Spironolactone reduces cerebral infarct size and EGF-receptor mRNA in stroke-prone rats

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Dorrance, Anne M., Heather L. Osborn, Roger Grekin, and R. Clinton Webb. Spironolactone reduces cerebral infarct size and EGF-receptor mRNA in stroke-prone rats. Am J Physiol Regulatory Integrative Comp Physiol 281: R944–R950, 2001.—Remodeling of the cerebral vasculature contributes to the pathogenesis of cerebral ischemia. Remodeling is caused by increased smooth muscle proliferation and may be due to an increase in the responsiveness of vascular cells to epidermal growth factor (EGF). Aldosterone is a risk factor for stroke, and the literature suggests it may play a role in increasing the expression of the receptor for EGF (EGFR). We hypothesized that mRNA for the EGF-stimulated pathway would be elevated in the vasculature of stroke-prone spontaneously hypertensive rats (SHRSP) and that this experimental ischemic cerebral infarct size would be reduced by aldosterone inhibition with spironolactone. We found that spironolactone reduced the size of cerebral infarcts after middle cerebral artery occlusion in SHRSP (51.69 ± 3.60% vs. 22.00 ± 6.69% of hemisphere-infarcted SHRSP vs. SHRSP + spironolactone P < 0.05). Expression of EGF and EGFR mRNA was higher in cerebral vessels and aorta from adult SHRSP compared with Wistar-Kyoto rats. Only the expression of EGFR mRNA was elevated in the young SHRSP. Spironolactone reduced the EGFR mRNA expression in the aorta (1.09 ± 0.25 vs. 0.56 ± 0.11 phosphorimaging units SHRSP vs. SHRSP + spironolactone P < 0.05) but had no effect on EGF mRNA. In vitro incubation of aorta with aldosterone ± spironolactone produced similar results, suggesting a direct effect of aldosterone. Thus spironolactone may reduce the size of cerebral infarcts via a reduction in the expression of the EGFR mRNA, leading to reduced remodeling.

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control (8). The constriction observed in response to EGF is thought to occur though the same MAP-kinase-stimulated pathway that stimulates cell proliferation. Recent studies have shown that the expression of mRNA for EGFR is increased in both the cerebral vasculature and in the aorta from DOCA-salt hypertensive rats (6, 7). Immunohistochemical studies suggest that in aorta from DOCA-salt rats, this increase in mRNA results in increased protein synthesis (13). This suggests that aldosterone may modulate the levels of EGFR at the genomic level.

Aldosterone has also been implicated in the pathogenesis of vascular disease in genetically hypertensive rats, and elevated plasma aldosterone is considered a risk factor for stroke. Administration of spironolactone, the aldosterone antagonist, to SHRSP led to reduced plasma aldosterone. In both of these studies, the SHRSP were fed a high-salt diet; this causes a paradoxical increase in plasma aldosterone activity and leads to the induction of hemorrhagic strokes. No attempt has been made previously to assess the effects of spironolactone on cerebral infarction, which has a different pathogenesis from hemorrhagic stroke and is more frequent in the human population.

Our hypothesis was that aldosterone has a deleterious effect on the vasculature by increasing the expression of EGFR. This increase in EGFR will lead to increased cell proliferation and vascular remodeling, resulting in a reduction in the internal diameter of the cerebral blood vessels, leading to the production of a large cerebral infarct when ischemia is induced. Our hypothesis would suggest that inhibition of the actions of aldosterone would reduce the size of the ischemic cerebral infarct and reduce the expression of mRNA for EGFR.

MATERIALS AND METHODS

Animals. Male SHRSPs were obtained from the breeding colony at the University of Michigan, Ann Arbor. Male WKY rats were purchased from Charles River. Rats were maintained on a 12:12-h light-dark cycle. They were housed two to a cage and allowed access to food and water ad libitum. Three separate groups of animals were used for this study: studies of cerebral blood vessels were carried out in adult (4–6 mo) and young (6 wk) rats, and a separate group of young rats was used for the spironolactone study. These studies complied with the protocols for animal use outlined by the American Physiological Society. All studies were approved by the Institutional Animal Care and Use Committee.

Spironolactone treatment protocol. At 6 wk of age, SHRS rats were implanted with time-release pellets containing either spironolactone (200 mg) or placebo (Innovative Research of America). Treatment continued for 6 wk, and WKY rats received placebo. Pellets were implanted subcutaneously in the nape of the neck. The pellets released 3.3 mg of spironolactone/day, providing a dosage greater than that reported to substantially reduce the actions and specific binding of endogenous aldosterone in rat tissues (5).

MCA occlusion. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and body temperature was maintained at 37°C during anesthesia using a microprocessor-controlled heat blanket and rectal temperature probe. The MCA was permanently occluded using the thread-occlusion technique of Zia Longa et al. (21). At 6 h postocclusion, the rats were anesthetized and decapitated and the brains were removed and sectioned coronally at 2-mm intervals from the frontal pole. The slices were stained with 2,3,5-triphenyltetrazolium chloride and fixed in paraformaldehyde solution (2%). The infarct volume was determined by image analysis using the public domain National Institutes of Health (NIH) Image program (developed at NIH and available at http://rsb.info.nih.gov/nih-image/). The percentage of hemisphere suffering infarction was assessed using the formula of Swanson et al. (18), taking cerebral edema into account.

RNA extraction and cDNA production. The ophthalmic artery was isolated under a light microscope and placed in guanidinium isothiocyanate buffer and snap frozen. This artery is between 100 and 150 μm in diameter and ~3 mm long; care was taken not to include any cerebral or connective tissue with the vessel. Analysis of cerebral vessels is difficult, because obtaining a homogeneous sample is troublesome. It is possible to use a sieving technique that provides a larger quantity of tissue for study, but samples produced in this way will contain veins that will dilute any response that is purely arterial. RNA was isolated from cerebral vessels using a CsCl cushion method described by Chen et al. (1). Because of the very small amount of RNA produced from these samples, the total RNA sample was used for first strand cDNA synthesis using oligo(dT) as a primer and avian myeloblastosis virus (AMV) reverse transcriptase (1). Occasional RNA samples were subjected to the PCR procedure without prior reverse transcription to control for the presence of contaminating genomic DNA in the sample. Thoracic aorta was also removed from rats and cleaned free of adherent fat, connective tissue, and blood before being snap frozen. RNA was extracted from aorta using a preparative kit (Qiagen) and quantified by spectrophotometry; 1 μg of RNA was used to produce cDNA using the above technique.

PCR amplification of EGF and EGFR cDNA. PCR amplifications were carried out on a portion of the cDNA produced. Each PCR reaction contained 5 μM of each oligonucleotide primer, 200 μM dNTP, 0.2 units Taq, 1.5 μM magnesium

| Table 1. Primer sequences for EGF, EGFR, and GAPDH |
|-------------------|-------------------|-------------------|-------------------|
| **Product**       | **Upstream Primer** | **Downstream Primer** |
| EGF               | GAC AGC AGA AGG GAT CAG TCA | CTG GAA GTT TGC AGA TGC CAA |
| EGFR peptide      | GCC TAG ATT GGA CGA TGG CAG C | GGT GGT CCT CTA GGA CCA CAA ACC |
| GAPDH             | TGC CTC AAG ATT GTC AGG AA | AGA TCC ACA AGG CAT AGA TT |

EGF, epidermal growth factor; EGFR, EGF receptor.
chlide, and 1 µCi of [32P]dCTP in the manufacturer’s buffer. Optimum annealing temperature was assessed using a gradient block thermal cycler. Cycle number and template dilution factor were determined for each amplicon before experimentation to ensure linearity. The cDNA produced was resolved on an 8% polyacrylamide gel, and the amount of DNA present was identified by phosphorimage analysis (Bio-Rad, Hercules, CA) and quantified using Multi-analyst software. The results were normalized to the expression of the constitutively gene GAPDH. Because of differences in the relative abundance of the cDNA for EGF, EGFR, and GAPDH, the PCR amplification of the cDNA for GAPDH was carried out on a 100-fold dilution of the cDNA used for analysis of EGF and EGFR. The specific oligonucleotide primers used are shown in Table 1; the primers were designed using an Internet base primer design program (GeneFisher). PCR products were sequenced by the Molecular Biology Core Facility at the Medical College of Georgia. BLAST analysis was carried out on the sequences produced to confirm the authenticity of the product.

**Aldosterone incubations.** To assess whether the observed effects were a direct effect of aldosterone, aortas were removed from normotensive rats and cleaned as described previously and incubated for 8 h under tissue culture conditions in PBS or PBS + aldosterone (10^{-8} M) ± spironolactone (10^{-5} M). RNA was extracted from the tissue, and RT-PCR was carried out for EGFR as above.

**Blood pressure and plasma aldosterone.** Systolic blood pressure was measured by tail-cuff plethysmography (pneumatic transducer). Blood was obtained for plasma aldosterone determination by cardiac puncture at the time of death. Plasma aldosterone was measured by radioimmunoassay using an iodinated tracer without prior extraction (11).

**Statistical analysis.** Results were analyzed by Student’s t-test (Bonferroni procedure was applied when multiple analyses were performed). A P value of <0.05 was deemed to be statistically significant. For the RT-PCR analysis, n = 8 for each group. The results for cerebral vessels are for single measurements from each sample; aortic samples were measured in triplicate. For the measurements of cerebral ischemia, n = 8 for placebo-treated SHRSP and WKY rats and n = 10 for SHRSP treated with spironolactone.

## RESULTS

**Effect of spironolactone treatment on blood pressure, body weight, and plasma aldosterone levels.** SHRSP had significantly higher blood pressure than the WKY rats. Spironolactone had no effect on blood pressure in the SHRSP over the treatment period. There was also no difference in body weight between the spironolactone-treated and control SHRSP. These results are similar to those observed previously for SHRSP rats fed a high-salt diet, in which spironolactone treatment also had no effect on blood pressure (16). The plasma aldosterone levels were not significantly different between any of the groups. These results are summarized in Table 2.

**Effect of spironolactone treatment on cerebral infarct size in SHRSP rat compared with WKY.** To assess the effect of aldosterone on the outcome of cerebral ischemia, rats were treated with spironolactone for 6 wk before induction of permanent focal ischemia by MCA occlusion. This 6-wk treatment period was chosen to encompass the time during which that blood pressure in the SHRSP is rising rapidly and vascular remodeling is an active process. Occlusion of the MCA produced a significantly greater cerebral infarct in placebo-treated SHRSP than WKY rats. The infarcts were restricted to the basal ganglia and the cortex. Treatment of the SHRSP with spironolactone significantly reduced the size of the cerebral infarct to levels similar to that in WKY rats (Fig. 1).

### Table 2. Blood pressure, body weight, and plasma aldosterone for SHRSP ± spironolactone and WKY rats

<table>
<thead>
<tr>
<th>Animal/Treatment</th>
<th>Systolic Blood Pressure, mmHg</th>
<th>Body Wt, g</th>
<th>Plasma Aldosterone, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHRSP</td>
<td>193 ± 2</td>
<td>282.8 ± 6</td>
<td>239.53 ± 47.12</td>
</tr>
<tr>
<td>SHRSP + spironolactone</td>
<td>203 ± 4*</td>
<td>272.7 ± 5.6</td>
<td>208.93 ± 29.14</td>
</tr>
<tr>
<td>WKY</td>
<td>118 ± 4*</td>
<td>303.4 ± 3.0</td>
<td>231 ± 51.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significant difference from stroke-prone spontaneously hypertensive rats (SHRSP) and SHRSP + spironolactone. WKY, Wistar-Kyoto.

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**Fig. 1. Spironolactone reduces the size of experimentally induced cerebral infarcts in stroke-prone spontaneously hypertensive rats (SHRSP).** A: representative brain slices from SHRSP ± spironolactone and Wistar-Kyoto (WKY) rats. The brains were sliced coronally (SHRSP). Cerebral infarcts in stroke-prone spontaneously hypertensive rats (SHRSP). A: representative brain slices from SHRSP ± spironolactone and Wistar-Kyoto (WKY) rats. The brains were sliced coronally and stained with TTC staining. The dark area is viable tissue, and the light area is the area damaged by the cerebral infarct. B: percentage of the hemisphere that has undergone a cerebral infarct. C: percentage of the hemisphere that has undergone a cerebral infarct. Infarct size was measured using National Institutes of Health Image program, and the %infarct was calculated using the equation of Swanson et al. (18). Infarcts were greater in SHRSP (n = 8) compared with both SHRSP + spironolactone (n = 10) and WKY (n = 8). *Significant difference from SHRSP.
EGF and EGFR mRNA from cerebral blood vessels.

Our hypotheses stated that expression of EGFR and EGF would be elevated in the vasculature of the SHRSP compared with WKY rats. For this elevation to be important in the pathogenesis of cerebrovascular disease, it is necessary that the differences occur in the small cerebral arteries. Studies were carried out in two sets of rats: adult rats that had prolonged hypertension and young rats that did not have maintained hypertension. This was done in an effort to dissect the genetic effects from the effects of prolonged hypertension. In the older rats, the levels of EGF and EGFR mRNA are increased in the cerebral vasculature of the SHRSP compared with WKY rats. The increase in the receptor mRNA expression was of a smaller magnitude than the increase in the peptide mRNA expression (Fig. 2). In the young rats, the mRNA for the EGFR was elevated in the SHRSP compared with the WKY; there was no difference in the mRNA for EGF. This suggests the expression of mRNA for EGR and EGFR is controlled differently over time (Fig. 3).

EGF and EGFR expression in aortas from spironolactone-treated rats. Previous studies in DOCA-salt hypertensive rats and the results described above suggest that aldosterone may affect EGFR at the genomic level in blood vessels. If this is the case, it is possible that the changes in expression of mRNA for EGFR will be seen in the aorta in the same fashion as in the cerebral vasculature. This appears to be true: differences in expression of mRNA for EGF and EGFR are similar in the aorta and cerebral vessels from adult SHRSP and WKY rats. EGFR mRNA expression was higher in the aorta from SHRSP than WKY rats. This elevated expression could be reversed by treatment with spironolactone. EGF expression was also higher in the SHRSP compared with the WKY rat; however, spironolactone treatment has no effect on EGF expression (Fig. 4).

The effect of aldosterone incubation on EGFR expression. The studies carried out above and those previously reported for DOCA-salt hypertensive rats suggest that mineralocorticoids may modulate EGFR at a genomic level, but in vivo, the effect of flow and pressure cannot be ruled out. Therefore, to assess whether
aldosterone is capable of having a direct effect on the EGFR mRNA, aortas from normotensive rats were incubated with aldosterone for 8 h under tissue culture conditions. Aorta incubated with aldosterone (10$^{-8}$ M) had a small but significant increase in mRNA for the EGFR. This increase could be inhibited with spironolactone (Fig. 5).

DISCUSSION

When cerebral ischemia is induced experimentally, SHRSP have larger cerebral infarcts than WKY rats (4). Here, we found that spironolactone administration was effective at reducing the size of the infarct in SHRSP independent of a reduction in blood pressure. This suggests that aldosterone has a deleterious effect on the cerebral vasculature. This is the first study to show that spironolactone administration has a beneficial effect on the outcome of ischemic stroke. Previous studies have shown that spironolactone is capable of reducing the number of spontaneous cerebral infarcts suffered by SHRSP (16). However, spontaneous cerebral infarcts are generally hemorrhagic in nature; these strokes have a different pathogenesis from ischemic strokes. It is most probable that the beneficial effects of spironolactone are occurring at the level of the vasculature. When cerebral ischemia is induced, the collateral vessels around the blockage dilate in an effort to increase blood flow to the area and reduce the amount of damage produced. The vessels from the SHRSP have a reduced ability to dilate in response to ischemia. It is possible that spironolactone treatment improves the ability of the vessels to dilate, therefore reducing the infarct size.

It is possible that vascular remodeling is at least partially responsible for the impaired ability of the blood vessels from SHRSP to dilate in response to ischemia. The literature suggests that the EGF-stimulated pathway is important in hypertension and vascular remodeling (2, 17), but the previous studies were carried out in cell culture, where the effects of flow, pressure, and circulating factors are absent. For the EGF-stimulated pathway to be important in the pathogenesis of cerebrovascular disease, it is important that the pathway be upregulated in the cerebral vessels themselves. Our initial experiments were to assess the levels of the mRNA for both EGFR and EGF in the cerebral vasculature in rats with developed hypertension and in young rats. Cerebral vessels from the older SHRSP had higher levels of EGF and EGFR mRNA compared with WKY. In the younger animals, only the expression of mRNA for EGFR was increased in the cerebral vasculature. This suggests that the expression of the mRNA for EGF and EGFR is being controlled in different ways. It is possible that EGFR is controlled by a circulating factor, whereas EGF is being controlled by blood pressure or flow because it only appears to be increased after hypertension is fully developed. Given

Fig. 4. Expression of EGF (A) and EGFR (B) mRNA from aortic tissue was elevated in SHRSP compared with WKY. Spironolactone reduced the expression of EGFR in SHRSP. mRNA expression was measured by RT-PCR and corrected for GAPDH expression. *Significant difference from SHRSP ($n=8$ for each group; all measurements were made in triplicate).

Fig. 5. Incubation of aorta with aldosterone increased the expression of EGFR in vitro. This could be inhibited with spironolactone. Aortas were incubated with aldosterone (10$^{-8}$ M) + spironolactone (10$^{-5}$ M) for 8 h. RNA was measured by RT-PCR and corrected for GAPDH. *Significant difference from control ($P<0.05$; $n=8$ for each group; all measurements were made in triplicate).
the small amount of tissue obtained from the cerebral vessels it is, as yet, unclear whether the increase in mRNA leads to an increase in protein expression. However, it is interesting to consider that once hypertension is developed, the vessels themselves may produce aldosterone that may act locally within the blood vessels to exacerbate the growth and proliferation.

A variety of factors may be responsible for the increase in EGFR mRNA in SHRSP. Because similar differences were observed in conduit and cerebral vessels, as others have seen similar changes in tissue culture (2, 17), it is unlikely that they are due to a pressure- or flow-mediated effect. A possible candidate for a circulating factor would be aldosterone. Aldosterone is a known risk factor for both stroke and hypertension, and other studies have suggested that mineralocorticoids affect the EGF-stimulated pathway. Vasoconstriction in response to EGF is augmented in DOCA-salt hypertensive rats (8). Recently, we have shown that EGFR and EGFR mRNA are increased in the aorta and cerebral vasculature of DOCA-salt hypertensive rats (6, 7), and immunohistochemical studies have suggested that this increase in mRNA in the aorta results in an increase in protein expression (13). Thus it is possible that mineralocorticoids play a role in the elevation of the mRNA for EGFR in the vasculature. If this is the case, then one would expect that by inhibiting the actions of aldosterone, it would be possible to reduce the deleterious effects. This appears to be the case because inhibition of aldosterone binding with spironolactone was effective at reducing the size of the cerebral infarcts after ischemic insult and reducing the EGFR mRNA expression in the SHRSP. Similar results were observed in vitro. Incubation of segments of aorta with aldosterone for 8 h caused a small but significant increase in the expression of EGFR mRNA. This increase could be inhibited by spironolactone, suggesting that aldosterone is acting at the classical mineralocorticoid receptor and not at the nongenomic receptor as previously suggested by Macleod et al. (12). The promoter region of the EGFR gene does contain the consensus sequence for mineralocorticoid binding.

If aldosterone is affecting the EGFR mRNA at a transcriptional level in all rat strains one would expect the elevation in EGFR mRNA seen here to be due to an increase in circulating aldosterone in the SHRSP. The literature concerning aldosterone levels in SHRSP is contradictory and complicated by the fact that many of the studies have been carried out on a background of high salt (10, 20). Under these conditions, SHRSP show a paradoxical increase in plasma renin activity and therefore plasma aldosterone. Here, we saw no difference in plasma aldosterone between SHRSP and WKY rats. However, it is not only the level of circulating aldosterone that is of interest here; vascular smooth muscle and endothelial cells express the 11β-hydroxylase and aldosterone synthase enzymes (9, 19). Vascular synthesis of aldosterone from the mesenteric bed has been suggested to be increased in SHRSP compared with WKY rats (20). Thus the possibility that locally produced aldosterone is responsible for the increase in EGFR mRNA warrants further investigation.

Spironolactone inhibits the action of both mineralocorticoids and androgens. Recent studies have shown that testosterone is capable of increasing the expression of EGFR in prostate epithelial cells (15). However, we do not believe that testosterone is the mediator of the effect seen here, because the incubation studies with aldosterone and spironolactone suggest that this is a direct effect of aldosterone on the vasculature.

In summary, it would appear the expressions of EGF and EGFR mRNA are both increased in the cerebral blood vessels and aortas in SHRSP. The increase in cerebral vessels may be responsible for the “stroke proneness” of the SHRSP as it may contribute to vascular remodeling. The aldosterone antagonist spironolactone is capable of reducing the infarct size after ischemic insult in SHRSP. This may be due to a reduction in EGFR mRNA and therefore a reduction in vascular remodeling.

Perspectives

This proposed pathway in which mineralocorticoids increase the expression of EGFR may be of clinical importance in situations where plasma aldosterone levels are elevated and there is an increased risk of stroke, such as in patients with dexamethasone-suppressible hyperaldosteronism. It may be possible, in some cases, to use spironolactone as a preventive medicine for patients with a family history of stroke and hypertension. This study adds significantly to the handful of recent studies that suggest that spironolactone has a beneficial effect on the cardiovascular system independent of changes in blood pressure. It is also possible that this proposed mechanism for increasing the expression of growth factor receptors will prove to be important in other disease states in which aberrant cell proliferation is occurring, such as cancer.

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