IJMS
Vol 34, No 1, March 2009

Original Article

Effect of Angiotensin II on Blood Flow in Acute and Chronically Inflamed Knee Joints of Rabbits: The Role of Nitric Oxide

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Abstract

Background: Angiotensin converting enzyme (ACE) upregulation in stromal cells of joints affected by rheumatoid arthritis may lead to higher tissue angiotensin II that is a vasoconstrictor and mitogen factor. To date, the role of angiotensin II on regulating blood flow in inflamed joints has not been studied.

Methods: Acute and chronic joint inflammation was induced in rabbits by intra-articular injection of carrageenan and antigen-induced arthritis method, respectively. The ACE level of synovial fluid and the response of joint blood flow to angiotensin II, angiotensin II receptor antagonist, and the role of nitric oxide (NO) in modulation of the effects of angiotensin II on joint blood vessels were examined.

Results: The synovial fluid level of ACE was significantly increased during the process of inflammation and angiotensin II increased joint vascular resistance dose-dependently in both acute and chronically inflamed joints. The angiotensin 1 receptor antagonist losartan completely blocked the vasoconstrictor effect of angiotensin II on joint blood vessels and induced vasodilatation. Nitric oxide synthase inhibitor N-omega -nitro L- arginine methyl ester (L-NAME) increased joint vascular resistance and augmented vascular response of inflamed joints to angiotensin II.

Conclusion: Angiotensin II receptors in joint blood vessels are angiotensin -1 subtype, and inflammation significantly increases the activity of synovial fluid ACE. Nitric oxide plays a significant role on regulating joint blood flow and in modulation of angiotensin 1 receptor-mediated vasoconstriction of inflamed joint blood vessels.

Iran J Med Sci 2009; 34(1): 36-45.

Keywords ● Angiotensin II receptors ● regional blood flow ● vascular resistance ● nitric oxide ● angiotensin converting enzyme ● acute and chronic inflammation

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Submitted: 9 July 2008
Revised: 15 November 2008
Accepted: 14 December 2008

Introduction

The regulation of synovial blood flow is of critical importance to the maintenance of a stable intra-articular environment. A high correlation has been found between synovial PO2 and synovial blood flow in both normal and acutely inflamed rabbit knee joints,[1] suggesting the importance

of joint blood flow regulatory mechanisms in supplying oxygen and nutrients to the synovial tissues.[2,3] To date, the effects of some factors such as sympathetic nervous system,[4] neuropeptides,[5] nitric oxide,[6] and prostaglandins,[7] on regulating joint blood flow have been investigated. However, the effect of angiotensin II has not been fully understood. Angiotensin II that is a potent vasoconstrictor in peripheral tissues with proinflammatory effects,[8] has been implicated in vascular intimal hyperplasia following endothelial damage.[9,10] This humeral factor is also formed in many tissues by the activity of local angiotensin converting enzyme (ACE).[11] A higher renin and ACE have activity has also been found in synovial fluid of inflammatory arthritis.[12,13] Therefore, local ACE upregulation in joints affected by rheumatoid arthritis may contribute to the pathogenesis of this disease through a higher tissue concentration of angiotensin II. Inflammation also activates inducible nitric oxide (NO) synthase leading to high production of NO that may exert both inflammatory and vasodilator effects. A recent study using an experimental model of arthritis has shown that AT1 receptor antagonist losartan reduced joint swelling, suggesting interaction between angiotensin II and NO production pathway,[14] but the changes in joint blood flow were not examined. Our previous study on the effect of inflammation on adrenoceptor profile of joint blood vessels has shown that inflammation alters the receptor profile from α2 to α1 and from β1 to β2 subtypes.[15,16] Such alteration may happen in angiotensin II receptors as well.

In our previous work, we studied; the effects of angiotensin II on joint blood vessels, angiotensin II receptor subtypes in these blood vessels, the role of NO on regulating joint blood flow, and the effect of NO on modulation of the effects of angiotensin II on joint blood flow in the normal joint.[17] The present study extended the previous work to acute and chronically inflamed conditions and investigated the alterations that may occur due to the inflammation process on those four variables. The level of ACE activity in synovial fluid was also assessed during the course of inflammation as a mechanistic tool.

Materials and Methods

This study was done in accordance with the national guidelines for conducting animal studies and the Ethic Committee of Kerman University of Medical Sciences approved the protocol. The study was performed on 68 Dutch White rabbits (2 -3 kg). The animals were kept two in one cage in animal room with free access to water and standard rabbit chow at temperature 20-23 °C and 12-hour light/12-hour dark conditions. During the course of animal preparation (sensitization, induction of arthritis), each animal was placed in a separate cage. At the day of experiment, the animals were anaesthetized by sodium thiopental [50 mg/kg intraperitoneally (ip)]. The animals were divided into three groups. In the first group (group 1, n=28), ACE level in synovial fluid was measured during the course of inflammation. In group 2 (rabbits with acute joint inflammation, n=20) and group 3 (rabbits with chronic joint inflammation, n=20) the response of joint blood vessels to angiotensin II, in control conditions and in the presence of losartan and N-omega-nitro L- arginine methyl ester (L-NAME) were studied.

Induction of Inflammation

Acute Inflammation

Acute knee joint inflammation was induced by intra-articular injection of 0.5 ml of a 2% solution of carrageenan 24 hours prior to the experiment.[18] A 28 G needle was inserted through the mid-patellar tendon into the joint cavity. Half of the solution was injected deeper into the posterior space and the other half into the anterior space.[15]

Chronic Inflammation

Chronic inflammation was induced by antigen-induced arthritis method,[19] an experimental model that mimics human rheumatoid arthritis.[20] The rabbits were first sensitized by intradermal injection of 1 ml mixture of methylated bovine serum albumin (MBSA, 4mg/ml) homogenized 1:1 with complete Freund's adjuvant. The detailed method for making this solution has been reported elsewhere.[16] Injection was performed in five points (0.2 ml in each point) on the back of the shaved neck on days -28 (first booster) and -14 (second booster) while the animals were anaesthetized temporarily by a gaseous mixture of 3% halothane in 30% O_2 – 67% N_2O. On day -7, a skin test was performed to assess the immune status of the animal by examining the delayed-type hypersensitivity reaction to subcutaneous injection of 0.2 ml MBSA (0.2 $mg.ml^{-1}$) on the leg. The skin thickness was measured using a caliper (abd Co, China) prior to and 24 hours after delayed-type hypersensitivity test. A minimum of 100% increase in thickness was considered sensitization. Sensitized animals received right knee intra-articular injection of MBSA (0.5 ml of 2 mg/ml solution) at the day 0 as mentioned above for intra-articular injection of carrageenan. The duration for chronic inflammation was the subsequent four weeks of day zero.[16,19]

The results obtained from these two groups were compared with the results of our study on normal joints,[17] performed in the same lab, using the same animal species, age, gender, and same techniques and methods.

Measurement of Knee Joint Diameter

The knee-joint diameter was measured 24 hours after induction of inflammation in group 2,[15] and regularly measured during the next 28 days post intra-articular antigen injection in group 3.[16,19] For this purpose, the medio-lateral diameter of the joint was measured by the caliper.

Detection of Synovial Fluid ACE Activity

Animals of the first group were divided to 5 subgroups of days 0 (before) and 7, 14, 21, and 28 after intra-articular injection of MBSA. At the day of experiment, the animals were euthanized under deep anesthesia induced by sodium thiopental (60 $mg.kg^{-1}$ ip) followed by intracardiac injection of saturated KCl. The joint space was opened and synovial fluid was lavaged from right knee using 1 ml of heparinized buffered saline. The samples were centrifuged and the supernatants were stored at -70 °C prior to the assay. ACE activity was determined by high performance liquid chromatography (HPLC; Waters, UV visible, model 486).[13]

Animal Preparation for Joint Blood Flow Studies

At the day of experiment, rabbits of groups 2 and 3 were anaesthetized by sodium thiopental (50 $mg.kg^{-1}$ ip). During surgery supplement anesthesia was added by a mixture of 1% halothane in 30% O_2 and 69% N_2O delivered through tracheal cannula. Deep anesthesia was maintained throughout the surgery and judged by the absence of withdrawal response to a pinch stimulus applied onto the hind limb. Arterial blood pressure was monitored through a heparinized saline (8 units/ml) filled cannula inserted into the carotid artery and connected to a pressure transducer and physiograph (Beckman R611, USA). The jugular vein was cannulated for injection of L-NAME. The medio-lateral area of right leg was shaved and a 2-cm incision was made longitudinally in the skin to get access to the saphenous artery. The artery was cannulated retrograde and the cannula advanced until the tip reached close to the joint area. The test drugs (angiotensin II and losartan) were injected in the entrance of joint blood vessels through this cannula. The skin over the anterior part of the joint capsule was removed along with the underlying fascia and then covered with a cling film to prevent the tissue from drying. Occasionally 0.1 ml physiological saline at 37 °C was added under the cling film to maintain the tissue wet. A fiberoptic probe connected to a laser Doppler flow meter (laser beam 580 nm, maximum intensity 3 mW; model MBF3D, Moor Instruments, Axminster, UK) was positioned just above the medial portion of the anterior joint capsule to indicate joint blood flow continuously. Previous studies have validated the use of this technique for assessment of relative changes in blood flow in the brain and joint.[21,22] This is a special technique for assessment of alterations in microcirculatory flow in tissues.

Experimental Protocol

After all surgical procedures, the animals were allowed to rest at least one hour to minimize the surgical stress. The gaseous anesthesia was discontinued and a maintenance dose of sodium thiopental (10 $mg.kg^{-1}.hr^{-1}$) was injected through the jugular vein. Then the animals of each group were divided into three subgroups:

In the first subgroup (angiotensin II dose-response subgroup, n = 7), 0.1 ml of saline or the same volume of different concentrations of angiotensin II (10^{-14} to 10^{-5} M) was injected through the saphenous artery cannula to produce an angiotensin II dose-response curve. To prevent tachyphylaxis to the consecutive doses, 10 and 30 minutes were allowed to lapse between the low and high angiotensin II concentrations respectively. We have observed that 10 minutes should lapse between two consecutive doses of 10^{-7} M or lower of angiotensin II to produce equal vasoconstrictor responses to the same repeated dose (unpublished observations). For doses of 10^{-6} M and more, a 30 minutes interval is needed. The temperature of drug solutions was 37 °C and the cannula was washed by saline after each injection.

In the second subgroup (losartan subgroup, n=6), only one concentration of angiotensin II (10^{-6} M) was injected. From the results of the first subgroup, it was concluded that 10^{-6} M was the optimum concentration to produce nearly maximum vasoconstriction with no systemic effects (minimal change in blood pressure) (see dose- response curves for % change in flux and % change in blood pressure in result section).Thirty minutes later, this dose was repeated following injection of 0.3 ml losartan (1 mM).[17] This time interval was needed to prevent tachyphylaxy.[17] Losartan was used in higher concentration compared with the concentrations normally used in invitro experiments because the drug would dilute in arterial blood before reaching joint blood vessels.

In the third subgroup of animals (L-NAME subgroup, n = 7), following a control injection of 10^{-6} M angiotensin II, L-NAME was injected through the jugular vein (3 $mg.Kg^{-1}$). Forty-five

minutes later -as it was proved by previous studies-[17,23] when joint blood flow and blood pressure were stabilized at new levels, the experiment in the second group was repeated. In a previous study, this dose of L-NAME and the time were found sufficient for complete NO synthase inhibition in joint blood vessels.[6]

Joint blood flow values were recorded immediately before and after each injection when the blood flow values underwent maximum change. Mean arterial pressure (MAP) values were calculated from blood pressure trace on the physiograph paper by adding one third of pulse pressure to the diastolic pressure. Joint vascular resistance (JVR) was calculated by dividing MAP by joint blood flow based on the Ohm's equivalent formula (R = pressure / flow). This variable is preferred on joint blood flow variable because it eliminates the effect of blood pressure on blood flow. Alterations in blood flow, blood pressure, and vascular resistance were expressed as percentage change from control values occurring immediately before each test procedure. The biological zero values were measured as described previously,[1] and subtracted from the flow values before calculation of percentage changes in blood flow.

Termination of each experiment was achieved by intrajugular vein injection of KCl (1M).

Statistical Analysis

The values expressed in the figures and texts are Mean ± SEM. Repeated measure ANOVA was used for comparison between dose-response curves to angiotensin II. In cases such as responses to a dose of angiotensin II in different stages of experiment, or comparing a variable between subgroups, appropriately one or two-way ANOVA followed by Tukey's test as post hoc were performed. P values less than 0.05 were considered significant.

Drugs used were: L-NAME and angiotensin II (Sigma, England), Sodium thiopental (Darupakhsh, Iran), heparin (Leo Pharma, Ballerup, Denmark), halothane (ICI Pharmaceutical, Ennore, India) and losartan (Cipla, Mumbai, India).

Results

Effect of Inflammation on Joint Diameter

Joint diameter was increased because of inflammation. In the acutely inflamed joints it was increased by 14.7 ± 0.9 percent during 24 hours. In chronically inflamed joints the maximum increase was 22 ± 1.2 percent found at the day 2 and then gradually decreased towards the normal level during the course of study (day 28).

ACE Activity of Synovial Fluid

The ACE activity of synovial fluid significantly increased during the first week of inflammation (acute phase) and then returned gradually towards normal level up to the end of week four (chronic phase) (figure 1). The ACE activity of synovial fluid remained significantly higher than normal during three weeks post inflammation.

Effect of Angiotensin II on Joint Blood Flow and Joint Vascular Resistance

Figure 2 is a representative trace recorded from one of the animals in the acutely inflamed (A, B, D & E) and chronically inflamed groups (C).

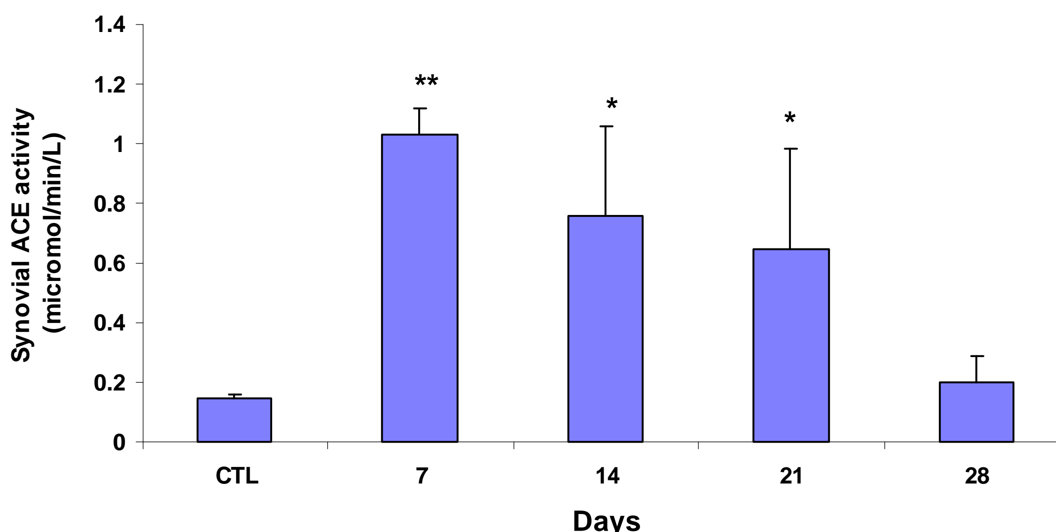

Figure 1: Alterations in the ACE activity of synovial fluid during the course of experiment. Each column belongs to a separate group of animals. * P<0.05 and ** P<0.01 compared with the control (non-inflamed) joints. (n = 5-6 in each group).

Joint blood flow was sharply reduced in response to close intra-arterial (intra-saphenous artery) injection of angiotensin II 10^{-6} M (figure 2A & C). This response was completely inhibited by pre treatment with losartan (figure 2B). Angiotensin II increased arterial blood pressure with some delay when it reached systemic circulation through the joint veins and this response was also significantly reduced by pre treatment with losartan (figure 2B). Basal joint blood flow significantly reduced by L-NAME (figure 2D) and pretreated vessels with L-NAME showed an augmented response to angiotensin II (compare figure 2E with figure 2A).

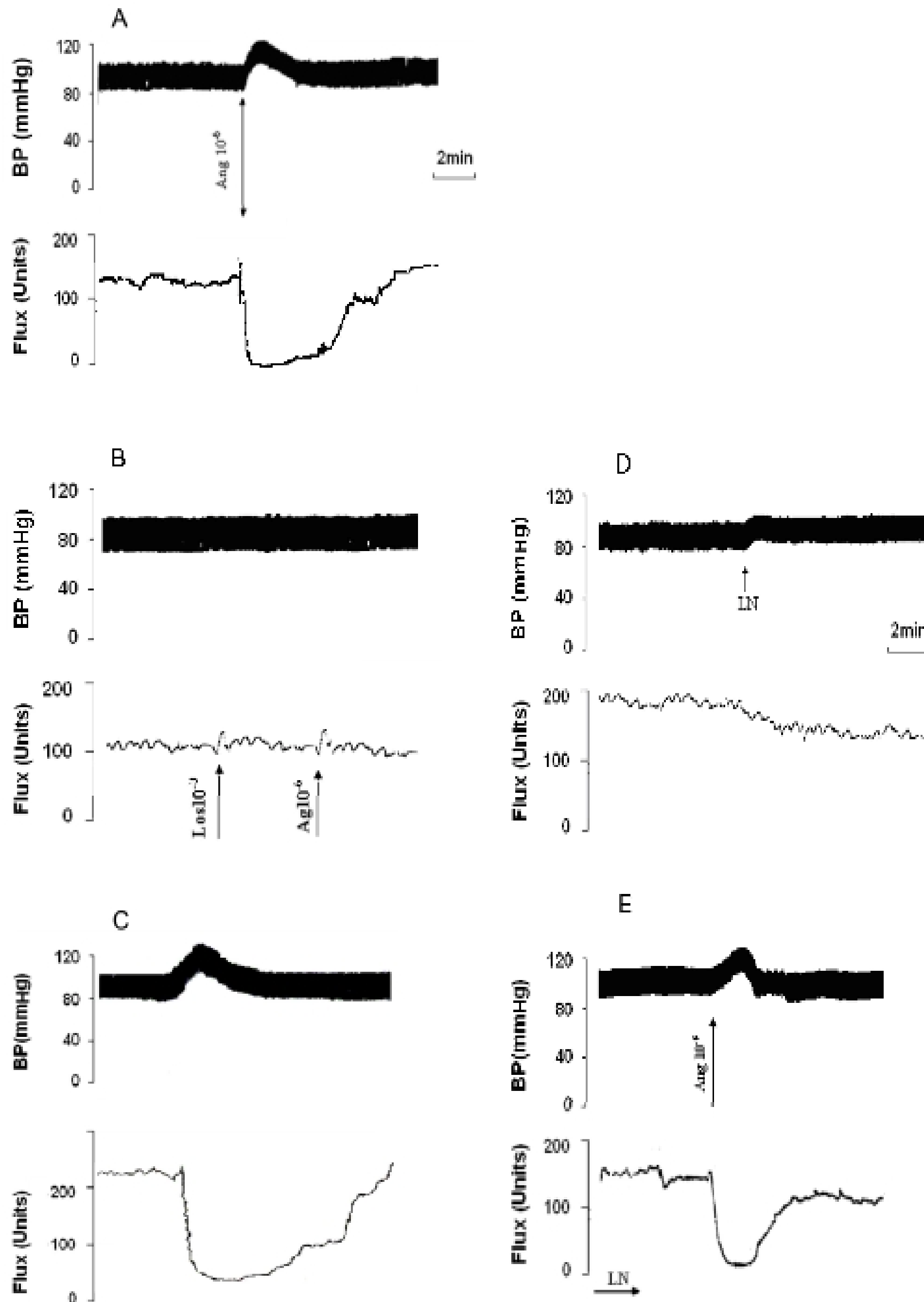

Figure 2: Knee joint blood flow (Flux) and responses of systemic arterial pressure (BP) to local intra-arterial injection of 0.1 ml of angiotensin II [10^{-6} M] in control (pre-LNAME or pre-losartan) conditions (A & C), after local intra-arterial injection of 0.3 ml of AT1 receptor antagonist losartan [1mM] (B), to intra-venous administration of L-NAME [3 mg.Kg^{-1}] (D) and 45 minutes following L-NAME administration (E). Joint blood vessels constricted and systemic blood pressure increased by angiotensin II. Losartan almost completely inhibited both effects. L-NAME reduced basal joint blood flow and enhanced joint blood vessel response to angiotensin II. Traces A, B, D and E belong to animals from acutely inflamed group, and trace C belongs to an animal in the chronically inflamed group. Ang = angiotensin II, Los = losartan, LN = L-NAME. The response of normal animals,[17] mirrored the response of chronic ones but with higher vasoconstriction due to angiotensin II.

Overall the effect of angiotensin II concentrations (10^{-14} to 10^{-5} M equal to 10^{-18} to 10^{-9} M doses respectively) on joint blood flow, MAP and joint vascular resistance in the acute and chronically inflamed groups (n = 7 in each group) is shown in figure 3. Figure 3A shows that angiotensin II, dose-dependently reduced joint blood flow with a 58.4 ± 8.4% decrease at 10^{-6} M dose in the acute and with a 63 ± 16 % decrease in chronically inflamed joints from the basal values of 169.3 ± 8.4 and 152.3 ± 10.4 arbitrary flux units respectively. The minimum dose to produce significant vasoconstriction was 10^{-11} M. No vasodilatation was found even with the dose of 10^{-14} M in either group.

Figure 3B indicates changes in MAP due to the above intervention. Systemic blood pressure did not show any change until the dose of 10^{-7} M. From that, a dose dependent increase was found in blood pressure with an increase of 31.5 ± 4.2% in dose of 10^{-5} M in the acute and 20.5 ±10.6% in chronic group. The basal MAP value was 88.9 ± 1.8 mmHg in the acutely inflamed and 86.9 ± 2.8 mmHg in the chronically inflamed group.

The maximum increase in joint vascular resistance was also observed at dose of 10^{-5} M (Figure 3C). This was 1251 ± 679% and 1424 ± 956% increase in acute and chronic joints, respectively. None of the doses administered showed vasodilator effect (decrease in joint vascular resistance).

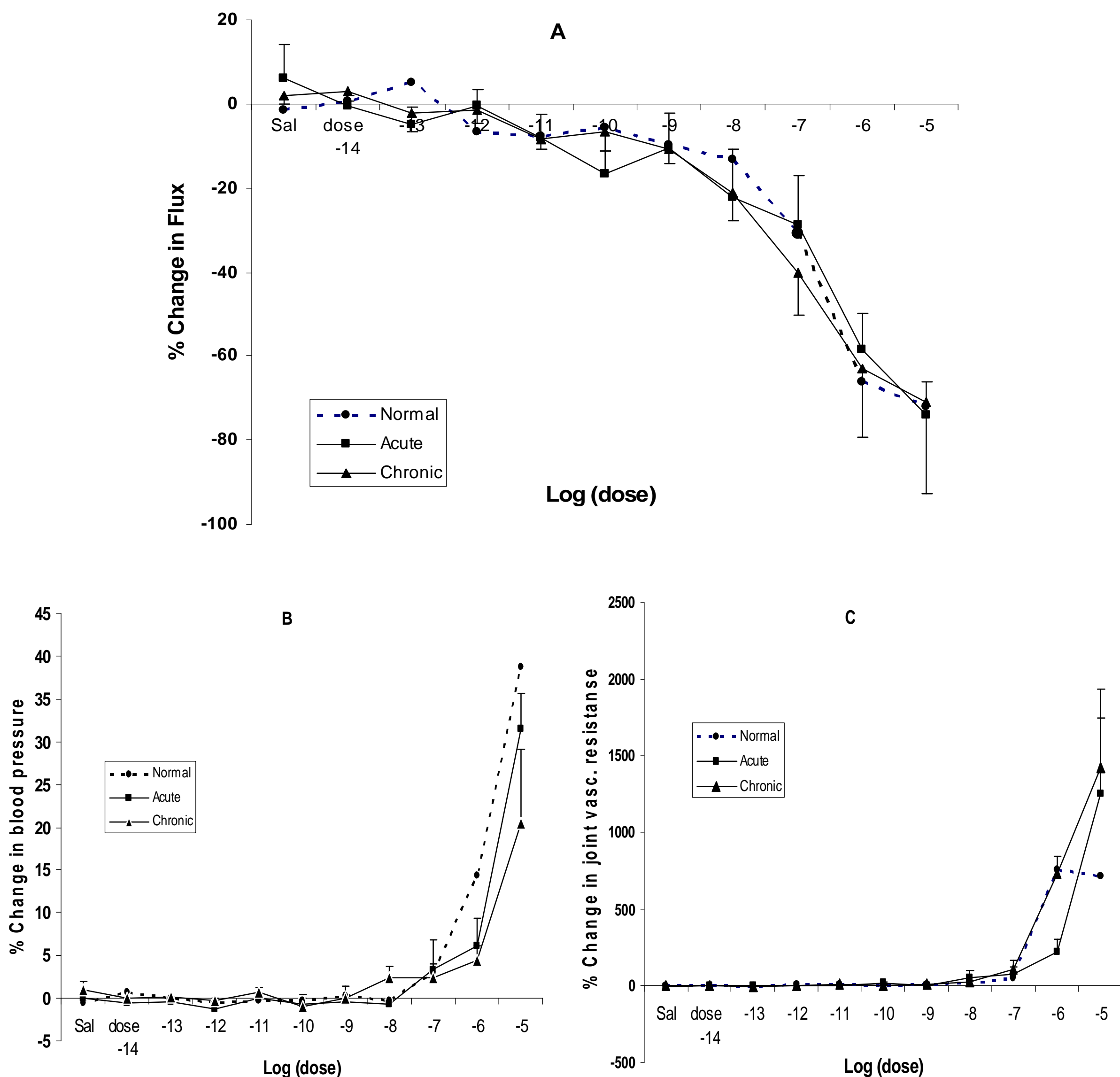

Figure 3: Alterations in joint blood flow (Flux) (**A**); Mean arterial blood pressure (**B**); and joint vascular resistance (**C**), due to local intra-arterial (close to joint) injection of 0.1ml volumes of different concentrations of angiotensin II in acutely inflamed and chronically inflamed joints. Joint blood vessels constricted by angiotensin II dose-dependently. Systemic blood pressure changed only with doses of 10^{-7} M and higher. The data for normal group belong to our previous study,[17] incorporated for comparison (n = 7 in each group).

In the second subgroups of animals, the effect of intra-saphenous administration of specific AT1 receptor antagonist losartan was investigated on responses of joint blood vessel to angiotensin II (10^{-6} M). Figure 4 shows that losartan per se had no effect on joint vascular resistance but completely blocked the effect of angiotensin II on joint vascular resistance in all subgroups (P< 0.01). No vasodilatation observed with angiotensin II administered after losartan.

Joint Blood Vessel Response to Angiotensin II in the Presence of L-NAME and Losartan

In the third group of animals, the effect of angiotensin II on joint vascular resistance was examined before and after intravenous administration of L-NAME and then after losartan (figure 5). L-NAME per se increased basal joint vascular resistance by 25.8 ± 8.7 percent in normal, 81.9 ± 12.9 percent in the acute, and 41.8 ± 8.7 percent in chronically inflamed joints (all were significantly different with the effect of saline) with significantly higher effect in the acutely inflamed joint compared with the other two subgroups. MAP was increased by 15 ± 3.1 percent in normal, 13 ± 1.7 percent in acute inflamed and 17.2 ± 2.6 percent in chronically inflamed subgroups after L-NAME administration (data not shown). In the presence of L-NAME, contrary to the normal joint, in both acute and chronically inflamed joints, the blood vessel response to angiotensin II was significantly increased (figure 5). Losartan, in the presence of L-NAME, completely blocked the joint blood vessel responses to angiotensin II in all subgroups.

Discussion

The results of the present study showed a dose-dependent reduction in joint blood flow in response to angiotensin II administration (figure 3A) which indicates that angiotensin II receptors are present in both inflamed joints blood vessels. Since this response was completely inhibited by losartan (figure 2B), it is concluded that this response is AT1-receptor mediated. As the systemic blood pressure was also changed by angiotensin II, it may be speculated that changes in blood flow are due to changes in blood pressure because one of the determinants of blood flow to a tissue is blood pressure. However this is not the case as the changes in blood flow are in the opposite direction of changes in blood pressure and the time course of changes in latter is much shorter than the time course of changes in the former (figure 2). On the other hand, when vascular resistance data in which the effect of blood pressure on blood flow is eliminated was presented (figures 4 and 5), the same results were concluded. It has also been previously shown that AT_1 receptors subtypes mediate vasoconstrictor effects of angiotensin II.[11] This finding is consistent with the results of Walsh and colleagues in human,[24] who utilized auto

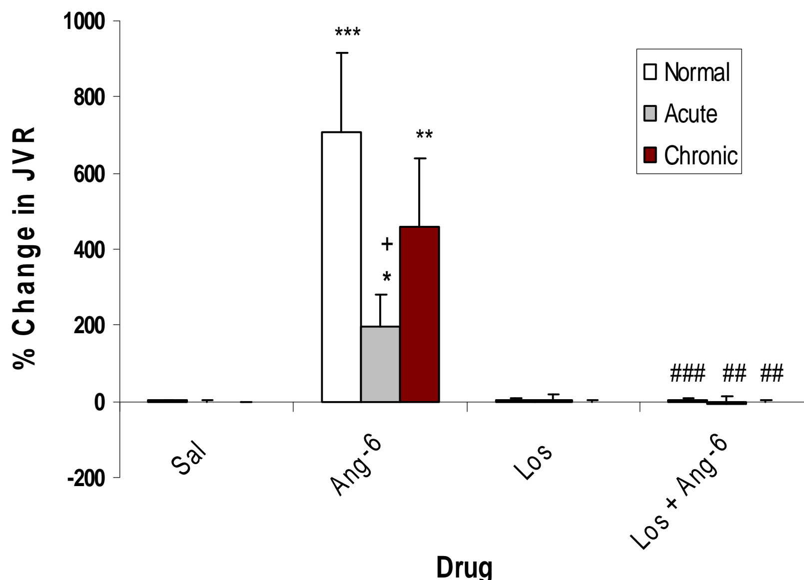

Figure 4: Alterations in vascular resistance of normal, acute and chronically inflamed joints due to local intra-arterial injection of 10^{-6} M angiotensin II before and after selective AT1 receptor antagonist losartan (1mM). Losartan completely blocked the vasoconstrictive effect of angiotensin II on joint blood vessels. * = P<0.05, ** = P<0.01 *** = P<0.001 compared with saline; += P<0.05 compared with normal joint; ## = P<0.01, ### = P<0.001 compared with angiotensin II [10^{-6} M] (n = 6-7 in each group). The data for normal group belongs to our previous study,[17] incorporated for comparison.

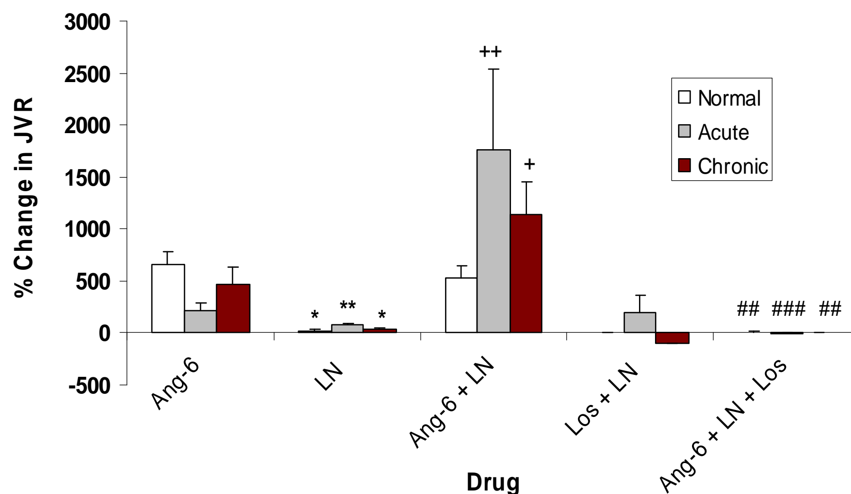

Figure 5: Alterations in vascular resistance of normal, acute and chronically inflamed joints after administration of NO synthase inhibitor L-NAME (3mg/kg) and after local intra-arterial injection of angiotensin II [10^{-6}M] before (Ang-6) and 45 minutes after L-NAME (Ang-6+LN), and after subsequent losartan 1mM (Ang-6+LN+Los). L-NAME increased basal joint vascular resistance (JVR) in all groups and enhanced the vessel response to angiotensin II significantly in inflamed groups (compare Ang-6+LN with Ang-6). Losartan completely blocked the vasoconstrictor effect of angiotensin II on joint blood vessels in the presence of L-NAME. * = P<0.05, ** = P<0.01 compared with saline (the data for saline are presented in figure 4); += P<0.05, ++ = P<0.01 compared with Ang-6, ## = P<0.01, ### = P<0.001 compared with angiotensin II [10^{-6} M] + LN; (n = 6-7 in each group. The data for normal subgroup belong to our previous study,[17] incorporated for comparison.

radiography technique in vitro and showed the presence of AT1 receptors in chronically inflamed knee joints. Similar dose response curves for blood vessels of normal, acute and chronically inflamed joints to angiotensin II (figure 3) may imply that angiotensin II receptor density did not change due to the process of inflammation. However this is not the case because inhibition of NO production by L-NAME (figure 5) significantly increased the response of blood vessels to angiotensin II in inflamed joints and this increase was bigger in acutely inflamed conditions in which NO production is higher. In fact, the response of acutely inflamed joint blood vessels was three times and of chronically inflamed blood vessels was twice as normal joints in the presence of L-NAME (figure 5). This means that NO had masked the higher response to angiotensin II anticipated in inflamed conditions and that is why the response of normal joint blood vessels to angiotensin II was shown stronger than the inflamed joints in the presence of NO (absence of L-NAME) (figure 4). In fact, the dose response curves in figure 3A would not be overlaid if NO production was inhibited before angiotensin II administration (as it happened in L-NAME group; figure 5). Price et al. showed that losartan was able to reduce joint swelling,[14] implying the involvement of angiotensin II in the process of joint inflammation. This effect of angiotensin II seems strange as this is a vasoconstrictor factor and it is anticipated that losartan increases joint swelling. In fact, this could happen considering that AT1 receptors are present on both endothelium and smooth muscle of blood vessels, the former group leading to vasodilatation on stimulation through NO production,[25] and the latter group leading to vasoconstriction by direct smooth muscle stimulation.[11] Normally, the effect of second group is predominated but during the process of inflammation (especially in acute form) the huge amount of NO production by inducible NO synthase weakens the presentation of direct effect. Consistent with this, it has recently been reported that angiotensin II also increase the release of another group of vasodilator and inflammatory mediators prostanoids.[26] Therefore, NO synthesis inhibition increased the vasoconstrictor effect of angiotensin II in inflamed joint groups (figure 5). As angiotensin II is a pro-inflammatory factor participated in inflammation cascade through its AT1 receptors,[27] blockade of these receptors have beneficial effect on inflammation.[14] The high level of synovial ACE activity during three weeks post inflammation especially in the first week (acute phase) (figure 1) could also intensify the local production of angiotensin II and its pro-inflammatory,[8] and NO producing,[25] effect. Similar increase in ACE activity was also reported in endothelium and synovial fluid of patients suffering from rheumatoid arthritis [12,13,28].

We did not measure the level of ACE prior to day 7, however, the work of Veale and colleagues has shown that the level of this enzyme is reached to maximum at the day seven in supernatants of cultured synovial membrane from patients with rheumatoid arthritis (17 ± 5 units in day 1 *v* 25 ± 3 units at day 7).[29]

Some investigations have shown the presence of angiotensin type 2 (AT2) receptors in vascular endothelium,[30,31] whose stimulation led to vasodilatation. No vasodilatation was found in this study in response to even low doses of angiotensin II (figure 3) or after AT1 receptor blockade (figures 2B and 4) implying the lack of AT2 receptor subtypes in inflamed joint blood vessels. The lack of AT2 subtypes was also found in normal joint blood vessels.[17]

L-NAME significantly increased joint vascular resistance (figure 5), indicating that along with its inflammatory actions, NO maintains the joint blood vessels dilation and plays a significant role in joint blood flow regulation in both acute and chronically inflamed conditions as it does in normal joint.[17] This role is more important in acutely inflamed compared with chronically inflamed or normal joints (figure 5) in where intense production of NO is present due to induction and up regulation of inducible NO synthase by the process of inflammation. This role of NO is reduced towards the chronic phase of joint inflammation.

Conclusion

Taken together, the results of the present study show that angiotensin II receptor profile in blood vessels of rabbit knee joints, which showed to be AT1 subtype, did not change during a period of four-week inflammation. NO plays a significant role in regulation of vascular tone especially in acute and chronically inflamed joints and counterbalance the AT1 receptor-mediated vasoconstrictor effect of angiotensin II in these conditions. This role is more prominent in acutely inflamed joints in which higher ACE activity is present.

Acknowledgement

Financial support of the present project by Kerman University of Medical Sciences and Health Services of Iran is acknowledged. Authors are grateful to Mrs. F. Esmaieli for her assistance in the laboratory. The results presented in this manuscript are parts of the data of one M.Sc. thesis in Physiology (Acute inflammation data) and one Ph.D. thesis in Physiology (chronic inflammation data) performed in Department of Physiology and Physiology Research Center of Kerman University of Medical Sciences.

Conflict of Interest: None declared

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