RESEARCH ARTICLE

Increased perfusion pressure enhances the expression of endothelin (ET_B) and angiotensin II (AT_1) receptors in rat basilar artery smooth muscle cells

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Abstract

Background:

It is known that the endothelin and angiotensin receptors ET_A/ET_B and AT_1 on smooth muscle cells induce contraction and stimulate proliferation and cell hypertrophy. In hypertensive animals and in situations of altered blood flow, e.g. in stroke, there is an up-regulation of ET- and AT-receptors due to de novo transcription. In order to simulate this situation we hypothesized that changes in perfusion pressure may result in altered expression of protein encoding for the receptors ET_A, ET_B, AT_1, and AT_2 in the rat basilar artery.

Methods:

Segments of the rat basilar artery (n = 6) were cannulated with glass micropipettes, pressurized and luminally perfused in a perfusion chamber. After exposure to normal (80/70 mm Hg) or high (140/130 mm Hg) pressure at constant flow for 16 hours the vessel segments were immersed in a fixative solution, dehydrated, frozen, cut in a cryostat and immunohistology-stained for ET- and AT-receptor protein.

Results:

ET_B- and AT_1-receptor proteins were significantly up-regulated after high perfusion pressure compared to normal perfusion pressure at 16 hours ($p < 0.05$). Immunohistochemistry showed that the up-regulated proteins were located mainly in the smooth muscle cells in the arterial wall.

Conclusion:

The results from our rat perfusion model show significant up-regulation of ET_B- and AT_1-receptor proteins after high perfusion pressure. Since the increased protein expression was located in the smooth muscle layer, the results of our study have suggested a shift in the role of the endothelin and angiotensin system towards a worsening of the pathophysiology in the early stages of hypertension.

Key words: artery, basilar, perfusion pressure, rat, angiotensin II, endothelin, receptors

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Introduction

Changes of the endothelin-1 (ET-1) and the angiotensin II (AT-II) systems are considered to play important roles in the pathophysiology of cardiovascular disease.[1,2] The relationship between the AT and the ET systems is complex, and their participation in pathophysiology of hypertension remains to be clarified. ET-1 is the most potent endogenous vasoconstrictive substance yet known. It exerts its effects through the ET_A- and ET_B-receptors. ET_A- and ET_B-receptors on SMC (smooth muscle cells) induce contraction and stimulate proliferation and cell hypertrophy. Endothelial ET_B-receptors stimulate the production of NO and prostacyclin and, accordingly, elicit vasorelaxation.[3] A similar relationship is thought to exist between the AT_1- and AT_2-receptors; whereas AT-II induces vasoconstriction via AT_1-receptors on SMC, it promotes vasodilatation via AT_2-receptors on endothelial cells.[4]

It has been shown that elevated perfusion pressure up-regulates ET-1 and ET_B-receptor expression in the rabbit carotid artery.[5] Examination of the expression of preproET-1 mRNA in small arteries from gluteal subcutaneous biopsies, normotensive subjects and mildly hypertensive patients revealed a similar expression of endothelial prepro-ET-1 mRNA, whereas in moderately to severely hypertensive patients, the ET-1 expression in the endothelium of small arteries was significantly increased.[6] Also, there has been seen enhanced ET_B- and ET_A-receptor contraction and expression in the left internal mammary artery from patients with hypertension as compared to normotensive patients and increased ET_B- and ET_A-receptor contraction in arteries from both human patients with hypertension and spontaneously hypertensive rats.[7,8]

The ET_B-receptor has been shown to be up-regulated in atherosclerotic human arteries in one study while, in another study, a reduction of ET_A- and AT_1-receptors and an increase in AT_2-receptorswere seen in human coronary artery SMC with atherosclerotic lesions.[9,10] Furthermore, the ET_B-receptor has been up-regulated in congestive heart failure, ischemic heart disease, subarachnoid haemorrhage and ischemic stroke.[11-15] One study revealed that AT_1- and AT_2-receptors presented a wide distribution in all layers of the mesenteric artery in normotensive rats, while in spontaneously hypertensive rats its distribution was not homogenous: AT_1-receptors were evident in the intima and adventitia but were rare in vascular SMC; the AT_2-receptor distribution pattern was vastly reduced.[16] The AT_1- and AT_2-receptors have been shown to be down-regulated in heart failure, the AT_1-receptor was also reduced in ischemic heart disease, while the AT_1-receptor was up-regulated in ischemic stroke.[17,18,15]

We have previously shown that there was an enhanced expression of endothelin and angiotensin receptors in rat mesenteric arteries exposed to different perfusion pressures.[19] Our aim this time was to see if there similarly was a change in expression of ET_A-, ET_B-, AT_1- or AT_2-receptors in rat basilar arteries exposed to elevated perfusion pressure while maintaining constant flow, thus increasing the shear stress.

Methods

Tissue preparation

Male Wistar Hannover rats (body weight 350 - 400 g) were anesthetized with CO2, exsanguinated, and decapitated. The brains were quickly removed and immersed into cold buffer solution (for composition, see below). The basilar arteries were freed of

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adhering tissue under a dissection microscope.

The Lund University Animal Ethics Committee approved the experimental protocol.

Buffer solutions

Standard buffer solution (mM): NaCl 119; NaHCO3 15; KCl 4.6; MgCl 1.2; NaH2PO4 1.2; CaCl2 1.5; glucose 5.5. Analytical grade chemicals and double distilled water were used for preparing all solutions.

In vitro perfusion pressure and shear stress

An approximately 3 mm long segment of the basilar artery was selected and cut clean. It was cannulated on either end with two glass micropipettes, fixed using fine suture, luminally perfused and immersed into a temperature controlled (37°C) perfusion chamber/tissue bath. As many branches as possible on the vessel were tied using the same suture. The vessel segments were immersed and perfused with the same bicarbonate buffer solution mentioned above. The solution was continuously gassed with 5% CO_2 in O_2, resulting in a pH of 7.4. The perfusion pressure in the vessels was set to either 80 mmHg (normal) or 140 mmHg (high) by leveling or lowering the syringe containing the ingoing buffer solution. The syringe containing the outgoing buffer solution was set to a height representative of 10 mmHg less than the ingoing, making a constant flow 0,1ml/min. The vessels remained in the perfusion chamber for 16 hours and were monitored every hour. Regular refilling of the baths with buffer solution was necessary, since they were sensitive to evaporation, making sure that there was enough buffer solution in the system continuously. Since the buffer solution was supposed to run through the vessel at all times, a check to control that the vessel was not occluded was performed regularly.

Immunohistochemistry

After the basilar artery segments had been exposed to normal or high perfusion pressure, they were immersed in a fixative solution consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at 4°C. After fixation, the specimens were dehydrated in a 20% sucrose phosphate buffer (0.1 M, pH 7.4) for 24 h at 4°C, and then frozen in Tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, Netherlands) and stored at - 80°C. Sections were cut at 10-μm thickness in a cryostat and mounted on SupperFrost Plus slides (Menzel GMBH & COKG, Braunschweig, Germany). Immunohistochemistry were carried out using standard protocols, i.e. the sections were incubated with the primary antibody overnight at 4°C and the secondary antibody for 1 h at room temperature in the dark. The primary antibodies and their dilutions were the following: AT_1 receptor (1:100 rabbit, Santa Cruz SC-1173); AT_2 receptor (1:100 rabbit, Santa Cruz SC-9040); ET_A receptor (1:50 rabbit Santa Cruz SC-33535); ET_B receptor (1:150 goat Santa Cruz SC-21196). The fluorescent secondary antibodies were goat anti-rabbit IgG (1:100 FITC) or donkey anti-goat IgG (1:200 Alexa488). Negative controls were performed where only the secondary antibody was used. The stained specimens were observed under a confocal microscope (Nikon, C1plus, Nikon Instruments Inc., NY, USA).The fluorescence was measured and analysed by Image J software (http://rsb.info.nih.gov/ij). For each specimen 2 randomly selected sections were observed. At each section fluorescence intensity was measure at 4 pre-set areas with the size of 400 pixels.

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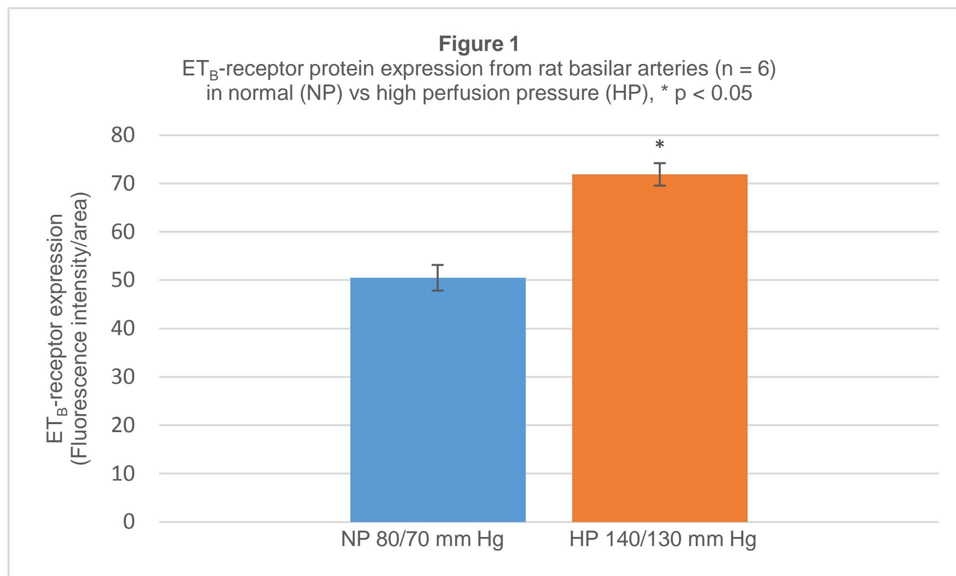
Data analysis

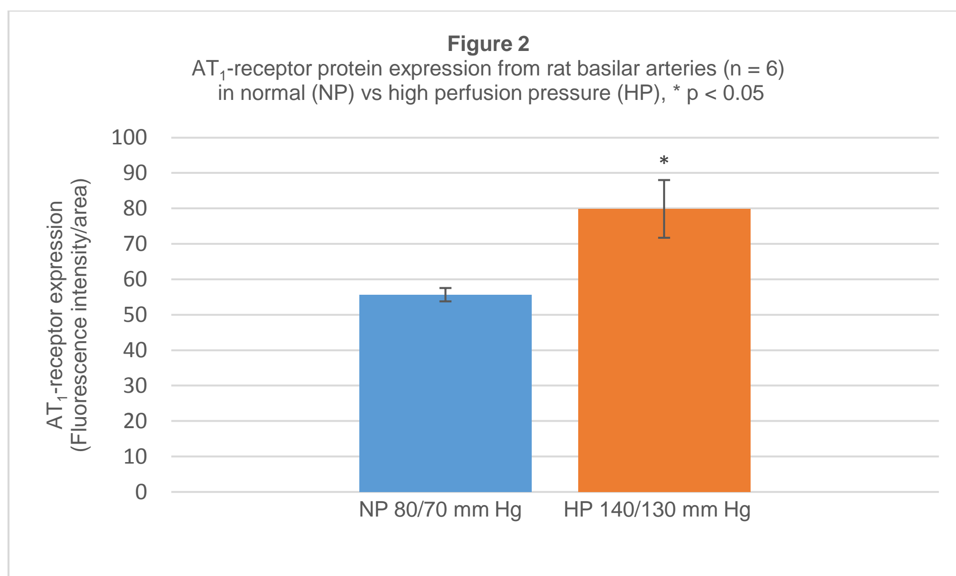
Statistics were performed using the IBM SPSS Statistics 22 program. The mean value and standard error of the mean (SEM) of each specimen was calculated. Statistical analysis was performed using the unpaired Student's t-test to compare two sets of data. A p-value <0.05 was considered significant. Fluorescence intensity was expressed as arbitrary units (AU) of fluorescence intensity/area.

Results

Receptor protein expression

See Figure 1 and 2. ET_B- and AT_1-receptor proteins were significantly up-regulated after high perfusion pressure compared to normal perfusion pressure at 16 hours. ET_B-receptor protein: High perfusion pressure 71.8 AU (Mean) ± 3.2 (SEM) compared to normal perfusion pressure 50.5 AU (Mean) ± 5.3 (SEM) ($p = 0.006$). AT_1-receptor protein: High perfusion pressure 79.8 AU (Mean) ± 10.2 (SEM) compared to normal perfusion pressure 55.6 AU (Mean) ± 3.4 (SEM) ($p = 0.048$).





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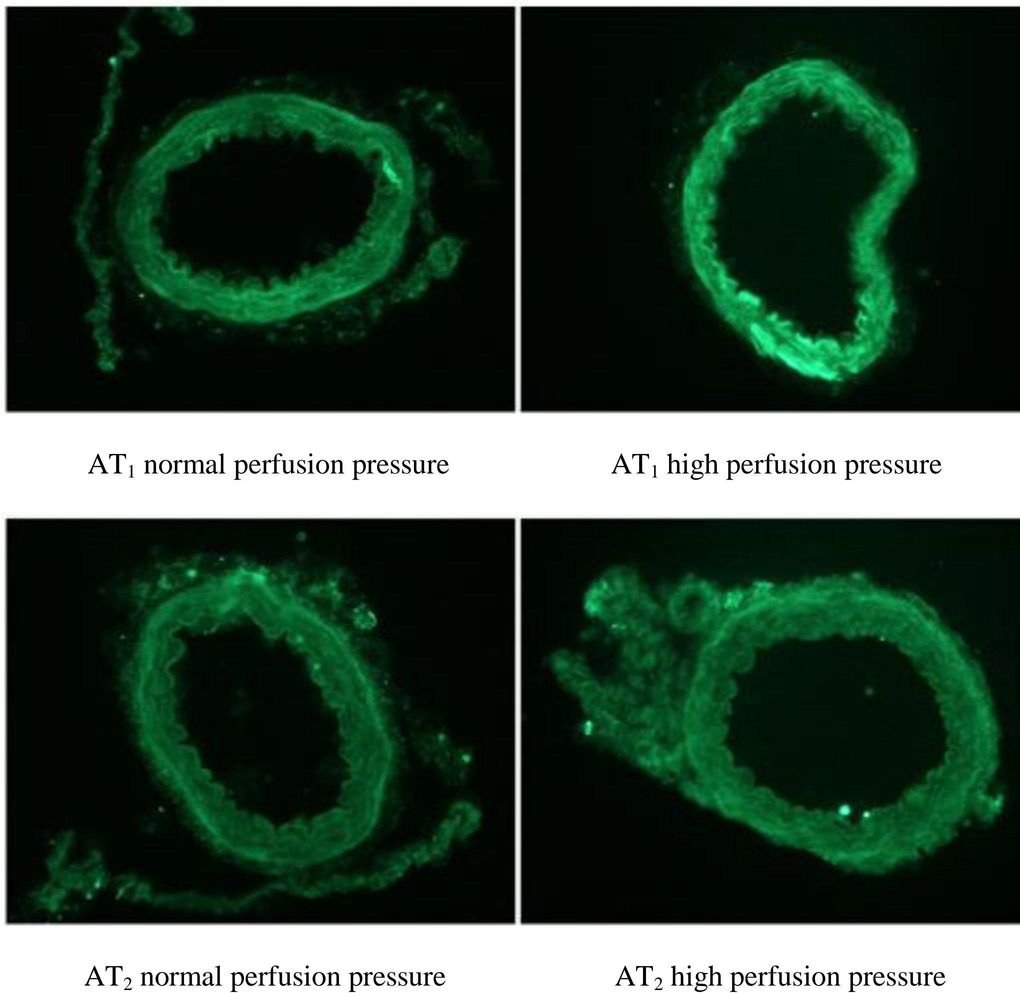
ET_A- and AT_2-receptor proteins were not significantly up-regulated after high perfusion pressure compared to normal perfusion pressure at 16 hours. ET_A-receptor protein: High perfusion pressure 74.5 AU (Mean) ± 5.9 (SEM) compared to normal perfusion pressure 59.2 AU (Mean) ± 7.7 (SEM) ($p = 0.149$). AT_2-receptor protein: High perfusion pressure 38.5 AU (Mean) ± 5.2 (SEM) compared to normal perfusion pressure 29.0 AU (Mean) ± 5.2 (SEM) ($p = 0.225$).

Localization of receptor expression

See Illustration 1. We evaluated the precise site of the enhanced expression of endothelin- and angiotensin receptors with high-power confocal microscopy. It can be clearly seen that the enhanced protein expression is located in all the layers of the vessel, but especially in the media layer.

Illustration 1. *Images showing the immunohistochemistry at 20x enlargement*



AT_1 normal perfusion pressure

AT_1 high perfusion pressure

AT_2 normal perfusion pressure

AT_2 high perfusion pressure

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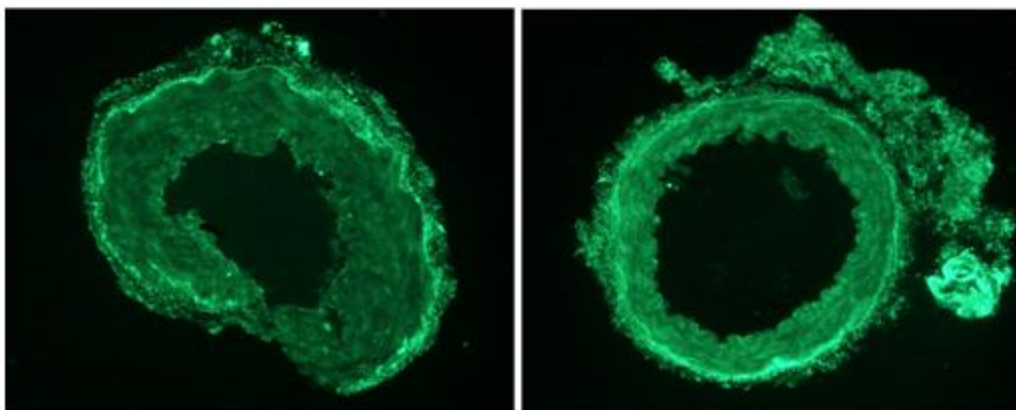
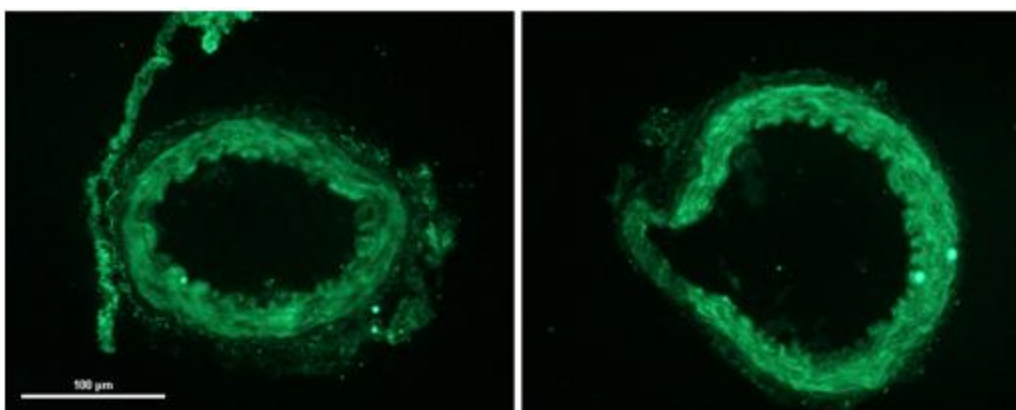


ET_A normal perfusion pressure ET_A high perfusion pressure



ET_B normal perfusion pressure ET_B high perfusion pressure

Discussion

We have shown that there was an up-regulation of ET_B- and AT_1-receptors in rat basilar arteries exposed to elevated perfusion pressure. As a matter of fact, all studied endothelin and angiotensin receptors showed an up-regulation in this environment, although not all expressions were significant at the < 0.05 p level. While similar studies have been performed previously, this is to the best of our knowledge the first study examining the consequences of perfusion pressure on endothelin and angiotensin receptors in rat basilar arteries. The results were quite similar to the results of our previous study on mesenteric rat arteries where we found an up-regulation of ET_B- and AT_1/AT_2-receptors in high perfusion pressure using the same method.[19] Our findings support the theory of receptor regulation in vessels exposed to elevated perfusion pressure at a constant flow i.e. increased shear stress, although the exact mechanisms involved still remains unknown.

In this study the majority of the up-regulation of ET_B-receptors was located in the SMC which strongly suggests a vasoconstrictive action of ET_B. In the same manner, we found increased AT_1-receptors in the basilar vessels in the high perfusion pressure environment further indicating a worsening of the vasoconstrictive state. The AT_2-receptor, which is known to cause vasodilation, was not significantly up-regulated. All together, the results thus pointed towards a situation where high perfusion pressure caused an up-regulation

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of mainly pathological phenotypes. ET_B-receptors have a dual role meaning they also cause vasodilatation if situated on endothelial cells. It is likely that this dual role plays a part in blood pressure regulation in healthy individuals. One hypothesis could be that the up-regulation of ET_B- and AT_1-receptors served as a feedback mechanism from the increased perfusion pressure. While intended to protect the vessels from the increased tension, it on the contrary worsened the already high blood pressure by constricting the vessels even further. The fact that the up-regulation occurred after only 16 hours also suggested that this feedback mechanism could happen at an early stage in hypertensive disease. On the other hand, we do not know what would have happened if the experiment lasted longer than 16 hours, for example 24 or 48 hours. It is not impossible that a compensatory up-regulation of the AT_2-receptor would have occurred alleviating the negative consequences of vasoconstriction. In our previous perfusion experiment, we saw an up-regulation of the AT_2-receptor in mesenteric rat arteries at 17 hours of high perfusion pressure.[19] On the contrary, in spontaneously hypertensive rats that are constantly in a hypertensive state, the AT_2-receptor pattern was vastly reduced throughout the entire mesenteric artery as compared to normotensive rats [16]; a finding that might suggest that the AT_2-receptor expression stays attenuated for the long run.

Our results on endothelin receptors in rat basilar arteries are in line with previous research on arteries in a rabbit perfusion model,[5] in spontaneously hypertensive rats,[8] and arteries from hypertensive humans.[6-8] Additionally, the up-regulation of ET_B-receptors from rat basilar arteries are in line with the results from studies on different arteries in cardio- and cerebrovascular diseases.[11-15] We saw an up-regulation of AT_1-receptors in rat basilar arteries which was in line with previous research on AT_1-receptor up-regulation in the middle cerebral artery in ischemic stroke,[15] while other studies have shown evidence of decreased AT_1-receptors in spontaneously hypertensive rats and cardiovascular disease.[16-18] It is possible that the discrepancies that existed in expression of endothelin and angiotensin proteins in different cardiovascular diseases could be the result of different expressions in different stages of the disease process. This can also be appreciated considering data showing up-regulation of rat endothelin receptors in metabolic diseases like dyslipidemia and hyperhomocystein-emia.[20,21] One might speculate that the expression might differ in early stages of hypertensive disease from that in later stage hypertension with organ damage, in the acute phase of ischemic heart disease and stroke, to that of organ failure in later stages of the diseases such as heart failure and cerebrovascular sequela with damaged autonomic vascular regulation.

We have previously shown that there was no up-regulation of endothelin or angiotensin receptors in rat mesenteric vessels exposed to pressure only, i.e. without perfusion.[19] We did not attempt such a comparison in our rat basilar experiment, but it is important to mention that if such a result would be true for the rat basilar vessels also, which is highly likely, shear stress could be a contributing factor to the expression of ET_B- and AT_1-receptors. Data regarding the regulation of the endothelin and angiotensin systems by shear stress forces are controversial and most of the research has been done on the cellular level rather than in whole arteries [22-28]; circumstances that make our results novel. Presuming that the actual outcome of shear stress was to worsen a hypertensive state even further, blocking the receptors in question could be a potential treatment for hypertension.

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We investigated the protein expression after perfusion pressure for 16 hours. It would have been pertinent to have done additional studies of the expression of endothelin- and angiotensin mRNA in order to verify the transcription – translation pathway. We have previously shown that the expression of ET_B and AT_2mRNA was significantly increased in rat mesenteric artery segments exposed to high perfusion pressure, compared with normal perfusion pressure after 4 h.[19] This has also been the case in other studies, for example Lauth et al whom found a significant gradual increase in prepro-ET-1 mRNA and ET_BmRNA in rabbit carotid artery exposed to high perfusion pressure at 160 mm Hg after 4, 8, and 12 hours.[5] Based on these data we might have expected a similar result in the rat basilar artery. Also, a better way of showing where the receptor up-regulation was located in the arterial wall would be by performing co-localization immunohisto-chemistry targeting for example actin filaments in the smooth muscle cells and endothelial cells.[19] However, visual judgment from the pictures alone strongly suggested that a majority of the up-regulation took place in the SMC.

Strengths: Most of the research done before has shown changes in receptor expression on the cellular level, in animal models or vessels taken from human subjects with various cardio- and cerebrovascular endpoints. Our research results are novel considering we used an experimental model that could be seen as depicting events in early hypertension and a model that has further implications when it comes to shear stress. Furthermore, we found changes in endothelin and angiotensin receptors in the rat basilar artery. Studies on early blood pressure changes in the rat basilar system are scarce and have potential important implications for the development of pathophysiological events in cerebrovascular disease.

Limitations: It is not known for sure how long it would take to optimally expose basilar rat vessels to high perfusion pressure before an altered protein expression occurred. It is possible that prolonging the experiment would cause an even further receptor up-regulation. Furthermore, this study is quite small, including only 6 rat vessels. Adding more vessels to the study could potentially alter the results. There are also some potential sources of error in the experimental design. One being the fact that the basilar artery is quite small and there is a possibility of occlusion of the artery while performing the experiment. This could potentially alter the receptor expression in the vessels; for example there might be different outcomes in protein expression from the part of the vessel that lies before the occlusion compared with the part of the vessel that follows the occlusion. Also, the number of small perpendicular vessel branches that leaves the basilar artery could also affect the outcome of the study if not completely tied up. Both these factors could lead to a temporary lowering of the shear stress. Lastly, the viability of the vessel after 16 hours of perfusion could have been altered.

In conclusion, we have demonstrated a significant up-regulation of ET_B- and AT_1-receptors in rat basilar arteries under elevated perfusion pressure.

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