LACK OF ADRENOMEDULLIN, BUT NOT COMPLEMENT FACTOR H, RESULTS IN LARGER INFARCT SIZE AND MORE EXTENSIVE BRAIN DAMAGE IN A FOCAL ISCHEMIA MODEL

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Abstract—Adrenomedullin (AM) and its binding protein, complement factor H (FH), are expressed throughout the brain. In this study we used a brain-specific conditional knockout for AM and a complete knockout for FH to investigate the effect of these molecules on the pathophysiology of stroke. Following 48 h of middle cerebral artery permanent occlusion, there was a statistically significant infarct size increase in animals lacking AM when compared to their wild type littermates. In contrast, lack of FH did not affect infarct volume. To investigate some of the mechanisms by which lack of AM may augment brain damage, markers of nitrosative stress, apoptosis, and autophagy were studied at the mRNA and protein levels. There was a significant increase of inducible nitric oxide synthase (iNOS), matrix metalloproteinase-9 (MMP9), fractin, and Beclin-1 in the peri-infarct area of AM-deficient mice when compared to their wild type counterparts and to contralateral and sham-operated controls. These data suggest that AM exerts a neuroprotective action in the brain and that this protection may be mediated by regulation of iNOS, matrix metalloproteases, and inflammatory mediators. In the future, substances that increase AM actions in the central nervous system may be used as potential neuroprotective agents in stroke. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: adrenomedullin, complement factor H, conditional knockout, focal ischemia, infarct size, neuroprotection.

Stroke is one of the main causes of death and a major cause of long-term disability in the western world. More than 80% of all strokes are caused by cerebral ischemia, resulting in devastating neurological sequelae accompanied by severe morphological and molecular alterations (Kunz and Iadecola, 2008). Since stroke is common and current drug therapies for the management of stroke patients are limited, there is a need for the identification of new targets and the development of new drugs (Willmot et al., 2005).

Adrenomedullin (AM) is a regulatory peptide with structural homology to calcitonin gene-related peptide and amylin. Many functions have been ascribed to this peptide, including vasodilatation, bronchodilatation, hormone secretion regulation, neurotransmission, growth regulation, apoptosis inhibition, and antimicrobial activity, among others (Lopez and Martinez, 2002). AM binds to plasma membrane receptors composed of calcitonin receptor-like receptor, a member of the seven transmembrane domain receptor superfamily, and receptor activity modifying protein type 2 or 3 (McLatchie et al., 1998). An AM binding protein was found in plasma (Elsasser et al., 1999) and later identified as complement factor H (FH), a component of the complement cascade (Pio et al., 2001). Interestingly, FH protects AM from protease degradation, thus prolonging its half life and improving its activity (Martinez et al., 2004b).

In the CNS, AM expression and distribution has been reported throughout the whole brain (Serrano et al., 2000). The components of the AM receptor (Li et al., 2004) and the AM binding protein (Serrano et al., 2003) have also been found throughout the CNS of experimental animals. It has been shown that the levels of AM increase following ischemic insults to the brain (Serrano et al., 2002), and this response has been interpreted as a neuroprotective mechanism. There has been some controversy on this issue since the first article where external addition of AM was studied resulted in a worsening of the brain damage following experimental stroke (Wang et al., 1995), but all subsequent studies have shown that external infusion of AM reduces brain ischemic damage (Dogan et al., 1997; Watanabe et al., 2001; Xia et al., 2006). In the same line, brain damage caused by ischemia was reduced in a mouse model where AM was genetically overexpressed in the liver (Miyashita et al., 2006). A formal demonstration of the protective role played by AM in brain ischemia would require a genetic model where expression of the peptide or the receptor has been eliminated. Several attempts have shown that regular knockouts of the gene coding for AM or its receptor result in embryo lethality, thus preventing studies of adult brain physiopathology (Caron and Smithies, 2001; Dackor et al., 2006; Shimosawa et al., 2002; Shindo et al., 2001). A recent study has used heterozygous mice...
and has shown that this partial lack of AM increases brain damage in a stroke model (Miyamoto et al., 2009). Nevertheless, this study uses a systemic detection of the gene and the results could be due to a reduction in the AM fraction produced in the CNS or in circulating AM. We have recently generated a brain-specific conditional knockout mouse model for AM (Fernandez et al., 2008). These animals lack AM expression in all their neurons but produce enough peptide from other sources to lead a normal life under regular conditions, although they are more sensitive to stress than their wild type counterparts (Fernandez et al., 2008), thus constituting an appropriate model to test the involvement of AM in ischemia. In this study we have used this conditional knockout for AM plus a regular knockout for the AM binding protein, FH (Pickering et al., 2002), to investigate the contribution of these molecules to brain damage prevention during stroke. Since AM has been implicated in the regulation of nitric oxide synthase (NOS) expression (Ikeda et al., 1996) and is important in modulating the inflammatory response (Gonzalez-Rey et al., 2006; Talero et al., 2008), several markers of these phenomena have been analyzed in the context of focal ischemia.

**EXPERIMENTAL PROCEDURES**

**Genetically modified mice**

Conditional brain AM knockout mice (AM KO in short) were produced in our laboratory as described (Fernandez et al., 2008). Briefly, a mouse strain where the complete adrn gene was surrounded by two LoxP sequences (floxed AM) was crossed with a transgenic line expressing Cre recombinase under the tubulin α-1 promoter, generating a CNS conditional knockout for AM. Quantitative data of this model show almost complete abrogation of AM expression in the CNS whereas normal levels of AM are present in peripheral organs (Fernandez et al., 2008). Complement factor H deficient mice were a generous gift from Prof. M. Botto (Imperial College, London, UK) and have been thoroughly characterized as well (Pickering et al., 2002). Both strains have been backcrossed at least seven times into a C57BL/6J genetic background. Heterozygous mice were crossed to obtain gene-different mice and wild type animals from the same litter. Genotyping was performed by PCR of tail clip DNA with primers and conditions described in the original papers (Fernandez et al., 2008; Pickering et al., 2002). Twelve-week-old male littermates were used for further experiments. All procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC) and reviewed by the Ethics Committees on Animal Welfare of all institutions involved. A special effort was made to reduce the number of animals used in the study and to provide them with the most comfortable conditions possible.

**Permanent focal ischemia model**

Mice (six to nine per group) were anesthetized with 3% isoﬂurane (Isova® Vet, Schering-Plough, Middlesex, UK) (in 70% N2O, 30% O2) for induction and with 1.5% isoﬂurane for maintenance. Rectal temperature was maintained at 36.5 °C with use of a heating pad. Middle cerebral artery (MCA) was exposed and occluded permanently by electrocoagulation as previously described (Caso et al., 2008). Briefly, for the MCA occlusion (MCAO), an incision perpendicular to the line connecting the lateral canthus of the left eye and the external auditory canal was made to expose and retract the temporalis muscle. A burr hole was drilled and the MCA was exposed by cutting and retracting the dura. The MCA was elevated and cauterized, thus producing a permanent MCA occlusion (pMCAO). Following surgery, subjects were returned to their cages and allowed free access to water and food. The survival rate of the animals until the end of the experiment was 80% and this rate was the same in all groups of animals.

**Determination of infarct size**

For infarct size four groups of animals were used, corresponding to AM KO (n=9), Factor H KO (n=8), and their wild type counterparts (n=6–9). Two days after pMCAO, animals were killed by an overdose of sodium pentobarbital to assess infarct outcome. Brain was removed and cut into seven 1 mm-thick coronal brain slices (Brain Matrix, WPI, UK) and stained with 2,3,5-triphenyltetrazolium chloride (1% TTC in 0.1 M phosphate buffer). Infarct volumes were calculated sampling each side of the coronal sections with a digital camera (Nikon Coolpix 990), and the images were analyzed using Image J 1.33u (National Institutes of Health, Bethesda, MD, USA).

The digitized image was displayed on a video monitor. With the observer masked to the experimental conditions, the contralateral hemisphere perimeter was overlapped onto the ipsilateral hemisphere to exclude edema, and infarct borders were delineated with an operator controlled cursor. The area of infarct, which was unstained, was determined by counting the pixels contained within the outlined regions of interest and expressed in square millimeters. Infarct volumes (in mm3) were integrated from the infarct areas over the extent of the infarct calculated as an orthogonal projection. All animals displayed infarcts after the occlusion procedure, which included the cortex, subcortex, and striatum, depending on the intensity of the lesion.

**Gene expression quantification**

Additional animals (n=6 each for group) were used to obtain RNA 5 h after pMCAO. Following deep anesthesia, the animals were sacrificed and brains quickly removed. Tissue samples were dissected out from both the peri-infarct area, the contralateral side, and the homologous area in the sham group. For this, the most rostral and caudal 1-mm thick coronal slices were excluded to avoid the chance presence or lack of infarct due to inter-animal variability. Then, the left MCA was identified, the territory around this vessel was excluded (core) and 1 mm of tissue around the ischemic core was dissected and immediately frozen in liquid N2.

Similar tissue samples were collected from the right side (contralateral), and from sham operated animals. The RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using SuperScript reverse transcriptase (Invitrogen). Real time PCR was performed using the Chromo4 (MJ Research, Hercules, CA, USA) thermocycler and software. Amplification was done in a final volume of 25 µl, containing 2 µl cDNA (diluted 1:10), 2 µl of primer mixture (at 10 nM), and 12.5 µl of 2×SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Values were determined by interpolation within a standard curve. At the end of the PCR, a melting curve was generated to ascertain an amplon quality. All gene expression values were normalized according to the 18S rRNA concentration of each sample. Primers are shown in Table 1.

**Western blotting**

For determination of protein levels of inflammatory and apoptotic mediators, brain tissue was collected from the peri-infarct area of mice and the homologous contralateral area (n=6 each group) killed 24 h after pMCAO. Similar tissue samples were also collected from mice that had been sham operated. Protein concentration was determined by NanoDrop ND1000. Equal amounts of total protein (10 µg) were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (HybondTM-P, Amersham Biosciences Europe GmbH, Freiburg, Germany). Immunodetection was performed by standard procedures. The
membranes were blocked with 5% non fat milk in TBS-T (0.05% Tween 20 in TBS) and probed with specific primary antibodies: rabbit anti-iNOS (1:200; Santa Cruz Biotechnology), rabbit anti-MMP-9 (1:2000; Millipore Bio-science Research Reagents), rabbit anti-fractin (1:4000; BD Pharmingen), and mouse anti-

Table 1. Primers used for quantitative real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>nNOS</td>
<td>Sense: CTGCTGAGGAAAGCGGTCAG</td>
</tr>
<tr>
<td></td>
<td>Antisense: CGCATATTGCAGAGGAAT</td>
</tr>
<tr>
<td>eNOS</td>
<td>Sense: GCC TGG GTT TAG GCC TGT G</td>
</tr>
<tr>
<td></td>
<td>Antisense: CTT AGG GTG TCG TAG GTG ATG</td>
</tr>
<tr>
<td>iNOS</td>
<td>Sense: GCTTCTGACCCCAACTACAAAGA</td>
</tr>
<tr>
<td></td>
<td>Antisense: GTGAGCCGCTTCATGTCAC</td>
</tr>
<tr>
<td>COX-2</td>
<td>Sense: ACTGGGCCCATGGAATGCACTAAA</td>
</tr>
<tr>
<td></td>
<td>Antisense: ACATGCAGTTCTACAGGATGTA</td>
</tr>
<tr>
<td>MMP9</td>
<td>Sense: CCATGGCAATTCTCTGCGGCTG</td>
</tr>
<tr>
<td></td>
<td>Antisense: TAGAGACTGTCATCGACGGTGA</td>
</tr>
<tr>
<td>Beclin-1</td>
<td>Sense: ATGGAGGGGTCTAAGGCGTC</td>
</tr>
<tr>
<td></td>
<td>Antisense: TCTTTCCTGTAGTGAGCTCT</td>
</tr>
<tr>
<td>VEGF</td>
<td>Sense: ATCTTCAGCCCGTCTGCTG</td>
</tr>
<tr>
<td></td>
<td>Antisense: GCATTCACTCAGTCGCTG</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Sense: ATGGCTTATGCTGAGCTGCCC</td>
</tr>
<tr>
<td></td>
<td>Antisense: ATCCTAGCTCCGCGATCCAG</td>
</tr>
</tbody>
</table>

Statistical analysis

Results are expressed by mean ± SEM of the indicated number of experiments. Groups were compared by either Student’s t-test or by ANOVA followed by post hoc Bonferroni’s test to compare paired experimental points, using Graphpad Prism 4.0 software. Results were considered significant at P<0.05.

RESULTS

Focal ischemia in AM-null, FH-deficient, and wild type animals

Animals lacking brain AM (AM KO) and their wild type counterparts (WT) were subjected to focal ischemia by permanent cauterization of the middle cerebral artery. The infarct volume, after 48 h, was larger in AM KO mice compared with WT mice (P<0.05) (Fig. 1A).

A similar approach was taken with the knockout model for FH. In this case, no statistically significant differences were observed when comparing the FH-null animals and their wild type counterparts (Fig. 1B).

Gene expression variations in brain damage marker genes

We analyzed gene expression for markers of nitrosative stress, inflammation, brain damage, and angiogenesis to gain further information in the potential mechanisms underlying the differences observed in the AM KO model (Figs. 2 and 3). Since we did not find any variation in the FH animals, we did not pursue this model any further. First, we analyzed the expression for the three isoforms of the NOS since all of them have been involved in regulating brain damage during stroke (Rodrigo et al., 2005). For nNOS, a decrease in expression was observed following pMCAO in the WT animals when compared to sham operated counterparts. This difference disappeared in the KO animals, which also expressed less nNOS in the sham controls when compared to their WT counterparts (Fig. 2A). No statistically significant changes were found among genotypes for endothelial NOS (Fig. 2B), but a very significant increase was found in the AM KO animals for inducible nitric oxide synthase (iNOS) when compared with their WT littermates or sham operated mice (Fig. 2C).

MMP9 is a matrix metalloproteinase that has been proposed as a marker of brain ischemia (Cuadrado et al., 2009) and cyclooxygenase-2 (COX-2) is an inflammation marker that is involved in the pathogenesis of stroke (Ahmad et al., 2009). MMP9 did not present differences between the genotypes (Fig. 3A). COX-2 increased its expression following pMCAO, as expected, but no significant differences were found when comparing animals containing or lacking brain AM (Fig. 3B). Beclin-1 is a molecule involved in the autophagy process and, as such, is a useful marker of brain damage (Rami et al., 2008). This is clearly shown by the steep increase in Beclin-1 expression following pMCAO (Fig. 3C).
ing pMCAO. In addition, we observed a significant increase in Beclin-1 expression in the animals lacking AM when compared to their wild type counterparts (Fig. 3C).

Vascular endothelial growth factor (VEGF), an important angiogenesis mediator (Shibuya, 2009), did not suffer important changes, only a modest increase in the brain of animals lacking AM was observed after pMCAO (Fig. 3D). In all cases, the area contralateral to the stroke was analyzed and produced results undistinguishable from the sham operated brains (data not shown).

Changes in the levels of apoptotic and proinflammatory proteins

We have analyzed the expression of apoptotic and proinflammatory markers in brain homogenates from peri-infarct tissue of pMCAO-injured mice. Acute expression of highly reactive mediators, such as iNOS, and MMP9, participates in brain damage after stroke [for review, see (Del et al., 2000)]. We have not found statistically significant differences in protein levels for iNOS between genotypes at the time point studied, although there is a statistically significant increase in expression following pMCAO in AM KO animals (Fig. 4A). For MMP9, we found increased expression following pMCAO in both genotypes, but the increase was larger in animals lacking CNS AM (Fig. 4B). COX-2 also showed increased expression following pMCAO but we did not see significant differences between genotypes at this time point (Fig. 4C). We have determined fractin protein in brain homogenates and we have found that fractin levels increased after pMCAO in the animals lacking AM, whereas no changes were found in the WT mice (Fig. 4D). As with the mRNA experiments, contralateral samples offered similar results to sham operated tissues (results not shown).

DISCUSSION

In this study we have shown that lack of AM in the mouse brain results in a worsening of the consequences due to focal ischemia. On the other hand, the lack of FH had no effect on the consequences of ischemia in the brain. Data concerning AM impact in stroke were confirmed by molecular and biochemical assays. Something we need to take into account is that our conditional knockout eliminates the whole gene, which codes for both AM and proadrenomedullin N-terminal 20 peptide (PAMP), therefore our phenotypic observations may be due to the lack of both peptides.

Our present data are in agreement with previous studies that suggested a protective role for AM in stroke and other diseases of the CNS (Dogan et al., 1997; Miyamoto et al., 2009; Serrano et al., 2002). AM expression is readily elevated following hypoxia exposure both in vitro (Garayoa et al., 2000) and in vivo in CNS models (Serrano et al., 2002). There are several mechanisms by which this elevated AM may exert its neuroprotective role. These include vasodilatation, suppression of apoptosis and induction of angiogenesis, promotion of astrocyte migration and survival, and protection from oxidative stress (Chen et al.,
AM is also important for the proliferation and differentiation of neural stem/progenitor cells (Vergano-Vera et al., 2010), suggesting that some of the effects here reported might be mediated by resident or circulating stem cells. In comparison with a previous paper where the effects of stroke in AM heterozygous mice were studied (Miyamoto et al., 2009), we obtained comparable results but, since our mouse model lacks AM specifically from the neural component while maintaining systemic levels, it indicates that the neuroprotective effect is caused by the fraction of AM that is produced within the CNS, rather than by circulating AM.

Several markers of nitrosative stress, inflammation, and brain damage have been studied in the peri-infarct area of wild type and mice lacking AM by both quantitative real-time PCR and Western blotting to gain some insight into the mechanisms of action that participate in the neuroprotective effect of AM. In some cases discrepancies between mRNA and protein data were observed but this is not surprising given that samples were tested at different time points (5 h and 24 h post-ischemia, respectively). We observed a clear up-regulation of iNOS mRNA in mice lacking brain AM when compared to their wild type counterparts. AM has been shown to mediate part of its cardiovascular effects through nitric oxide regulation (Ueda et al., 2005). iNOS expression is regulated in a negative feed-back fashion by nitric oxide (Rodrigo et al., 2005), so we can expect that a lack of AM in the brain may trigger an uncontrolled up-regulation of iNOS, which in turn will be responsible for extensive brain damage (Rodrigo et al., 2005). In consequence, we can suggest that NOS regulation would be another mechanism by which AM exerts its neuroprotective action.

The pattern observed for the expression of the neuronal form of NOS is a little more complex. First, there is a clear downregulation of nNOS mRNA following pMCAO in the WT animals. Similar behaviour has been described previously in the ischemic mouse hippocampus (Luo et al., 2007), indicating that this diminution in nNOS expression seems to be the habitual response to brain ischemia. Second, there is a significant decrease on nNOS expression in the sham KO mice when compared to the sham WT. Previous studies have shown that infusion of AM results in a marked increase of nNOS expression in the

Figure 3. mRNA expression levels after cerebral ischemia for inflammatory, brain damage, and angiogenesis markers. Quantitative real time PCR values for MMP9 (A), COX-2 (B), Beclin-1 (C), and VEGF (D) in the peri-infarct area of wild type (WT) and littermate mice lacking AM in their CNS (KO) 5 h after pMCAO. All values were divided by the 18S rRNA contents of each sample (normalized expression). Bars represent mean±SEM of three independent values. Statistical significance was evaluated by ANOVA followed by post hoc Bonferroni’s test to compare paired experimental points (* P<0.05; **P<0.01; ***P<0.001).

2005; Xia et al., 2004).
kidney (Yoshihara et al., 2005), thus we can expect a reduction of nNOS in mice lacking AM.

Brain damage after stroke has been shown to be mediated by acute expression of several inflammatory enzymes, such as iNOS (Del et al., 2000), MMP9 (Cuadrado et al., 2009), and COX-2 (Ahmad et al., 2009), among other pro-inflammatory cytokines. Therefore, the increase of the infarct volume found in AM-deficient mice might be explained, at least in part, by the increased expression of these molecules. Sham AM-deficient mice showed similar expression levels of MMP9, iNOS, and COX-2 to sham WT animals. Moreover, it has been reported that increased NO production is necessary for MMP9 activation (Marcet-Palacios et al., 2003), what might constitute a potential extracellular proteolysis pathway to neuronal cell death in cerebral ischemia. So, the dual increase in iNOS and MMP9 expression found after

**Fig. 4.** Expression of ischemia-inducible inflammatory and apoptotic markers in AM conditional knockout mice. Representative immunoblots showing expression of iNOS (A), MMP9 (B), COX-2 (C), and fractin (D) protein in peri-infarct cortical tissue of wild type (WT) and AM conditional knockout (KO) mice subjected to a pMCAO for 24 h. Equal protein loading was confirmed by measuring β-actin in all blots. Densitometric analysis of five to six independent experiments are shown as the ratio between the subject protein and β-actin (normalized expression). Statistical significance was evaluated by ANOVA followed by post hoc Bonferroni’s test to compare paired experimental points (*P<0.05; **P<0.01; ***P<0.001).
pMCAO in AM-deficient mice is likely to be one of the main mechanisms responsible for the increase in infarct volume. We also studied markers of two independent mechanisms of brain damage, apoptosis and autophagy. Apoptosis is essential in maintaining cellular homeostasis and for normal development. Morphological as well as biochemical changes are a hallmark of apoptosis, and include membrane blebbing, chromosomal condensation and DNA fragmentation. These changes are caused by a class of cysteine proteases called caspases, which upon activation can cleave multiple cytoplasmic and nuclear substrates. Actin is a 45 kDa caspase substrate that has been shown can cleave multiple cytoplasmic and nuclear substrates.

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