risk for stroke, but the benefit of hormone replacement therapy (HRT) in stroke remains unclear. Recent clinical trials demonstrated a lack of benefit and potential harm from HRT, in contrast to earlier epidemiological studies demonstrating reduced risk and better outcome from stroke with HRT (1). In experimental models of stroke, 17β-estradiol has consistently been shown to reduce neuronal cell death and brain lesion size (2). The apparent disagreement among observational studies, preclinical data, and clinical trials underscores the complexity of the actions of estradiol in brain, and it emphasizes the importance of elucidating and understanding the diverse and widespread actions of the hormone in the CNS beyond its effects on reproductive centers. Estradiol is a pleiotropic hormone that exhibits acute and chronic effects, and it acts by genomic and nongenomic mechanisms. Our previous work demonstrated that chronic estradiol replacement confers protection against cerebral ischemia, which is in part linked to the transcription of neuroprotective genes (3). In the current study, using DNA microarrays, we identified a gene product, cocaine- and amphetamine-regulated transcript (CART), that is highly induced by estradiol in the ischemic cerebral cortex and that exhibits potent neuroprotective effects in models of neural injury both *in vitro* and *in vivo*. The *cart* gene encodes a neuropeptide that has been implicated in food intake, drug addiction, and the neuroendocrine response to stress (4–8). However, the role of CART as a neuroprotective peptide and its regulation by estrogen in ischemic brain have not been examined. Our findings contribute to the understanding of the effects of HRT on CNS injury, and they suggest that CART is an endogenous neuroprotectant and may serve as a therapeutic agent against ischemic and other forms of neurodegenerative diseases.

Results

CART Expression and Regulation by Estradiol in Ischemic Cerebral Cortex. Female rats were ovariectomized at 8–10 weeks of age and left untreated or implanted s.c. with 21-day release pellets containing 25 μg of 17β-estradiol for 1 week. We previously demonstrated that this regimen yields physiological levels of plasma estradiol found in cycling females, and it is associated with protection from ischemic brain injury (3, 9). Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) for 2 h by using the intraluminal filament insertion technique, as previously described (10). At 6 and 24 h after MCAO, rats were decapitated under deep halothane anesthesia, and the brains were quickly removed and frozen ($n = 6$ per group at each time point). These animals were used for the microarray studies, as described in the Supporting Text, which is published as supporting information on the PNAS web site. Total RNA was prepared from the ischemic and contralateral cerebral cortex and striatum between coronal levels 2 and 3 mm relative to bregma, representing the core of middle cerebral artery territory. Additional animals were subjected to CART mRNA and protein analysis by Northern blotting, Western blotting, *in situ* hybridization, and immunohistochemistry studies. Each analysis was performed at least three times on samples from different animals (a total of 24 animals, 12 per group, 3 used in each of 4 analyses). As seen in Tables 1 and 2, which are published as supporting information on the PNAS web site, microarray data analysis revealed that CART mRNA was increased by an average of 4-fold in the ischemic cerebral cortex at 6 h after MCAO in estradiol-treated compared with untreated samples. Additional studies were conducted to confirm CART up-regulation and elucidate its functional significance.

Fig. 1*A* is a representative Northern blot, demonstrating that CART mRNA was expressed at a higher level in estradiol-treated ischemic cerebral cortex (+) compared with untreated animals (−) at 6 and 24 h after MCAO. CART mRNA was barely detectable in the contralateral hemisphere, with no apparent differences between groups (data not shown). Fig. 1*B* is a Western blot analysis of brain tissue with anti-CART peptide antibody. The figure demonstrates that CART peptide is strongly induced at 6 h after MCAO in the ipsilateral cerebral cortex of estradiol-treated (+, ipsilateral), but not untreated (−, ipsilateral) animals. Peptide levels in naïve brain (no surgical manipulation) and in the contralateral cerebral cortex were

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Abbreviations: CART, cocaine- and amphetamine-regulated transcript; CRE, cAMP response element; CREB, cAMP response element-binding protein; ER, estrogen receptor; ERE, estrogen response element; HRT, hormone replacement therapy; MAPK, mitogen-activated protein kinase; MCAO, middle cerebral artery occlusion; OGD, oxygen–glucose deprivation; PI, propidium iodide; TTC, 2,3,5-triphenyltetrazolium chloride.

Data deposition: The microarray data in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE5315).

‖To whom correspondence should be addressed at present address: Department of Anesthesiology and Perioperative Medicine, Oregon Health and Science University, 3181 S.W. Sam Jackson Park Road, UHS-2, Portland, OR 97239-3098. E-mail: alkayedn@ohsu.edu.

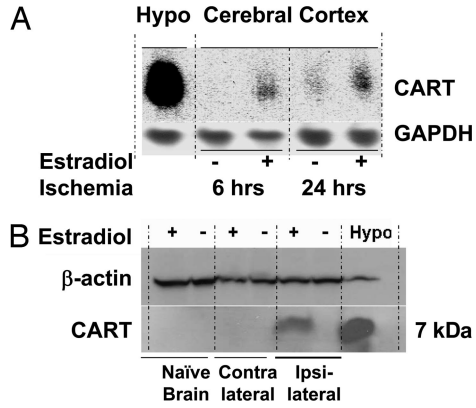

Fig. 1. Estradiol induces CART expression in ischemic cerebral cortex. (*A*) Northern blot analysis of RNA samples prepared from the ipsilateral cerebral cortex of estradiol-treated (+) and untreated (−) animals at 6 and 24 h after MCAO by using CART cDNA probe. RNA from the hypothalamus (Hypo) was used as a positive control. The blot was reprobed for GAPDH as an internal loading control. (*B*) Western blot analysis of protein extracts from non-ischemic, naïve cerebral cortex and from ipsilateral and contralateral cortices of estradiol-treated (+) and untreated (−) animals by using anti-CART peptide antibody. The hypothalamus was used as a positive control and β-actin as an internal control for equal loading.

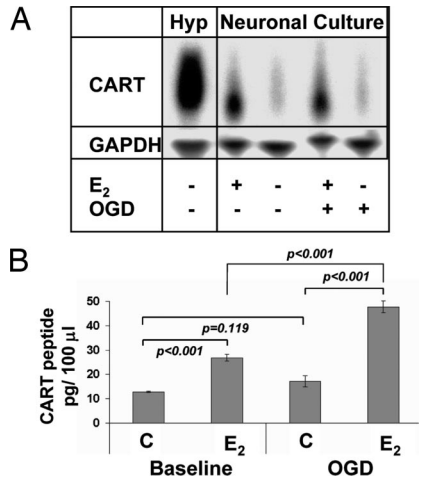

Fig. 2. Estradiol enhances CART expression and secretion in primary cultured cortical neurons after oxygen-glucose deprivation (OGD). (*A*) Northern blot analysis of CART mRNA expression in cortical neurons after OGD and in hypothalamus (Hyp). Estradiol (E_2, 10 nM, +) or vehicle (−) was applied 24 h before 2-h OGD, and RNA was isolated at 24 h after OGD and probed with a CART cDNA probe. Blots were reprobed for GAPDH to control for loading. (*B*) Estradiol increases CART peptide release into culture medium. CART concentrations were measured at baseline and at 24 h after OGD in estradiol-treated and untreated cultures (control; C) by using RIA with [^{125}I]CART and anti-CART peptide antibody.

undetectable, with no apparent differences between estradiol-treated (+, contralateral) and untreated (−, contralateral) samples. CART mRNA up-regulation in ischemic brain was localized by using *in situ* hybridization to layer 4 of the somatosensory cerebral cortex, and immunohistochemistry studies with anti-CART antibody localized CART peptide immunoreactivity in neuronal cell bodies and processes but not in astrocytes (see the Supporting Text and Figs. 6 and 7, which are published as supporting information on the PNAS web site).

Role of CART in Estradiol-Mediated Neuroprotection. To determine the role of CART up-regulation in estradiol-mediated neuroprotection, we used an *in vitro* model of ischemic neuronal cell death. In agreement with our *in vivo* observation, Fig. 2*A* demonstrates that estradiol treatment (10 nM, +) increases CART mRNA expression in primary cultured cortical neurons in the presence (+) or absence (−) of OGD. The induction of CART by estradiol was abolished by actinomycin D, suggesting that estradiol likely increases CART mRNA by a transcriptional mechanism of action. To determine whether CART mRNA is translated into secreted peptide, we measured CART peptide concentration in culture medium by using an RIA with [^{125}I]-CART and anti-CART peptide antibody. Fig. 2*B* demonstrates that estradiol increases the CART peptide concentration in culture media. The figure also demonstrates that the effect of estradiol to induce CART is more pronounced in postischemic neurons. Fig. 3 demonstrates that this concentration of estradiol (10 nM) significantly reduces ODG-induced neuronal cell death. However, the protective effect of estradiol is reduced by estrogen receptor (ER) antagonist ICI 182780 (100 nM), suggesting that estradiol reduces cell death, at least in part, by a mechanism involving the ER. More importantly, Fig. 3 demonstrates that the protective effect of estradiol is attenuated by preincubation with a CART-blocking antibody (2 μg/ml), suggesting that CART plays an important role in estradiol-mediated neuroprotection. Antibody alone had no effect on cell death, suggesting that the antibody does not exert nonspecific toxic effects on neurons and that the increase in cell death is specific to estradiol treatment. To confirm specificity and effectiveness of CART antibody in precipitating CART peptide, we measured the CART peptide concentration in culture media by an RIA. CART-blocking antibody completely precipitated CART peptide in medium, resulting in CART concentrations below the limit of detection (0 pg/μl).

Neuroprotective Property of CART. To evaluate directly the effect of CART peptide on OGD-induced cell death, we applied a naturally occurring and biologically active rat $CART_{55–102}$ fragment (numbers refer to the order of amino acids within the rat pro-CART polypeptide, 0.4 nM final concentration; American Peptide Company, Sunnyvale, CA) 30 min before the 2-h OGD. The next day, cells were stained with propidium iodide (PI) and calcein AM to identify dead vs. viable cells, respectively. Fig. 4*A* demonstrates that rat $CART_{55–102}$ reduces the number of PI-positive cells (dead cells, red dots). At concentrations of 0.2 and

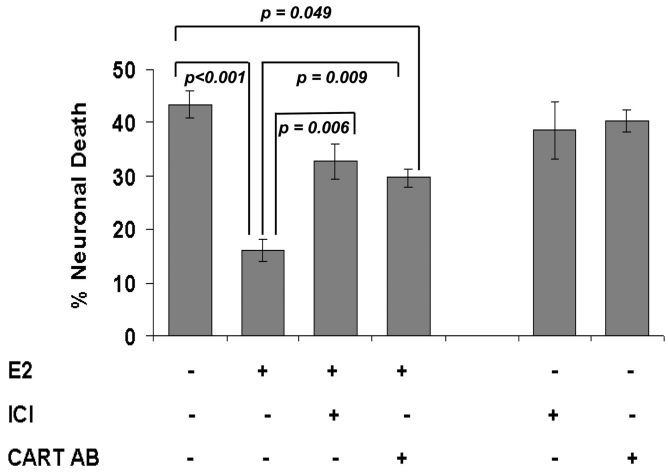

Fig. 3. CART inhibition attenuates the neuroprotective effect of 17β-estradiol. The ability of 17β-estradiol (E_2, 10 nM, +) to reduce ODG-induced neuronal cell death is reduced by coincubation of ER antagonist ICI 182780 (ICI, 100 nM) or CART-blocking antibody (AB, 2 μg/ml overnight). Antibody or ICI 182780 alone had no effect on cell death.

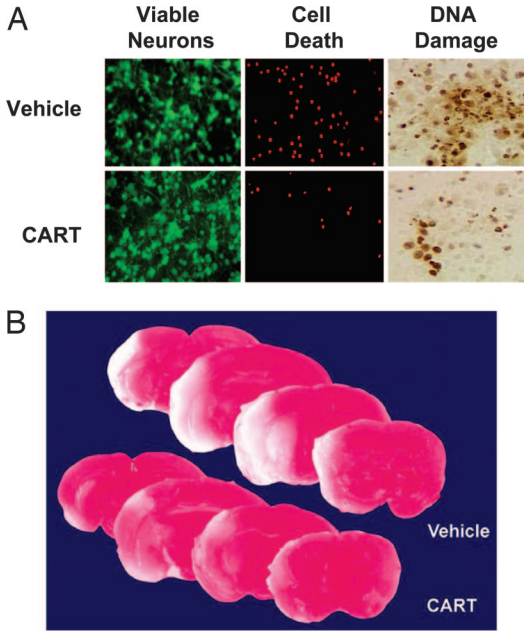

Fig. 4. CART peptide reduces ischemic cell death in cultured cortical neurons and brain damage after MCAO in mice. (*A*) Rat $CART_{55–102}$ peptide reduces OGD-induced neuronal cell death and DNA damage. Cell death was assessed by PI (red, middle), and DNA damage was assessed by TUNEL staining (brown, right) at 24 h after OGD. (*B*) Rat $CART_{55–102}$ reduces hemispheric infarct size after MCAO in mice. Infarct size was determined by the absence of TTC staining in successive coronal sections at 24 h after 2-h MCAO.

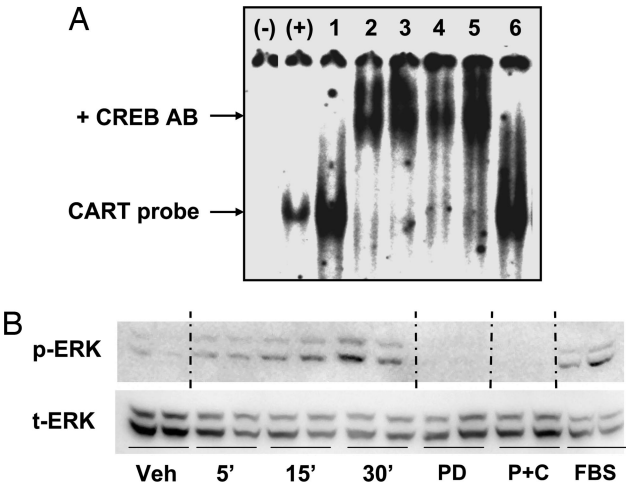

Fig. 5. Mechanisms of neuroprotection by CART and regulation by estradiol. (*A*) Estradiol increases CREB binding to CART promoter DNA. EMSA in CART promoter DNA after incubation with brain nuclear extract at 3 h after MCAO (lane 1) was similar to that of a CRE sequence (+), eliminated by mutating the CRE site (−), and supershifted with an antibody against CREB (+ CREB AB, lanes 2–5), but not STAT3 (lane 6). The shift was stronger in estradiol-treated (lanes 3 and 5) compared with untreated animals (lanes 2 and 4). (*B*) CART increases the phosphorylation of ERK1/2 (*p*-ERK) in primary cortical neurons. ERK activation by CART (0.2 nM) starts at 5 min (5′) after application, and it continues for at least 30 min (30′). Baseline (PD) and CART-induced (P+C) ERK phosphorylations were inhibited by MAPK inhibitor PD98059 (5 μM) and stimulated by FBS.

0.4 nM, CART reduced OGD-induced cell death in primary cortical neuronal cultures by 37% and 59%, respectively ($n = 5$ per group). We confirmed this finding by using a TUNEL assay, which detects DNA strand breaks in injured cells. The rightmost panel demonstrates that CART reduces the number of TUNEL-positive cells after OGD in primary cortical neuronal cultures. There were no significant differences in the percentage of cell death at concentrations lower than 0.1 nM; and at concentrations higher than 10 nM, there was a tendency for increased cell death. To determine the effect of CART peptide against ischemic brain injury *in vivo*, male mice (20–26 g) were subjected to 2-h intraluminal MCAO, and rat $CART_{55–102}$ was administered at concentrations ranging from 1 to 100 μg/kg of body weight as a single bolus injection into the jugular vein at the onset of reperfusion (right after filament withdrawal). Using 2,3,5-triphenyltetrazolium chloride (TTC) staining, we measured the infarct size at 24 h of reperfusion, as previously described (3). Fig. 4*B* demonstrates that rat $CART_{55–102}$ peptide administration at a concentration of 2.5 μg/kg of body weight in 100 μl reduced the hemispheric infarct by 38%, from 39 ± 2% in vehicle (saline)-injected mice ($n = 10$) to 26 ± 4% in CART-injected animals ($n = 11$, $P = 0.011$). In agreement with *in vitro* data, at higher concentrations, CART seemed to have a deleterious effect because most mice did not survive the 24-h period after MCAO when CART was administered at concentrations exceeding 25 μg/kg of body weight.

Mechanism of CART Up-Regulation by Estradiol. We then examined the role of cAMP response element-binding protein (CREB) in mediating CART up-regulation by estradiol. We used an EMSA to determine whether estradiol enhances CREB binding to CART promoter DNA in ischemic brain. An oligonucleotide probe derived from the CART promoter bearing the CRE consensus sequence was radiolabeled and incubated with nuclear extracts from estradiol-treated and untreated ischemic brain. Fig. 5*A* demonstrates that incubation of the CART promoter probe with brain nuclear extract retards migration of the CART probe (lane 1), suggesting probe binding to nuclear proteins. This upward shift was similar to the shift induced by a consensus CRE-positive control probe (+), eliminated by mutating the CRE site nucleotide sequence (−), and further shifted (supershift) by coincubation with anti-CREB (lanes 2–5) but not with antibody against another transcription factor, STAT3 (lane 6), suggesting a specific binding between CREB and the CART promoter CRE site. Interestingly, CREB/CART binding was higher in brain nuclear extracts from estradiol-treated (lanes 3 and 5) compared with untreated (samples 2 and 4) ovariectomized females, suggesting that estradiol enhances CREB/CART DNA binding.

Mechanism of Neuroprotection by CART. To determine whether the mechanism of neuroprotection by CART is linked to the activation of the ERK/MAP kinase (MAPK) pathway, we measured the phosphorylation of ERK1/2 in primary cortical neurons. Fig. 5*B* demonstrates that a neuroprotective concentration of CART (0.2 nM rat $CART_{55–102}$) activates the ERK/MAPK pathway, as indicated by increased ERK1/2 phosphorylation. CART increased ERK phosphorylation as early as 5 min, and activation continued to increase for up to 30 min after the application of CART. ERK phosphorylation was inhibited by MAPK inhibitor PD98059 and stimulated by FBS, which was used as a positive control.

Discussion

The major findings of this study are: (*i*) CART expression is induced in cerebral cortical neurons after focal cerebral ischemia; (*ii*) estradiol enhances CART expression in ischemic cerebral cortex, which contributes to estradiol-mediated neuroprotection; and (*iii*) rat $CART_{55–102}$ peptide administration is protective against ischemic neural injury *in vitro* and *in vivo*. These observations suggest that CART up-regulation is an

important endogenous mechanism of neuroprotection in brain that is further augmented by estradiol treatment. The findings contribute to our understanding of the effects of HRT in stroke-injured brain, and they point to an unidentified role of CART as a neuroprotective agent.

The name CART, or cocaine- and amphetamine-regulated transcript, describes its discovery in 1995 by Douglass *et al.* (11), who found, by using differential display, that CART mRNA was up-regulated in rat brain after acute administration of cocaine and amphetamine. However, CART peptide has been implicated in a variety of brain functions, including drug addiction (12) and appetite control (8, 13), and the precise role of CART in brain function and disease continues to be investigated (5, 6, 8, 13–15).

Our findings suggest that CART is a neuroprotective peptide that plays an important role in the protection afforded by estradiol against ischemic brain injury. We previously demonstrated that estradiol is neuroprotective in part through a genomic mechanism of action (3). Gene expression profiling with DNA microarrays revealed that CART mRNA was strongly induced in the ischemic cerebral cortex of estradiol-treated animals. We confirmed this observation by using Northern and Western blot analysis, which demonstrated that the level of CART expression in the ischemic cerebral cortex was significantly higher in estradiol-replaced compared with untreated ovariectomized female rats. CART mRNA and protein were undetectable in the contralateral hemisphere regardless of treatment, suggesting that the effect of estradiol to induce CART is specific to the ischemia-injured brain or that the effect of estradiol was not apparent because of a low level of CART in the uninjured cerebral cortex.

CART is constitutively expressed almost exclusively in neural and endocrine tissues. In brain, the highest levels are found in the hypothalamus; moderate levels in the midbrain and thalamus; and lower but detectable levels in the hindbrain, hippocampus, ventral striatum, and cerebral cortex (4, 5, 11, 16). In agreement with studies in humans (17, 18) and rats (11, 16), our study localizes CART mRNA expression in ischemic brain within layer IV of the somatosensory cerebral cortex. The distribution of CART immunoreactivity was more widespread, and it tended to be stronger in cells and processes adjacent to the infarct zone. Expression of CART within the periinfarct zone is consistent with a neuroprotective role for CART. Furthermore, CART immunoreactivity colocalized with neuronal nuclei (NeuN) but not glial fibrillary acidic protein (GFAP) immunoreactivity, suggesting that, similar to its constitutive pattern of expression, inducible CART is also expressed exclusively in neurons. In agreement with ultrastructural localization of CART (19), we observed CART expression in cell bodies, varicosities, and neuronal processes, suggesting that CART maybe secreted by neurons within the periinfarct zone during cerebral ischemia.

To determine whether estradiol-induced CART plays a role in mediating the effect of estradiol on neuronal survival, we used primary cultured cortical neurons. In agreement with our *in vivo* finding, CART mRNA expression and CART peptide release into neuronal culture medium were increased by estradiol. In contrast with the *in vivo* data, the increase in CART was apparent under baseline conditions, although the effect was further enhanced after OGD. The discrepancy between the *in vivo* and *in vitro* data may be because the culture is enriched in neurons. Alternatively, higher baseline CART in cultured cells may reflect a degree of cellular stress caused by cell dissociation and culture conditions. As previously reported (20), estradiol reduced neuronal death by a mechanism involving ER because ER antagonist ICI 182780 attenuated the neuroprotective effect of estradiol. More importantly, a CART-neutralizing antibody blocked the ability of estradiol to reduce cell death, suggesting that CART up-regulation by estradiol is an important event in the mechanism of neuroprotection by estradiol and that CART peptide itself may have neuroprotective properties. To test this idea directly, we pretreated primary cultured neurons with biologically active rat $CART_{55–102}$ peptide at concentrations equivalent to those measured in brain *in vivo* (21). We observed that, at subnanomolar concentrations, CART significantly reduced OGD-induced DNA damage and cell death in primary cultured cortical neurons. This neuroprotection is in agreement with previous reports suggesting that CART may exhibit neurotrophic properties (22). The therapeutic range for the CART protective effect was narrow because higher concentrations of CART increased cell death. This dual effect of CART on neuronal survival may be explained by the presence of two types of CART receptors or binding sites with different affinities and opposing effects on neuronal survival. Interestingly, we previously observed that for estradiol itself, although clearly protective against ischemic brain damage at low physiological doses, protection was lost at supraphysiological concentrations (9). We further confirmed these observations *in vivo* by using the MCAO model of stroke in mice. At lower concentrations, rat $CART_{55–102}$ peptide administration significantly reduced infarct size after MCAO, demonstrating a protective effect of CART against cerebral ischemia *in vivo*. We administered CART i.v. based on previous studies demonstrating that CART easily crosses the blood–brain barrier (23). The concentrations of CART used in this study were similar to those previously given to animals *in vivo* (23, 24), and they were equivalent to the concentrations of CART measured in brain tissue (21). Furthermore, in pilot studies, these doses exhibited no adverse effects on hemodynamic and gross neurobehavioral parameters. However, higher CART concentrations led to premature death of mice after MCAO, a finding that is in agreement with our *in vitro* data, although additional mechanisms may be at play *in vivo*. Premature death may not necessarily be mediated by the effects of CART on brain tissue or neuronal cells. For example, CART has been shown to constrict isolated cerebral blood vessels (25). It is possible that, although this effect is not apparent under baseline conditions, its significance is amplified in ischemia-compromised cerebral circulation, exacerbating injury and hindering recovery from stroke.

Regulation of CART by estradiol has not been demonstrated, although a gender difference in CART mRNA expression has been reported (26). The mechanism of estradiol-induced CART is likely transcriptional because the effect was abolished by the transcription inhibitor actinomycin D. A search of the published CART promoter revealed no perfect estrogen response element (ERE), although there were multiple half-EREs. There are, however, other potential DNA-binding sites within the CART promoter that can potentially mediate CART up-regulation by estradiol, including binding sites for activating protein 1, STAT, and CREB (27). These factors are known to mediate non-ERE-mediated transcription by estradiol (28). In agreement with previous reports suggesting that CART gene transcription is regulated by CREB (29–31), we observed specific binding between CART promoter DNA and CREB, a transcription factor known to be activated by estrogen (32) and to contribute to estrogen-mediated neuroprotection (33). These findings suggest that CART up-regulation by estradiol is likely mediated through CREB activation.

The molecular mechanism of action of CART peptide is unknown. Specific binding sites have recently been reported for CART in brain and cell lines (34, 35), although no receptor or binding partner has yet been identified. There are multiple actions of CART that can potentially explain its neuroprotective effect, including inhibition of calcium signaling (36), CREB phosphorylation (37), and activation of the ERK/MAPK pathway (38). Our data demonstrate that CART increases the phosphorylation of ERK1 and ERK2, which correlates with their activities. ERK1 and ERK2 are serine–threonine kinases that play an important role in cell survival, proliferation, and differ-

entiation (39). The ERK/MAPK pathway has previously been shown to be activated by CART in a pituitary-derived cell line (38) and to contribute to the mechanism of neuroprotection by estrogen (40).

In summary, we identified CART as an integral player in the mechanism of protection by estradiol against brain ischemia, and we determined that CART itself is a neuroprotective peptide. These findings have important clinical implications relevant to the understanding of the effects of HRT on stroke outcome and to the development of therapeutic agents for the treatment of stroke and other neurodegenerative diseases based on homology to CART.

Methods

Ovariectomy and Estrogen Replacement. Ovariectomy was performed in Wistar female rats (Harlan, Indianapolis, IN) at the age of 8–10 weeks, and estradiol was replaced by 21-day-release s.c. pellets containing 25 μg of 17β-estradiol (Innovative Research of America, Toledo, OH) for 7 days, as previously described (3, 9). Plasma 17β-estradiol was measured with a commercially available RIA kit (Coat-A-Count Estradiol-6; Diagnostic Products, Los Angeles, CA).

Experimental Stroke in Rats and Mice. Transient focal cerebral ischemia was induced in spontaneously breathing rats and mice under halothane anesthesia (1% in O_2-enriched air by mask) by intraluminal MCAO, as previously described (3, 9). Briefly, a surgical nylon monofilament with a heat-rounded tip was inserted through the external carotid artery and advanced into the internal carotid artery to occlude the origin of the middle cerebral artery. Adequacy of vascular occlusion and reperfusion was assessed by laser Doppler monitoring of cerebral cortical perfusion, and body and head temperatures were monitored and controlled with heating lamps and water pads. Arterial blood pressure and gases were monitored through a femoral catheter. After 2 h of occlusion, the filament was withdrawn to allow for reperfusion, and animals recovered from anesthesia and survived for 6 or 24 h. At the appropriate time point, animals were killed under deep halothane anesthesia, and brains were quickly frozen for CART expression analysis or sliced into thick sections for infarct measurement. To determine the effect of CART on infarct size, rat $CART_{55–102}$ peptide was administered as a single bolus injection into the jugular vein at the onset of reperfusion at 1–100 μg/kg of body weight. Tissue infarction was identified by TTC staining in thick (2-mm) coronal sections. Slices were photographed, and images were studied with image-analysis software (MCID; Imaging Research, St. Catharines, ON, Canada). Infarct volume in all slices was expressed as a percentage of the contralateral hemisphere after correcting for edema.

Northern Blotting. The CART cDNA probe was prepared by amplifying a 300-bp CART cDNA fragment from rat brain by RT-PCR (GenBank accession no. U10071, base pairs 101–404). The probe was labeled with ^{32}P by using a Prime-It II random primer labeling kit (Stratagene, La Jolla, CA). Total RNA was isolated by using an RNA preparation kit (RNeasy; Qiagen, Valencia, CA), and samples (50 μg) were denatured, separated by gel electrophoresis in Mops buffer, and blotted to a nylon membrane. The blot was prehybridized in ExpressHyb (Clontech, Mountain View, CA) at 68°C for 1 h and then incubated in the same solution with 1×10^6 cpm/ml probe at 68°C for 1 h. The blot was rinsed twice for 40 min each in 2× SSC/0.05% SDS at room temperature and once in 0.1× SSC/0.1% SDS at 50°C. The blot was apposed to an x-ray film at −70°C overnight. The membrane was stripped and reprobed with GAPDH to control for loading.

Western Blotting. Frozen brain tissue was thawed in lysis buffer (250 mmol/liter sucrose/1 mmol/liter EDTA/10 mmol/liter KPO_4/0.1 mmol/liter PMSF, pH 7.7) in the presence of protease inhibitors, and the homogenate was sonicated, incubated on ice for 30 min, and centrifuged at 14,000 × *g* for 15 min at 4°C. The protein concentration was measured in the supernatant, and samples of 100 μg were loaded on 10–20% Tris/*N*-tris(hydroxymethyl)methylglycine/peptide gel, separated, and transferred overnight at 4°C to a PVDF membrane. Blots were blocked for 2 h with PBS containing 5% nonfat dry milk and 0.1% Tween 20 (PBST) at room temperature and incubated overnight with anti-rat CART antibody (G-003-61, 1:1,000; Phoenix Pharmaceuticals, Belmont, CA) in 5% milk in PBST. Membranes were washed with PBST and then incubated with HRP-conjugated anti-rabbit IgG antibody (1:5,000) for 1 h. The signal was detected by Enhanced Chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and film autoradiography. Blots were stripped and reprobed with anti-β-actin antibody (1:5,000; Sigma, St. Louis, MO). Phospho-specific antibody for ERK1/2 (Thr-202 and Tyr-204, 9106; Cell Signaling, Beverly, MA) was used at 1:1,000 dilution in 5% nonfat dry milk/Tween 20–Tris-buffered saline overnight at 4°C with mild agitation. Total ERK levels were measured with anti-p42 MAPK antibody (1:5,000 dilution, 9108; Cell Signaling).

CART RIA. The concentration of CART in culture medium was measured with a $CART_{55–102}$ RIA kit (RK-003-62) from Phoenix Pharmaceuticals according to the manufacturer's protocol.

Neuronal Culture and OGD. Primary cultured cortical neurons were prepared from brains of rat embryo brain on gestational day 17–19, as previously described (41). To eliminate potential sources of estradiol, cells were grown in serum-free neurobasal medium with B-27 supplement (Invitrogen, Carlsbad, CA) without phenol red. On day 12 *in vitro*, cells were treated with 10 nM 17β-estradiol or vehicle overnight. The next day, cells were subjected to combined OGD for 2 h to simulate ischemia *in vitro*. Briefly, cultures were switched from normal feeding medium to oxygen-depleted, glucose-free medium containing 120 mM NaCl, 25 mM Tris·HCl (pH 7.4), 5.4 mM KCl, and 1.8 mM $CaCl_2$. Cells were incubated in a hypoxia chamber (Billups-Rothenberg, Del Mar, CA) previously flushed for 15 min with 5% CO_2/95% N_2 at 2 psi (1 psi = 6.89 kPA). Valves were closed, and chambers were incubated at 37°C for 2 h. At the end of OGD, cells were returned to normal feeding medium and incubated under normoxic conditions at 37°C for 24 h. Culture medium was aspirated by RIA for measurement of extracellular concentrations of CART peptide, and cells were either homogenized for RNA extraction and CART mRNA measurement by Northern blotting or stained with markers of cell death and viability. Cell death was assayed by staining cells with PI (0.625 μg/ml; Sigma) in physiological salt solution for 10 min at 37°C. Viable cells were determined by 0.25 ng/ml calcein AM (Molecular Probes, Eugene, OR), and the ratio of PI-positive cells to total PI and calcein-positive cells was used as a measure of cell death. Cell damage was also assessed with terminal deoxynucleotidyltransferase-mediated TUNEL by using a commercially available kit (ApopTag; Q-Biogene, Irvine, CA). Control cultures and cultures incubated without the nucleotide were used as negative controls. ERK inhibitor PD98059 was purchased from Sigma and applied to cultures at 5 μM final concentration 15 min before CART application.

EMSA. A double-stranded oligonucleotide probe with the following sequence was used to evaluate potential CREB binding to the CART promoter: 5′-cgggcatTGACGTCAaacggcagcg-3′, where the capitalized sequence is a putative CREB-response element [nucleotides −153 and −127 within the proximal CART promoter (27)] and the underlined nucleotides were changed to CC in the negative control probe. As a positive control, we used a probe (5′-acgctgctgcgtcagcaaat-3′) derived from the rat NMDA

receptor 1 (NMDAR1) gene promoter previously demonstrated to bind CREB specifically in brain tissue (42). Probes were labeled with [γ-^{32}P]ATP by T4 kinase kinase, and DNA-binding reactions were carried out in a 35-μl volume containing 33 mM Tris·HCl, 167 mM NaCl, 17% glycerol, 3 mM EDTA, 3 mM DTT, and 2 μg of the nonspecific competitor poly(dI-dC) (Sigma). DNA-binding reactions were carried out by incubation of 1×10^5 cpm of labeled probe for 30 min at room temperature with 20 μg of nuclear protein extracted from the cortex at 3 h after MCAO. Supershift was induced by coincubation with anti-CREB antibody (1:50 dilution; Chemicon, Temecula, CA). Samples were run on a 5% (wt/vol) polyacrylamide gel [acrylamide/bisacrylamide, 37:1 (wt/wt)] at 4°C. Gels were subsequently dried and exposed to x-ray film at −70°C.

Statistical Analysis. Differences among treatment groups in infarct volumes, cell death, and CART levels were analyzed with a *t* test for two groups and ANOVA with a post hoc Student–Newman–Keuls test for multiple groups. Repeated measurements such as physiological variables were subjected to two-way ANOVA; one factor was group treatment, and the second factor was time. The criterion for statistical significance was set at $P < 0.05$. All values are reported as the mean ± SEM.

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