Increased angiotensin II type 1 receptor expression in temporal arteries from patients with giant cell arteritis.

Dimitrijevic, Ivan; Malmsjö, Malin; Andersson, Christina; Rissler, Pehr; Edvinsson, Lars

Published in:
Ophthalmology

DOI:
10.1016/j.ophtha.2008.12.021

2009

Corticosteroids are the only proven treatment for giant cell arteritis. This study shows upregulated angiotensin II type 1 receptors in both smooth muscle cells and inflammatory cells in the temporal arteries from patients with GCA.

Increased angiotensin II type 1 receptor expression in temporal arteries from patients with giant cell arteritis

[a]Ivan Dimitrijevic M.D., [a,b]Malin Malmsjö M.D., PhD, [c]Christina Andersson M.T., [c]Pehr Rissler M.D., [a]Lars Edvinsson M.D., PhD.

[a]Department of Emergency Medicine, [b]Department of Ophthalmology and [c]Department of Pathology, Clinical Sciences, Lund, Lund University, Sweden

Short title: Angiotensin II and giant cell arteritis

Corresponding author:

Ivan Dimitrijevic

Division of Experimental Vascular Research

BMC A13

SE-221 84 Lund

Sweden

Telephone number +46 46-176 749

Fax number +46 46-222 0616

e-mail: ivan.dimitrijevic@med.lu.se

KEY WORDS: AT_1 receptor; AT_2 receptor; immunohistochemistry; temporal artery, giant cell arteritis, vascular

FINANCIAL SUPPORT: This study was supported by the Swedish Research Council and the Heart and Lung Foundation (Sweden).

No authors have any financial/conflicting interests to disclose.

Purpose

Currently, giant cell arteritis (GCA) is primarily treated with corticosteroids or immunomodulating agents, but there is interest in identifying other non-corticosteroid alternatives. Similarities exist in the injury pathways between GCA and atherosclerosis. Angiotensin II is a vasoactive peptide involved in vessel inflammation during atherosclerosis, and angiotensin II receptor inhibitors are effective in preventing atherosclerosis. The present study was performed to elucidate the role of angiotensin type 1 (AT_1) and type 2 (AT_2) receptors in GCA.

Participants

Ten patients with GCA and ten control patients, who were clinically suspected of having GCA but were diagnosed as not having GCA, were included.

Methods

Immunohistochemistry, using anti-AT_1 and anti-AT_2 antibodies, was performed on formalin-fixed and paraffin-embedded temporal arteries.

Main outcome measures

AT_1 and AT_2 receptor immunostaining intensity was quantified.

Results

Hematoxylin-eosin-stained sections of temporal arteries from patients with GCA showed intimal hyperplasia, internal elastic lamina degeneration and band-shaped infiltrates of inflammatory cells, including lymphocytes, histocytes, and multinucleated giant cells. AT_1

receptor staining was primarily observed in the medial layer of the temporal arteries, and was higher in the patients with GCA than in the control patients. This was a result of increased AT_1 receptor immunostaining of both vascular smooth muscle cells and of infiltrating inflammatory cells. Only faint immunostaining was seen for AT_2 receptors, primarily in the endothelial cells, and to a lesser extent on the smooth muscle cells. Immunostaining with antibodies for the AT_2 receptor was similar in the patients with GCA and in controls.

Conclusions

These results suggest that AT_1 receptors play a role in the development of GCA. Inhibition of the angiotensin system may thus provide a non-corticosteroid alternative for the treatment of GCA.

Introduction

Giant cell arteritis (GCA) is a granulomatous form of vasculitis involving large and medium-sized vessels.[1] The extracranial branches of the aorta are the main targets, and the major source of diagnostic biopsies is the temporal artery. The clinical manifestations of GCA are due to end organ ischemia and include blindness, jaw claudication, headaches, stroke and aortic arch syndrome. These symptoms are often accompanied by signs of systemic inflammation, such as malaise, anorexia, fever and myalgia. Excessive amounts of acute phase proteins are produced and the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and interleukin-6 levels are elevated.

GCA is a form of panarteritis with transmural inflammation, involving the media, intima and adventitia of the affected arteries. Active disease is characterized by a nodular granulomatous reaction that is centered around a fragmented internal elastic lamina with inflammatory cells in the intima or media.[2, 3] The typical lesions are formed by interferon-γ-producing $CD4^{+}$T cells and macrophages.[3, 4] Thrombosis may be present, although ischemic symptoms are usually the result of lumen narrowing resulting from mural thickening.

Currently, giant cell arteritis (GCA) is primarily treated with corticosteroids or immunomodulating agents. [5] However, there is interest in identifying other non-corticosteroid alternatives because of the numerous complications associated with long-term corticosteroid treatment.[6] The complications appear to be dose related, and non-corticosteroid medication may therefore be beneficial. Studies have been carried out on methotrexate, anti-TNF-a agents, and others, such as azathioprine and cyclophosphamide,[7] but none of these has yet

been recommended for GCA. Modulating the inflammatory response by inhibiting the renin-angiotensin system may be an alternative approach for the treatment of GCA.

Angiotensin II receptor inhibitors are effective in preventing atherosclerosis. GCA and atherosclerosis have similar injury pathways, both of which involve vessel inflammation. Angiotensin II is a vasoactive peptide that is known to play a role in vessel inflammation in atherosclerosis. Angiotensin II modulates the immune response via the production of NF-κB in the cytoplasm of mature phagocytes, resulting in the transcription of inflammatory cytokines and chemokines, including IL-1beta, IL-6, IFN-gamma and TNF-alpha, and IL-8, MCP-1 and CAM-1, respectively.[8-10] Angiotensin-converting enzyme inhibitors reduce the expression of monocyte chemo-attractant protein-1 and concomitant macrophage plaque infiltration in animal models of atherosclerosis.[11] In patients with carotid artery disease, the angiotensin receptor blocker irbersartan inhibits metalloproteinase activity, as well as T-cell and macrophage infiltration in the vascular wall.[12]

To our knowledge, no study has yet been performed to elucidate the possible role of angiotensin II receptors in GCA in humans. In the present study, angiotensin type 1 (AT_1) and type 2 (AT_2) receptor expression was investigated in temporal arteries, using immunohistochemistry. The immunostaining intensity in temporal arteries from patients with GCA was compared to that in arteries from control patients. The latter were clinically suspected of having GCA, but this diagnosis was ruled out.

MATERIALS AND METHODS

Tissue collection

Temporal artery vessel biopsy specimens from 20 patients were retrieved from the Department of Pathology, Lund University, Sweden, where they had been routinely examined for histopathology. All patients' symptoms and background characteristics are listed in Table 1. The original pathology reports were reviewed to verify the diagnosis GCA. The diagnosis was based on the American College of Rheumatology 1990 criteria for the classification of GCA [13]. The absence of inflammatory lesions in a sample led to the categorization of the sample as negative. Temporal arteries from ten patients with GCA were compared to arteries from ten patients who were clinically suspected of having GCA, but the diagnosis had been ruled out (control patients). The histopathological findings in the temporal arteries from all 20 patients are listed in Table 2.

Ethics

The samples were handled in accordance with permission obtained from the Lund University Human Ethics Committee (LU 818-01), and the study conformed to the principles outlined in the Declaration of Helsinki.

Immunohistochemistry

Four-µm sections were cut from formalin-fixed, paraffin-embedded blocks and dried at 60 °C for one hour. After dewaxing and rehydration, the sections were treated with 10 mM citrate buffer, pH 6.0, in a microwave oven for 15 min for antigen retrieval. Immunohistochemical staining with rabbit polyclonal antibody AT_1 receptor (sc-1173) and AT_2 receptor (sc-9034, both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:50, anti CD68 (Dako

M876) diluted 1:200, and anti CD45 (Dako M701) diluted 1:600, smooth muscle actin diluted 1:500 (Dako M0851) and CD31 diluted 1:50 (Dako M823) was performed in an automated immunostainer, TechMate 500 (Ventana Biotek, Tuscan, AZ, USA), using the biotin-streptavidin-peroxidase method with diaminobenzidine as the chromogen (Dako REAL™ Detection System, Peroxidase/DAB+, Rabbit/Mouse). Mayer's hematoxylin was used for counterstaining. Primary antibodies were omitted for negative control. Details and antibody specificity are given on the company's homepage. The samples were examined with a microscope (Olympus BX60, Tokyo, Japan) and photographed using a digital camera (Pixera Pro 600ES, Los Gatos, CA, USA).

Calculations and statistics

Calculations were performed on the results obtained from temporal arteries from 10 patients with GCA and 10 control patients. The immunofluorescence intensity was measured using ImageJ (http://rsb.info.nih.gov/ij/). Measurements were performed on three sections, at four different regions in each sample, and the mean values were calculated. Statistical analysis was performed using Student's t-test. Significance was defined as $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***). Values are presented as means ± standard deviation (SD).

Results

Hematoxylin-eosin staining

Hematoxylin-eosin-stained sections of temporal arteries from patients with GCA showed intimal hyperplasia with reduction of the lumen, internal elastic lamina degeneration and band-shaped infiltrates of inflammatory cells, including lymphocytes, histocytes, and multinucleated giant cells (Table 2 and Figure 1). Immunostaining for CD68 demonstrated strong labeling in the area of inflammatory cell infiltrates. Immunostaining for CD45 was also seen, but was not as prominent. In the more severe cases of GCA, transmural inflammatory infiltrates and necrosis were seen in portions of the vascular wall. The control patients were clinically suspected cases of GCA, in which the diagnosis of GCA had been ruled out. The temporal arteries from these control patients had intimal hyperplasia and sub-intimal fibrosis and focal loss of internal elastic lamina, suggestive of atherosclerotic disease.

AT_1 receptor immunostaining

Immunostaining for AT_1 receptors was observed in the smooth muscle cell layer and in the endothelial cell layer (Figure 2) in temporal arteries from control patients. In the smooth muscle cell layer of the temporal arteries from patients with GCA there was a 0.55 fold increase in the immunostaining intensity of AT_1 receptor in the smooth muscle layer compared to control patients (40 ± 22 in controls and 73 ± 28 in patients with GCA, $p<0.05$). No changes in immunostaining of AT_1 receptors were detected in the endothelial cell layer in temporal arteries from controls or GCA patients (56 ± 20 in controls and 60 ± 19 in patients with GCA, p=n.s.). In the temporal arteries from the patients with GCA, AT_1 receptor staining was observed in the lymphocytes, histocytes, multinucleated giant cells, and smooth muscle cells, as well as in the neointima (Figure 2).

AT_2 receptor immunostaining

Only faint immunostaining was seen for AT_2 receptors, primarily in the endothelial cell layer, and to a lesser extent in the smooth muscle cell layer. No difference was observed in the AT_2 receptor immunostaining intensity between the temporal arteries of patients with GCA and controls in the smooth muscle cell layer (36 ± 18 in controls and 41± 22 in patients with GCA, p=n.s.) or in the endothelial cell layer (49 ± 17 in controls and 47 ± 20 in patients with GCA, p=n.s., Figure 3).

Counterstaining with alpha-gamma actin and CD31 verified the presence of AT_1 and AT_2 receptors in smooth muscle and endothelial cells, respectively.

Discussion

Major findings

The objective of this study was to investigate the possible roles of AT_1 and AT_2 receptors in GCA. Temporal arteries from patients diagnosed with GCA were compared to those from control patients that were clinically suspected cases but the diagnosis of giant cell arteritis had been ruled out. The major finding of the present study was increased immunostaining intensities for AT_1 receptors in both smooth muscle cells and inflammatory cells in the temporal arteries from patients with GCA.

GCA diagnosis

The patients included in the present study were diagnosed as having GCA according to the American College of Rheumatology 1990 criteria for the classification of giant cell arteritis, which have a sensitivity of 94% and a specificity of 91%.[13] The criteria were initially developed as a research tool, and the presence of three or more of the following criteria are required for the diagnosis of GCA: age of onset greater than 50 years, onset of new headache, temporal artery abnormality (tender or reduced pulsation), elevated ESR, defined as 50 mm/h, and abnormal arterial biopsy. Table 1 gives the characteristics of the patients' clinical presentation.

Hematoxylin-eosin-stained sections of temporal arteries demonstrated features that are typical of giant cell arteritis,[14] including intimal hyperplasia with reduction of the lumen, internal elastic lamina degeneration and band-shaped infiltrates of inflammatory cells, including lymphocytes, histocytes, and multinucleated giant cells. In the more severe cases of GCA,

transmural inflammatory infiltrates and necrosis were seen in parts of the vascular wall. Table 2 provides a detailed presentation of the vascular wall lesions in study patients with GCA.

Angiotensin II receptors in the vascular wall

Two angiotensin II receptors have been identified and cloned in man: the AT_1 and AT_2 receptors.[15, 16] AT_1 and AT_2 receptors are distributed heterogeneously in human tissues and blood vessels.[17] In the present study, immunostaining for AT_1 receptors was seen on the smooth muscle cells of the media in the temporal arteries from the control patients. Only faint immunostaining was seen for AT_2 receptors, primarily in the endothelial cell layer, and to a lesser extent in the smooth muscle cell layer. This is in accordance with previous findings of AT_1 receptors on smooth muscle cells, and AT_2 receptors on endothelial cells.[18-21] Immunostaining for AT_2 on the smooth muscle cells of arteries has also been reported.[22]

In the present study, the levels of AT_1 receptors on smooth muscle cells were higher in the temporal arteries from the patients with GCA than in the temporal arteries from the control patients. Angiotensin II is a potent vasoconstrictor and mitogen of vascular smooth muscle cells. The stimulation of AT_1 receptors is known to result in progression of atherosclerotic lesions, inflammation and plaque rupture.[23, 24] The upregulation of AT_1 receptors in GCA could make the arteries prone to tonic contraction and spasm, resulting in the progression of vascular wall inflammation.

Angiotensin II receptors and vascular wall inflammation

High AT_1 receptor immunostaining intensity in arteries from patients with GCA was not only due to increased protein expression on the smooth muscle cells, but also to high levels of AT_1 receptor expression on inflammatory cells, including lymphocytes and macrophages

infiltrating the vascular lesions. AT_1 receptors have previously been demonstrated on both lymphocytes and macrophages.[25-27] Angiotensin II affects human T-lymphocyte function via AT_1 receptor activation, which results in increased production of pro-inflammatory cytokines such as interferon-γ and TNF-α.[28 29] The intense expression of the AT_1 receptor in inflammatory cells in the GCA vascular lesion may contribute to the progression of the disease. We suggest that inhibiting the angiotensin system may offer a new means of pharmacologically modulating the progression of GCA. Indeed, inhibitors of AT_1 receptors have been shown to modify the progression of atherosclerosis and stabilize atheromatous plaques.[11, 12] Atherosclerosis and GCA are both vascular diseases in which inflammation is a common entity.

Conclusions

The present study demonstrates high levels of AT_1 receptor expression in both the vascular smooth muscle cells and inflammatory cells in the temporal arteries of patients with GCA. Increased activity in the angiotensin II system may play a role in the progression of inflammation in GCA. We suggest that inhibition of the renin-angiotensin system may provide a non-corticosteroid alternative for the treatment of GCA inflammation.

References

1. Hunder GG. Clinical features of GCA/PMR. Clin Exp Rheumatol 2000;18:S6-8.
2. Weyand CM, Bartley GB. Giant cell arteritis: new concepts in pathogenesis and implications for management. Am J Ophthalmol 1997;123:392-5.
3. Poller DN, van Wyk Q, Jeffrey MJ. The importance of skip lesions in temporal arteritis. J Clin Pathol 2000;53:137-9.
4. Rauzy O, Fort M, Nourhashemi F, et al. Relation between HLA DRB1 alleles and corticosteroid resistance in giant cell arteritis. Ann Rheum Dis 1998;57:380-2.
5. Carroll SC, Gaskin BJ, Danesh-Meyer HV. Giant cell arteritis. Clin Experiment Ophthalmol 2006;34:159-73; quiz 94.
6. Proven A, Gabriel SE, Orces C, et al. Glucocorticoid therapy in giant cell arteritis: duration and adverse outcomes. Arthritis Rheum 2003;49:703-8.
7. Nuenninghoff DM, Matteson EL. The role of disease-modifying antirheumatic drugs in the treatment of giant cell arteritis. Clin Exp Rheumatol 2003;21:S29-34.
8. Ruiz-Ortega M, Lorenzo O, Ruperez M, et al. Angiotensin II activates nuclear transcription factor kappaB through AT(1) and AT(2) in vascular smooth muscle cells: molecular mechanisms. Circ Res 2000;86:1266-72.
9. Mezzano S, Droguett A, Burgos ME, et al. Renin-angiotensin system activation and interstitial inflammation in human diabetic nephropathy. Kidney Int Suppl 2003:S64-70.
10. Sadoshima J. Cytokine actions of angiotensin II. Circ Res 2000;86:1187-9.
11. Halkin A, Keren G. Potential indications for angiotensin-converting enzyme inhibitors in atherosclerotic vascular disease. Am J Med 2002;112:126-34.

12. Cipollone F, Fazia M, Iezzi A, et al. Blockade of the angiotensin II type 1 receptor stabilizes atherosclerotic plaques in humans by inhibiting prostaglandin E2-dependent matrix metalloproteinase activity. Circulation 2004;109:1482-8.

13. Hunder GG, Bloch DA, Michel BA, et al. The American College of Rheumatology 1990 criteria for the classification of giant cell arteritis. Arthritis Rheum 1990;33:1122-8.

14. Salvarani C, Cantini F, Boiardi L, Hunder GG. Polymyalgia rheumatica and giant-cell arteritis. N Engl J Med 2002;347:261-71.

15. Tsuzuki S, Ichiki T, Nakakubo H, et al. Molecular cloning and expression of the gene encoding human angiotensin II type 2 receptor. Biochem Biophys Res Commun 1994;200:1449-54.

16. Furuta H, Guo DF, Inagami T. Molecular cloning and sequencing of the gene encoding human angiotensin II type 1 receptor. Biochem Biophys Res Commun 1992;183:8-13.

17. Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. Pharmacol Rev 2000;52:11-34.

18. Weber KT. Fibrosis and hypertensive heart disease. Curr Opin Cardiol 2000;15:264-72.

19. Levy BI, Benessiano J, Henrion D, et al. Chronic blockade of AT2-subtype receptors prevents the effect of angiotensin II on the rat vascular structure. J Clin Invest 1996;98:418-25.

20. Brilla CG, Janicki JS, Weber KT. Impaired diastolic function and coronary reserve in genetic hypertension. Role of interstitial fibrosis and medial thickening of intramyocardial coronary arteries. Circ Res 1991;69:107-15.

21. Baker KM, Aceto JF. Angiotensin II stimulation of protein synthesis and cell growth in chick heart cells. Am J Physiol 1990;259:H610-8.

22. Zhuo J, Moeller I, Jenkins T, et al. Mapping tissue angiotensin-converting enzyme and angiotensin AT1, AT2 and AT4 receptors. J Hypertens 1998;16:2027-37.

23. Diet F, Pratt RE, Berry GJ, et al. Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. Circulation 1996;94:2756-67.

24. Schieffer B, Schieffer E, Hilfiker-Kleiner D, et al. Expression of angiotensin II and interleukin 6 in human coronary atherosclerotic plaques: potential implications for inflammation and plaque instability. Circulation 2000;101:1372-8.

25. Shimada K, Yazaki Y. Binding sites for angiotensin II in human mononuclear leucocytes. J Biochem 1978;84:1013-5.

26. Suzuki H, Shibata H, Murakami M, et al. Modulation of angiotensin II type 1 receptor mRNA expression in human blood cells: comparison of platelets and mononuclear leucocytes. Endocr J 1995;42:15-22.

27. Rasini E, Cosentino M, Marino F, et al. Angiotensin II type 1 receptor expression on human leukocyte subsets: a flow cytometric and RT-PCR study. Regul Pept 2006;134:69-74.

28. Hahn AW, Jonas U, Buhler FR, Resink TJ. Activation of human peripheral monocytes by angiotensin II. FEBS Lett 1994;347:178-80.

29. Ruiz-Ortega M, Ruperez M, Lorenzo O, et al. Angiotensin II regulates the synthesis of proinflammatory cytokines and chemokines in the kidney. Kidney Int Suppl 2002:12-22.

Table 1 Background characteristics of all patients included in the study.

	Controls	GCA
	n = 10	n = 10
Median age (range) (years)	70 (54-92)	74 (66-88)
Gender (men/women)	6 / 4	4 / 6
Headache	2	7
Jaw claudication	1	3
Polymyalgia rheumatica	1	1
Temporal artery pain	1	9
Abnormal temporal artery on palpation	2	4
Any visual symptom	4	6
Diplopia	2	0
Scalp tenderness	3	6
Weight loss	2	3
Anorexia	1	4
Fever	2	4
Myalgia	3	4
Arthralgia	1	0
ESR < 50 mm/h	4	0
ESR 50-100 mm/h	6	10

ESR > 100 mm/h	0	0
C-reactive protein	29 ± 26	137± 37
Anemia	2	4
Abnormal arterial biopsy	0	10

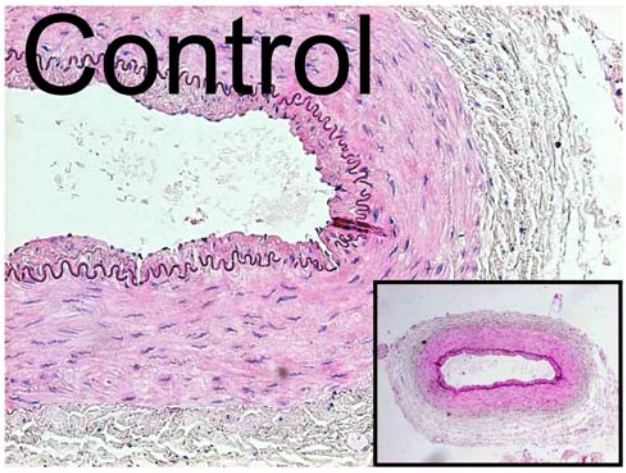
ESR = erythrocyte sedimentation rate, CRP = C-reactive protein, and GCA = giant cell arteritis

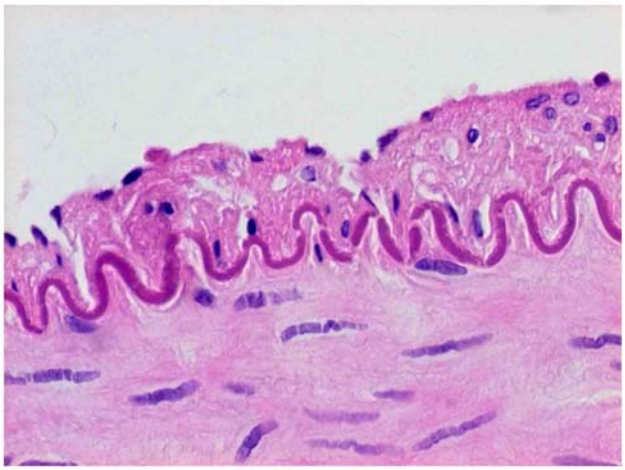
Table 2 Giant cell arteritis (GCA) was defined using The American College of Rheumatology (1990) criteria for the classification of giant cell arteritis.[13] The absence of both lymphocytes and multinucleated giant cell infiltrates in the sample led to the categorization of a sample as negative. The histopathological findings in arteries from control patients and arteries from patients with GCA are listed in this table.

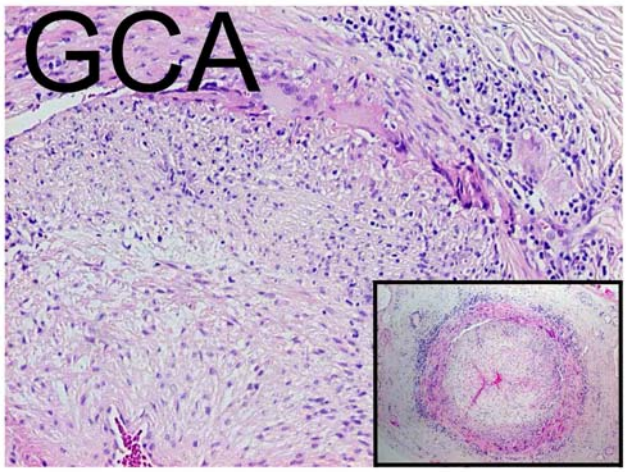
	Control n = 10	GCA n = 10
Presence of multinucleated giant cells		
Yes	0	9
No	10	1
Internal elastic lamina degeneration		
Intact	5	0
Focal rupture	3	2
Rupture < 50% of the vessel circumference	1	8
Rupture > 50% of the vessel circumference	1	0
Intimal hyperplasia		
Absent	1	0
0-25% lumen occlusion	8	1
25-50% lumen occlusion	1	9
> 50% lumen occlusion	0	0

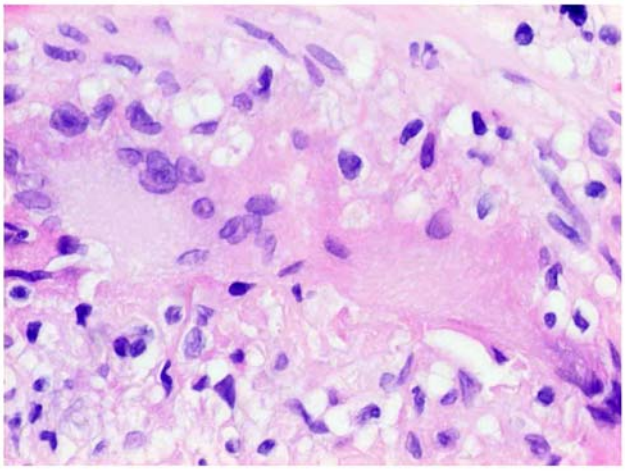
Calcification	0	0
Histocytes	0	10
Multinucleated giant cells	0	9
DAB staining intensity		
No	0	0
Mild	10	0
Moderate	0	4
Intense	0	6

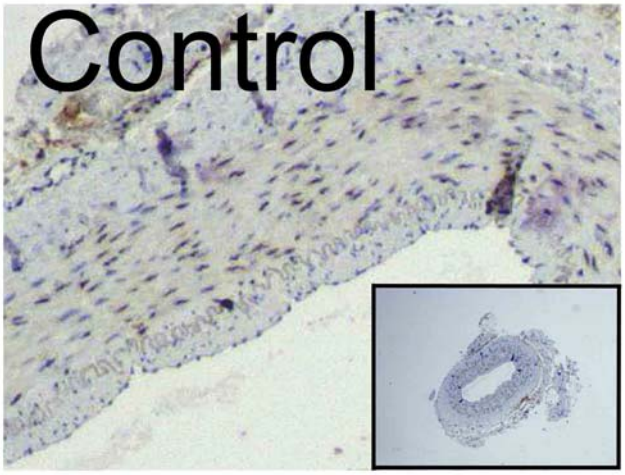
DAB = Diaminobenzidine and GCA = giant cell arteritis.

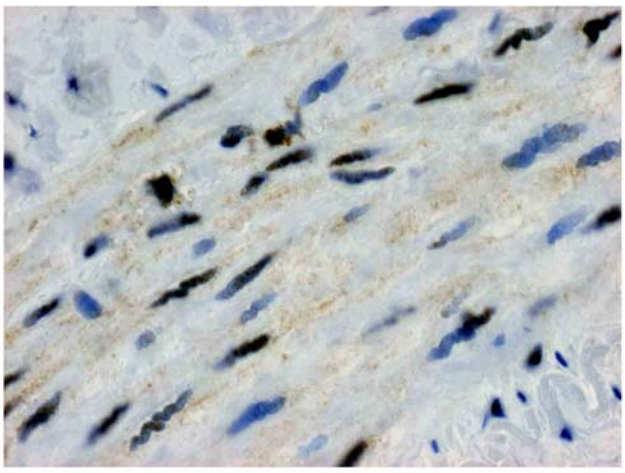

Control

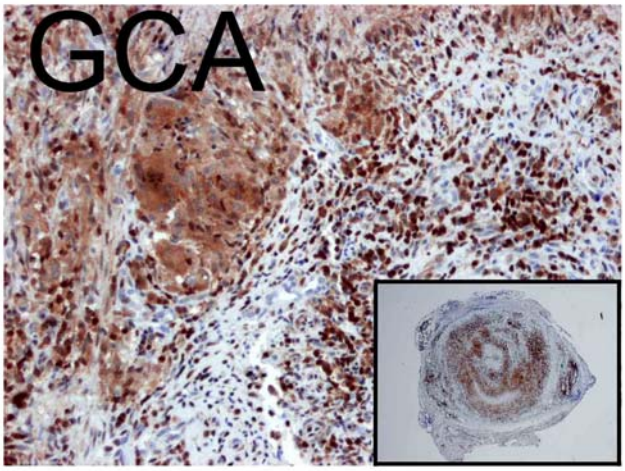


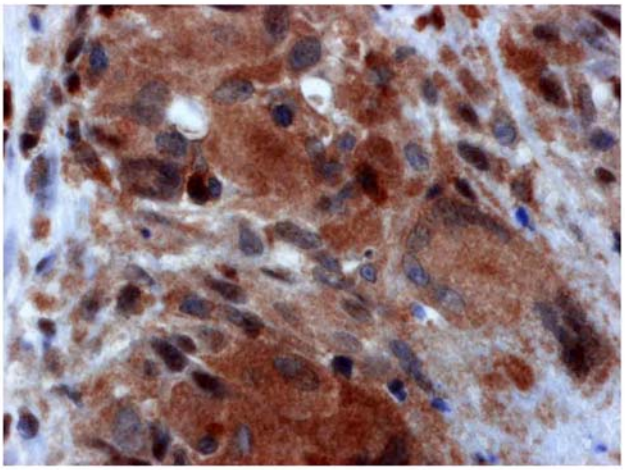

GCA

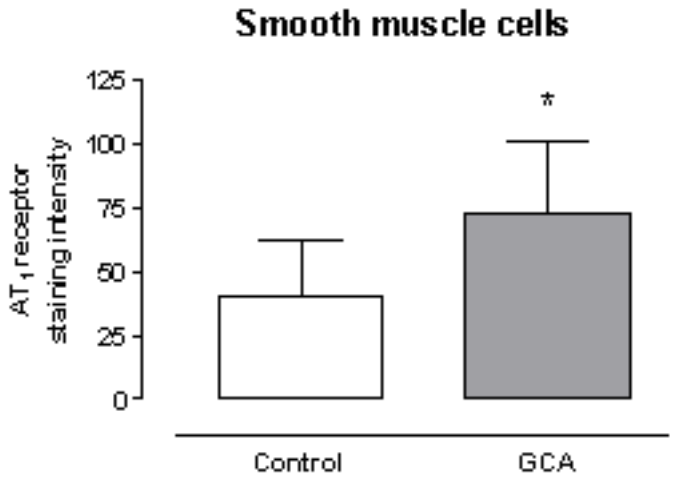


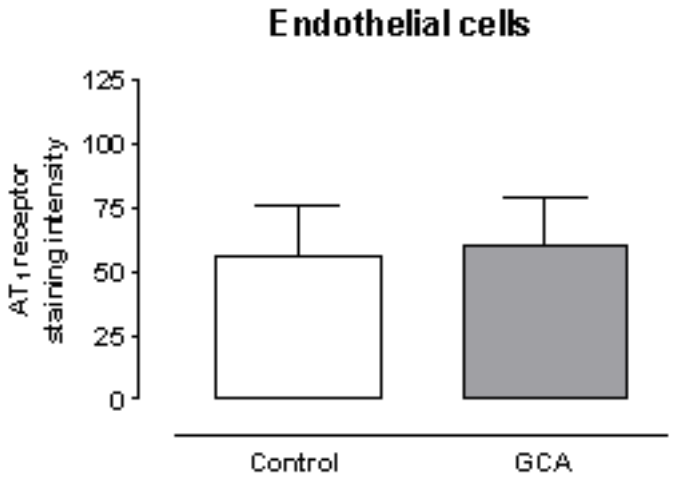

Control

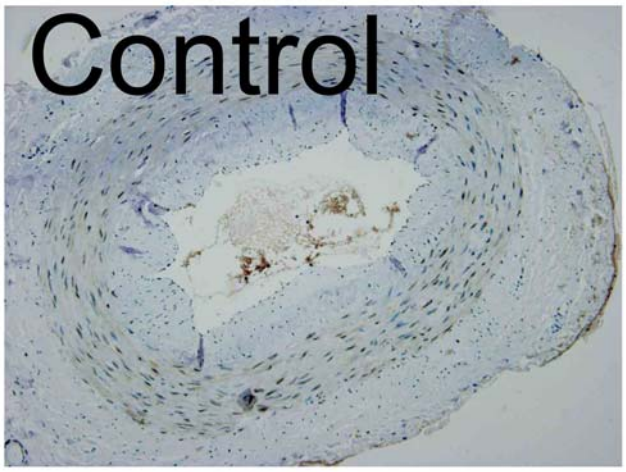


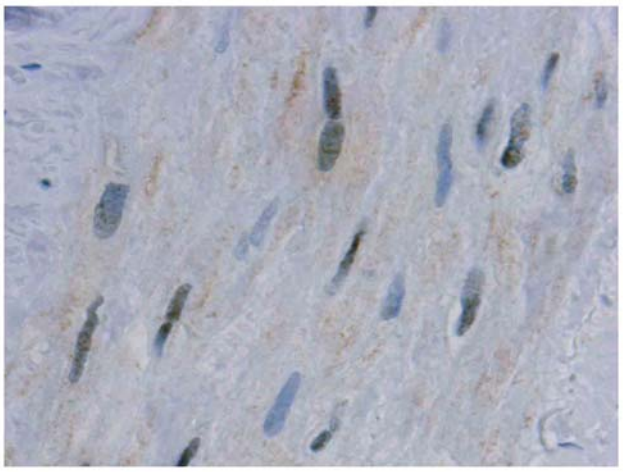

GCA

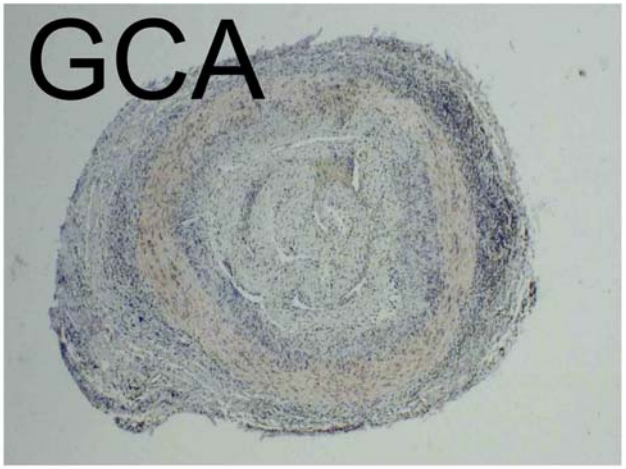


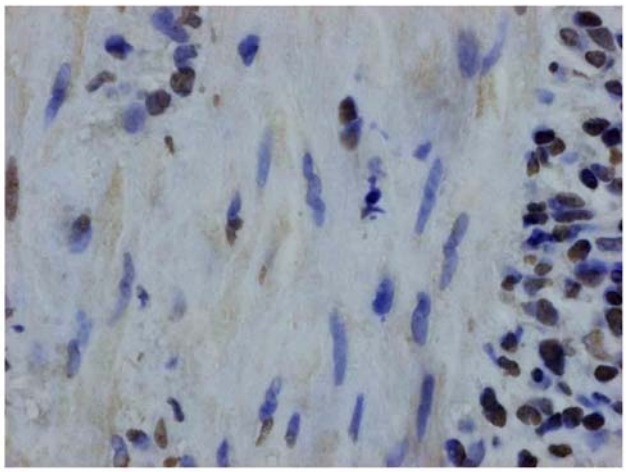

Smooth muscle cells
AT1 receptor staining intensity
125
100
75
50
25
0
*
Control
GCA

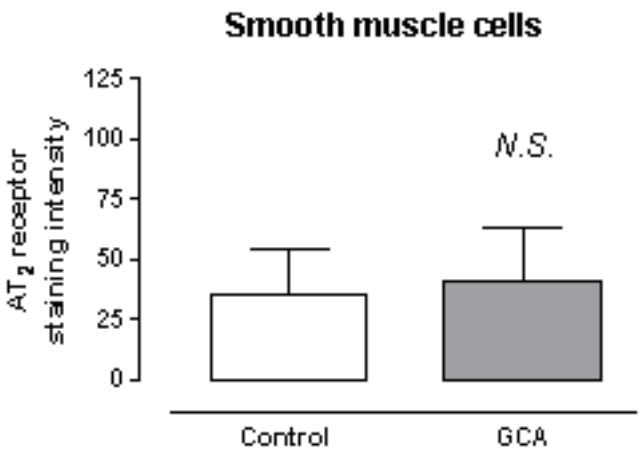

Endothelial cells
AT1 receptor staining intensity
125
100
75
50
25
0
Control
GCA


Control




GCA




Smooth muscle cells
125
100
75
50
25
0
AT2 receptor staining intensity
N.S.
Control
GCA

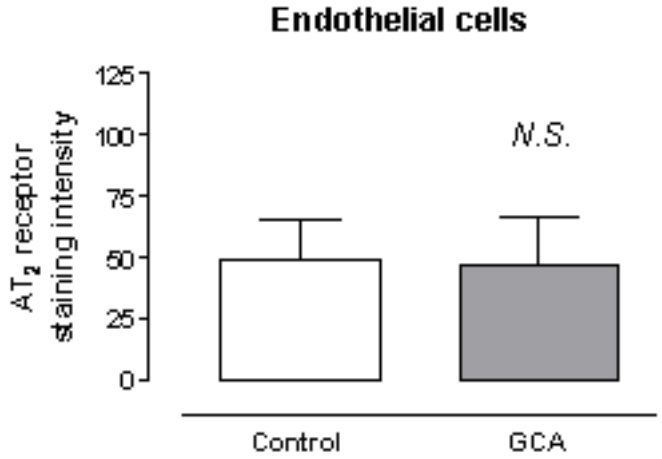

Endothelial cells
125
100
75
50
25
0
AT2 receptor staining intensity
N.S.
Control
GCA

Figure legends

Figure 1: Representative example of hematoxylin-eosin-stained sections of a temporal artery from (A) a control patient and (B) a patient with giant cell arteritis (GCA). Note the intimal hyperplasia with reduction of the lumen, internal elastic lamina degeneration, band-shaped inflammatory infiltrates of immune cells, and the focal necrosis in the artery with GCA. (Original magnification x 50, x 200 and x 400.)

Figure 2: Immunostaining with antibodies for angiotensin II type 1 (AT_1) receptors in temporal arteries from the control patients and the patients with giant cell arteritis (GCA). The top panels show representative examples of immunohistological images and the bottom panels show the AT_1 receptor immunostaining intensities, arbitrary units. The results are shown as mean values ± standard deviation (SD). Statistical analyses were performed using Student's t-test. Significance was defined as $P<0.05$ (*). Note that the AT_1 receptor expression is higher in both the smooth muscle cell layer and in the inflammatory cells in the temporal artery from the patients with GCA.
(Original magnification x 50, x 200 and x 400.)

Figure 3:
Representative examples of immunohistological images showing immunostaining for angiotensin II type 2 (AT_2) receptors in temporal arteries from the control patients and patients with giant cell arteritis (GCA). The bottom panels show the AT_2 receptor immunostaining intensities, arbitrary units. The results are shown as mean values ± standard deviation (SD). Statistical analyses were performed using Student's t-test in which

significance is defined as $p<0.05$. N.S. denotes no significant difference. (Original magnification x 50 and x400.)