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Mouse model of focal cerebral ischemia using endothelin-1

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Abstract

Intracerebral injection of the vasoconstrictor peptide, endothelin-1 (ET-1), has been used as a method to induce focal ischemia in rats. The relative technical simplicity of this model makes it attractive for use in mice. However, the effect of ET-1 on mouse brains has not been firmly established. In this study, we determined the ability of ET-1 to induce focal cerebral ischemia in four different mouse strains (CD1, C57/BL6, NOD/SCID, and FVB). In contrast to rats, intracerebral injection of ET-1 did not produce a lesion in any mouse strain tested. A combination of ET-1 injection with either CCA occlusion or N, G-nitro-L-arginine methyl ester (L-NAME) injection produced only a small infarct and its size was strain-dependent. A triple combination of CCA occlusion with co-injection of ET-1 and L-NAME produced a lesion in all mouse strains tested, and this resulted in a significant motor deficit. However, lesion size was still relatively small and strain-dependent. This study shows that ET-1 has a much less potent effect for producing an infarct in mice than rats.

Keywords

Endothelin-1; focal ischemia; mouse; receptor; endothelial nitric oxide

Introduction

The majority of research on cerebral ischemia has been done using rat models of stroke. However, the use of mice in stroke research offers certain advantages. For example, using knock-out mice offers the potential to ask more mechanistic questions about the molecules involved in stroke pathophysiology. Furthermore, with the advent of research into cell transplantation therapy for stroke, various immunodeficient mouse strains (e.g. NOD/SCID mice) are beneficial as they can support survival of xenogenic cell grafts without the need for daily immunosuppression regimes (Pflumio et al., 1996, Cashman et al., 1997, Cummings et al., 2005).

There are a number of rodent focal ischemia models, although the more commonly used models (suture model and distal middle cerebral artery occlusion) are technically challenging in mice given their small size. An alternative and simpler model is intracerebral injection of the vasoconstrictor peptide, endothelin-1 (ET-1). This has been reported as a simple and

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reproducible method of focal ischemia in rats (Fuxe et al., 1989, Sharkey and Butcher, 1995, Gilmour et al., 2004, Frost et al., 2006, Windle et al., 2006), and the lesions caused by a drop in blood flow in the injected area (Kurosawa et al., 1991). However, the vasoconstrictive effect of ET-1 in the mouse brain has not been fully reported. In this study, we examined the ability of ET-1 to induce focal cerebral ischemia in different mouse strains as it offers the potential of a technically simple stroke model for mice.

1. Animals and Methods

2.1. Animals

All experimental protocols performed on animals were approved by the Stanford University Administrative Panel on Laboratory Animal care. Adult male Sprague-Dawley rats (Charles-River, USA, 320 - 340g), CD-1 mice (Charles-River, USA, 22 - 25g), C57/BL6 mice (Jackson, USA, 20 - 25g), NOD/SCID mice (Stanford Colony and Taconic, USA, 20 - 25g), and FVB mice (Jackson, USA, 20 - 25g) were used in this study.

2.2. Surgical procedure

All animals were anesthetized with 2-3% isoflurane plus oxygen and air (ratio: 0.2/0.8 L/min) by facemask, and maintained with 1.5% to 2% isoflurane. Temperature was maintained at 37°C throughout the surgery using a self-regulating heating blanket. ET-1 (Calbiochem, USA) was dissolved in sterile saline at various concentrations (see Results Section) and different doses were delivered into the cortex or striatum by stereotaxic injection. ET-1 was injected at 0.3 µl/min by an infusion pump, and the needle left *in situ* for 5 minutes post-injection before being slowly removed. The stereotaxic coordinates (listed below) were determined from the Paxinos /Watson rat atlas and Paxinos/ Franklin mouse atlas (Paxinos and Watson, 1998, Paxinos and Franklin, 2004); all stereotaxic measurements are relative to bregma and with the depth determined from the brain surface:

1. Rat: cortex and striatum (triple injection): AP 0, ML +2.5, DV -2.3; AP +2.3, ML +2.5, DV -2.3; AP +0.7, ML +3.8, DV -7.0
2. Rat: cortex (triple injection): AP 0, ML +2.5, DV -2.3; AP +2.3, ML +2.5, DV -2.3; AP +0.7, ML +3.8, DV -2.3
3. Mice: cortex (single injection): AP +1.0, ML +1.0, DV -1.0
4. Mice: cortex (double injection): AP +1.0, ML +1.0, DV -1.0; AP 0, ML +1.0, DV -1.0
5. Mice: cortex (triple injection) : AP +2.0, ML +2.0, DV -0.7; AP +1.5, ML +1.0, DV -0.7; AP +1.0, ML +2.0, DV -0.7
6. Mice: striatum (single injection): AP+1.0, ML +1.0, DV -3.0
7. Mice: striatum (double injection): AP 0, ML +2.0, DV -3.0; AP +1.0, ML +1.0, DV -3.0

For some mice, the ipsilateral common carotid artery (CCAO) was permanently occluded just prior to the ET-1 injection in order to further reduce the cerebral blood flow. Some mice also received N, G-nitro-L-arginine methyl ester (L-NAME; Sigma, USA), a nitric oxide synthase (NOS)-inhibitor, via intracerebral injection (2.7 µg/µl) with ET-1.

2.3. Behavioral testing

Motor behavior was tested at two days post-surgery using the cylinder test to measure forelimb use during vertical exploration (Schallert et al., 2000). Mice were placed in a plexiglas cylinder, and the number of times the mouse reared and touched the cylinder in a weight-bearing fashion

with the left, right, or both forelimbs was counted. Approximately 20 of these limb-use movements were counted per trial. The behavior score was calculated using the equation (affected limb use + 'both' limb use)/ (unaffected limb use + 'both' limb use) which gives a ratio of affected to unaffected limb use. Statistical analyses were performed using Tukey post-hoc test following two way ANOVA (SigmaStat, SYSTAT, California, USA).

2.4. Sacrifice and 2, 3, 5-triphenyl-2H-tetrazolium chloride (TTC) staining

Two days after the surgery, the animals were anaesthetized with isoflurane and decapitated. The brains were immediately taken for TTC staining. Four equidistant sets of coronal sections (2mm thickness) were prepared. The sections were incubated in distilled water containing 2% TTC (Sigma) at 37°C for 20 min. The infarct size was defined as: small (<20% of the hemisphere); medium (20-60% of the hemisphere) and large (>60% of the hemisphere).

2. Results

We first tested ET-1 in rats since it is known to produce lesions in rats (Windle et al., 2006). Intracerebral injection of ET-1 (1µg or 2µg per site) into three cortical or striatal sites along the A-P axis resulted in a large and reproducible lesion similar to what has been previously reported (Windle et al., 2006) (Figure 1; Table 1). We then tested similar dose and injection paradigms in CD1, C57/BL6, NOD/SCID and FVB mouse strains. However, no infarct was found after injection of ET-1 into the cortex and/or striatum into one, two or three sites along the A-P axis in any of the mice tested, regardless of strain (Table 1). Increasing the dose of ET-1 per injection site to 4 or 5µg/site did not produce an infarct but did increase the rate of mortality; at doses of 4µg/site or higher, with one or two injection sites, the mortality rate was 75% (6 out of 8 animals treated with ET-1 alone died; Table 1). The animals that went on to die were very sick immediately after surgery possibly due to an ET-1 toxic effect, and some exhibited tonic-clonic seizure; most animals died within 24h of surgery. To try and further reduce cerebral blood flow and cause an infarct, we combined ET-1 injection with ipsilateral CCAo. With this paradigm, only FVB mice exhibited a lesion, but it was always small (Figure 1; Table 1). In all the strains, no lesion was found with CCAo alone in the absence of ET-1. The next paradigm tested was co-injecting ET-1 with L-NAME (10 mM). L-NAME is known to reduce the effect of injury-induced NOS which causes vasodilation (Chataigneau et al., 1999, Zhou et al., 2003). Striatal injection of ET-1/L-NAME resulted in a small infarct in NOD/SCID and FVB mice, the other two strains showed no lesion (Figure 1; Table 2). In FVB mice, a one site injection of ET-1/L-NAME gave a larger lesion than that seen in NOD/SCID mice receiving two injections. Two injections of ET-1/L-NAME into FVB mice, however, were lethal (Table 2). The final stroke paradigm tested was a triple combination of CCAo with co-injection of ET-1/L-NAME. This method produced an infarct in all four mouse strains (Figure 1, Table 2). The largest lesions were in the FVB mice, followed by C57/BL6 mice; the lesions were not restricted to the striatum where the ET-1/L-NAME was injected, but also extended into the cortex. In the other two mouse strains (CD1 and NOD/SCID), the lesions were much smaller and more localized to the area of injection.

This strain effect on lesion size is statistically significant ($p=0.0001$; Pearson's Chi-Squared tests). There is also a strain-dependent effect on mortality ($p=0.007$, 2X2 table and Fisher's exact test) with FVB mice being significantly more susceptible to death than the other strains. However, there is no significant difference in survival between ET-1/L-NAME treatment with or without CCAo ($P=0.5$; Pearson's Chi-Squared tests).

Regardless of lesion size, all mice subjected to ET-1/L-NAME co-injection (without CCAo) showed a significant motor behavior deficit on the cylinder test at two days after stroke (Figure 2); this was most pronounced in the FVB mice ($p < 0.001$ compared to all other strains as determined by Tukey post-hoc test following two way ANOVA) which exhibited the largest

lesion. When ET-1/L-NAME injections were combined with CCAo, a more severe behavior deficit was seen, but this was only significantly different from the deficit caused by ET-1/L-NAME alone in CD1 and C57/BL6 mice (Figure 2).

In summary, ET-1 alone produces a large, reproducible lesion in the rat brain, but has very little effect on the mouse brain. ET-1 plus L-NAME can produce a lesion in FVB mice but not other strains. A combination of ET-1, L-NAME plus CCAo is required to produce an infarct in most mouse strains.

3. Discussion

Previous reports have shown that intracerebral injection of ET-1 produces a reproducible infarct in rats (Fuxe et al., 1989, Sharkey and Butcher, 1995, Gilmour et al., 2004, Frost et al., 2006, Windle et al., 2006), however there are very few reports using ET-1 to induce cerebral ischemia in mice (Wang et al., 2007). Therefore, in this study we compared the ability of ET-1 to produce infarcts in rats and different mouse strains. As expected, we found reproducible infarcts in the rat. However, the same dose of ET-1 did not produce a lesion in any of the mouse strains we tested. A recent publication by Wang et al. reported that cortical injection of ET-1 (0.5 or 1 µg) did produce an infarct in C57/BL6 mice (Wang et al., 2007). However, they had only very minimal lesions, consistent with our results showing that ET-1 is significantly less potent in mice than rats. Moreover, in our study, increasing the dose of ET-1 only increased mortality of the mice and did not produce a lesion. Combining ET-1 injection with CCAo, or co-injecting ET-1 with L-NAME (an endothelial NOS inhibitor which prevents NOS-induced vasodilation), to further reduce blood flow did produce a small infarct, but this was strain-dependent. The most successful approach was the combination of CCAo with a co-injection of ET-1 and L-NAME. This produced infarcts in all mice strains tested, although the lesion size was strain-dependent, with the FVB mouse strain showing the greatest sensitivity. Consistent with this, the FVB mice showed the largest behavioral deficit after co-injection of ET-1 and L-NAME compared to the other mouse strains.

It is unclear why there is such a large discrepancy in the ability of ET-1 to cause infarcts in rats versus mice. ET-1 is a 21-amino acid peptide that acts through specific receptors which are widely distributed throughout the CNS on vascular endothelia, smooth muscle cells, neurons, astrocytes, and microglia (Hughes et al., 2003). The peptide sequence of ET-1 is very similar in rat and mouse, and is identical in the region covered by the commercial ET-1 peptide. Therefore, a difference in the ET-1 ligand is not responsible for the difference in potency in mice and rats.

The difference between the mouse and rat responses to ET-1 may be due to differences in the expression of receptor isoforms. The vascular effects of ET-1 are mediated by two main receptors, the endothelin-A receptor (ET_A-R) and the endothelin-B receptor (ET_B-R) (Webb et al., 1998). ET_A-R is mainly located on smooth muscle cells, and its activation is thought to be the major contributor to the vasoconstrictor effects of ET-1 (Zhou et al., 2004, Schaller, 2006). In contrast, activation of ET_B-R (which is found on both smooth muscle and endothelial cells) results in vasodilation, caused by release of nitric oxide (NO) and prostacyclin from endothelial cells (Webb et al., 1998). Wiley et al. (Wiley and Davenport, 2004) reported that the mouse brain is rich in the vasodilatory receptor ET_B-R, with twice as many ET_B-Rs than ET_A-Rs. This may account for the poor ET-1-mediated vasoconstriction in the mouse brain. Consistent with this, we found that co-injecting L-NAME (which inhibits NOS and thus NO production) with ET-1 increased the probability of an infarct, suggesting that ET_B-R induced vasodilation may have a significant effect in the mouse brain.

In summary, the ability of ET-1 to induce vasoconstriction in the mouse brain is significantly lower compared to its potency in the rat brain. Consequently, intracerebral injection of ET-1 as a model of focal ischemia is not very effective in mice. However, combining a co-injection of ET-1 and L-NAME with CCAo can produce a lesion and behavior deficit, although the lesion size is very strain-dependent. This model has the advantage of being technically much easier and faster than other rodent stroke models and thus may serve as a higher throughput model of stroke. However, it does not confer any other advantages over current models as it is not possible to truly localize the lesion to the site of injection, and the mortality rate is similar, if not worse, to that seen with other models.

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Figure 1. Summary of ET-1 induced lesions
TTC staining two days after ET-1 injection (+/- L-NAME and CCAo as indicated) shows the variability in lesion sizes between Sprague Dawley rats and the various mouse strains tested. The dose per injection site for ET-1 was 1.0µg, and 2.7µg for L-NAME. The schematic illustrates the injection coordinates used. The infarcts were reproducible within each group.

Figure 2. Behavior deficits measured by the cylinder test

Forelimb motor function measured 2 days after co-injection of ET-1/L-NAME alone shows a significant deficit in the affected side in all mouse strains ($p < 0.001$ by two way ANOVA). ET-1/L-NAME with CCAo produces a further deficit in CD1 and C57/BL6 mice (* $p < 0.05$ compared to pre-treatment; † $p < 0.05$ compared to ET-1/L-NAME injection alone, determined by Tukey post-hoc test; $n = 4-7$ per group). The bar graph shows mean values $\pm$ SEM.

Table 1

Infarct produced by ET-1 injection alone +/- CCAo

Summary of the ET-1 dose and injection regimes tested in Sprague Dawley rats and the mouse strains CD1, C57/BL6, NOD/SCID and FVB. Out of the 63 mice tested with the various dosages and injection sites of ET-1, infarcts were only found in 2 animals and these were both FVB mice.

Species	Strain	ET1 dose per injection site (µg)	CCAo	Location (# Injection sites)	N	Survival (%)	Infarct size
Rat Mouse	SD CD1	1.0 – 2.0	—	Cortex/Striatum (3) or Cortex (3)	5	5 (100)	Large
		0.5 – 1.0	—	Cortex (1 or 2) or Striatum (1)	3	3 (100)	None
		2.0	—	Cortex (1 or 2) or Striatum (1)	5	5 (100)	None
		1.0 – 2.0	+	Striatum (2)	2	2 (100)	None
Mouse	C57/BL6	4.0	—	Cortex (1) or Striatum (1)	2	0 (0)	—
		1.0	—	Cortex (1, 2 or 3) or Striatum (1 or 2)	9	9 (100)	None
		1.0	+	Striatum (2)	2	2 (100)	None
		2.0	—	Cortex (1, 2 or 3) or Striatum (1 or 2)	11	9 (82)	None
		2.0	+	Striatum (1 or 2)	2	2 (100)	None
		5.0	—	Cortex (3) or Striatum (1 or 2)	3	0 (0)	—
		5.0	+	Striatum (1 or 2)	2	2 (100)	None
		1.0	—	Cortex (1)	1	1 (100)	None
Mouse	NOD/SCID	1.0	+	Striatum (1 or 2)	3	3 (100)	None
		2.0	—	Cortex (3) or Striatum (1 or 2)	3	3 (100)	None
		2.0 – 3.0	+	Cortex (3) or Striatum (1 or 2)	3	1 (33)	None
		5.0	—	Striatum (1)	3	2 (66)	None
		5.0	+	Striatum (1)	1	0 (0)	—
		1.0	—	Striatum (2)	3	3 (100)	None
Mouse	FVB	1.0	+	Striatum (2)	5	2 (40)	Small

CCAo= common carotid artery occlusion. N= number of animals in the group

Infarct produced by co-injection of ET-1/L-NAME +/- CCAo
A cocktail of 1.0µg/µl ET-1 plus 2.7µg/µl L-NAME was injected into the striatum. This produced a very small lesion and only in FVB and NOD/SCID mice. However, a triple combination of CCA occlusion with co-injection of ET-1/L-NAME produced a lesion in all mouse strains tested. However, lesion size was still relatively small and strain-dependent.

Mouse	ET1dose per injection site (µg)	L-NAME dose per injection site (µg)	CCAo	Location (# Injection sites)	N	Survival (%)	Infarct size
CD1	1.0	2.7	—	Striatum (2)	4	3 (75)	None
	1.0	2.7	+	Striatum (2)	5	3 (60)	Small
C57/BL6	0.5	2.7	—	Striatum (2)	2	2 (100)	None
	1.0	2.7	—	Striatum (2)	4	4 (100)	None
NOD/SCID	1.0	2.7	+	Striatum (2)	5	4 (80)	Medium
	1.0	2.7	—	Striatum (2)	5	3 (60)	Small
	1.0	2.7	+	Striatum (2)	5	3 (60)	Small
	1.0	2.7	—	Striatum (1)	5	5 (100)	Small
FVB	1.0	2.7	+	Striatum (1)	7	5 (71)	Medium
	1.0	2.7	—	Striatum (2)	2	0 (0)	—
	1.0	2.7	+	Striatum (2)	3	0 (0)	—