doi:10.4149/endo_2014_01_9

Limbs' postischemic revascularization is not improved by losartan treatment in diabetic rats

[1]Fallahzadeh A, [2]Khazaei M

[1]Department of Physiology, Yasuj University of Medical Sciences, Yasuj, Iran; [2]Department of Physiology, Isfahan University of Medical Sciences, Isfahan, Iran
E-mail: khazaei@med.mui.ac.ir

Objective. Most physiological actions of angiotensin II (Ang II) on cardiovascular system are mediated by angiotensin type 1 receptor (AT1R). Since peripheral artery disease is one of the most important complications of diabetes, in this study, we aimed to investigate the effect of losartan, an AT1R blocker, on skeletal muscle angiogenesis in diabetic hind limb ischemic rats.

Methods. Twenty four male Wistar rats were randomly divided into four groups as follow: diabetic sham; diabetic sham + losartan (15 mg/kg/day); diabetic hindlimb ischemia; diabetic hindlimb ischemia + losartan. For induction of diabetes, streptozotocin was injected (55 mg/kg; i.p.). The animals were sacrificed after 21 days and the serum concentrations of vascular endothelial growth factor (VEGF), soluble VEGF receptor-1 (sFlt-1), nitric oxide (NO), capillary density, and capillary to fiber (cap/fib) ratio in ischemic legs were evaluated.

Results. The serum NO concentrations were significantly decreased, sFlt-1 concentrations increased, and VEGF concentrations did not significantly change after experiment in diabetic sham and diabetic hind limb ischemic rats. Administration of losartan did not induce significant changes in serum NO, sFlt-1, and VEGF concentrations ($p>0.05$). Capillary density and cap/fib ratio in ischemic leg of diabetic rats were not affected by losartan treatment ($p>0.05$).

Conclusion. AT1R blocker, losartan, was not able to restore neovascularization in the ischemic leg of diabetic animals. Therefore, based on the present data, the losartan cannot be considered for treatment or prevention of peripheral artery disease in diabetic subjects.

Keywords: diabetes, angiotensin, angiogenesis

Angiogenesis and collateralization of blood vessels are insufficient in diabetic patients and this may cause a peripheral organ ischemia (Silvestre and Levy 2006). Ang II plays a critical role in the function of blood vessels (Watanabe et al. 2005). It is a factor effective during angiogenesis as it has been recently reported in many studies (Walther 2003; Ribatti 2007). Angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) exert most of their useful effects through blocking Ang II effects (Watanabe et al. 2005). Most physiological actions of Ang II are mediated by AT1R (Watanabe et al. 2005; Munk et al. 2007). Angiotensin type 2 receptor (AT2R) is highly expressed during embryonic development but its expression is very low during growth and maturity (Munk et al. 2007).

Angiogenesis means sprouting of new blood vessels from the preexisting ones (Carmeliet 2003). Angiogenesis is also involved in some physiological and pathological conditions such as wound healing or tumor growth

Corresponding author: Majid Khazaei, M.D., Ph.D., Associate Professor of Physiology, Department of Physiology, Isfahan University of Medical Sciences, Isfahan, Iran; phone: 0098-3117922407; fax: 0098-3116688597; e-mail: khazaei@med.mui.ac.ir.

(Ribatti 2007). Diabetes is accompanied by chronic complications in which enhanced or reduced angiogenesis is involved in their pathogenesis (Simons 2005; Silvestre and Levy 2006). One of the most important complications of diabetes is peripheral artery disease (Silvestre and Levy 2006; Brem and Tomic-Canic 2007). The angiogenic effect of Ang II in different experimental models of hypoxia/ischemia is mostly mediated by AT1R (Emanueli 2002; Rakusan 2007). Some studies revealed that AT1R stimulation induces angiogenesis (Tamarat et al. 2002; Rakusan 2007), while others indicated reduced angiogenesis after AT1R agonists administration (Tamarat et al. 2002; Ribatti 2007). Nevertheless, only a few studies have indicated a role of AT1R in angiogenesis in diabetes and tissue ischemia during diabetes. Since the peripheral artery disease is a common complication in diabetic subjects, the purpose of this study was to investigate the effect of AT1R blocker, losartan, on the angiogenesis in ischemic legs of diabetic rats.

Materials and Methods

Animals and induction of diabetes. In this study, 24 male Wistar rats (10-12 weeks old) weighing 230±30 g, were examined. The animals were housed in an air conditioned room with 12h light/dark cycle and temperature between 20-25°C. The animals were fed with a standard pellet diet with free access to regular drinking water. All procedures and protocols used were approved by the Ethics committee of Isfahan University of Medical Sciences in accordance with the guiding principles for the care and use of laboratory animals. For induction of diabetes, single dose of streptozotocin (STZ, Sigma Co, 55 mg/kg) dissolved in a cold saline was injected intraperitoneally (Szkudelski 2001; Baluchnejadmojarad et al. 2004). After 48 h, the blood glucose level was measured. The animals with blood glucose level higher than 300 mg/dl were considered as diabetic (Baluchnejadmojarad et al. 2004).

Rat ischemic hindlimb model. The rats were anesthetized by intraperitoneal injection of 75 mg/kg ketamine and 7.5 mg/kg xylazine. The medial side of the left thigh was shaved and a small incision made. The left femoral artery and all its major branches were ligated with 3-0 surgical silk, as previously described (Fallahzadeh et al. 2011). Sutures were used to close the incisions. Sham-operated animals underwent the same surgical procedure without femoral artery ligation. The animals were observed during 48 h to check out that no sign of tissue necrosis occurred (Tamarat et al. 2002).

Experimental design. The diabetic rats were randomly divided into four groups (n=6 each). The experimental groups were as follow: group 1 – sham; group 2 – sham + losartan; group 3 – diabetic hindlimb ischemia and group 4 – diabetic hindlimb ischemia + losartan. Groups 2 and 4 received AT1R blocker, losartan, at the dosage of 15 mg/kg/day intraperitoneally for 21 days (Moosavi and Johns 1999). Groups 1 and 3 received an equal volume of drug solvent (saline). Before and after the experiment, blood samples were taken. After centrifugation at 3000xg for 20min, the aqueous phases were collected and extracted. Then, they were stored at -70˚C for serum VEGF, sFlt-1 and NO measurements. At the end of the experiment, the rats were sacrificed. The gastrocnemius muscles were removed from ischemic legs and fixed in formalin solution for immunohistochemical analysis.

Serum NO, VEGF, and sFlt-1 measurements. Serum NO concentration was determined by assessing serum nitrite (the main metabolite of NO) concentration (Nematbakhsh 2008) by griess reagent method (Promega, Madison, USA). The limit detection of the kit is 2.5 μM. Serum VEGF and sFlt-1 concentrations were measured using the quantitative sandwich enzyme immunoassay technique with the appropriate kits (R&D Systems, Minneapolis, USA). The minimum sensitivity of VEGF and sFlt-1 assays is 3.9 pg/ml and 3.8 pg/ml, respectively. The intra- and inter-assay coefficient of variation is less than 10% and 5%, respectively.

Angiogenesis analysis. Tissue blocks were prepared from formalin-fixed tissues. Then, tissue sections (5 μm) were made from each tissue sample. Endothelial cells were identified by immunohistochemical staining. Endothelial cells stained by anti-CD31 antibody were counted under light microscopy. Fifteen random microscopic fields (×400) from three different sections in each tissue were examined by two blinded observers and counted for the presence of capillary endothelial cells (CD31 positive cells). The capillary density was expressed as the number of capillaries per mm (Sasaki 2002; Watanabe et al. 2005). For higher accuracy, the number of muscular fibers (number per mm^2) was also counted and the cap/fib ratio was also calculated (Nematollahi et al. 2009).

Statistical analysis. The results are reported as the mean ± SE. Paired *t*-test was used to analyze paired data. One-way ANOVA using Tukey's post hoc test was used to compare data between groups. $p<0.05$ was considered statistically significant.

Table 1

Body weight (g) and blood glucose level (mg/dl) in experimental groups

Groups	n	Body weight (g)		Blood glucose level (mg/dl)	
		day 0	day 21	day 0	day 21
DS	6	232 ± 9.8	169 ± 10.8*	557 ± 20.6	539.2 ±50.6
DSL	6	233 ± 4.7	162 ± 10.1*	585.6 ± 7.4	567.4 ± 15.9
DH	6	245 ± 7.2	198.67 ± 9.5*	552.5 ± 21.7	566.5 ± 21.4
DHL	6	244 ± 5.0	204.2 ± 4.0*	510.8 ± 44.7	554.5 ± 30.6

Values are presented as mean ± SE.*p<0.05 versus day 0
DS – diabetic sham; **DSL** – diabetic sham rats treated with losartan; **DH** – diabetic hind limb ischemia; **DHL** – diabetic hind limb ischemic rats treated with losartan

Results

Body weight and blood glucose. The body weight was reduced during the experiment in all groups (p<0.05). The blood glucose level in diabetic rats was higher than 300 mg/dl during experiment. Administration of losartan caused no significant changes in the blood glucose level and body weight in hindlimb ischemic and diabetic sham animals (Table 1).

Serum NO, VEGF and sFlt-1 concentrations. Serum NO concentrations in the study groups are shown in Fig. 1A. The serum NO concentrations on

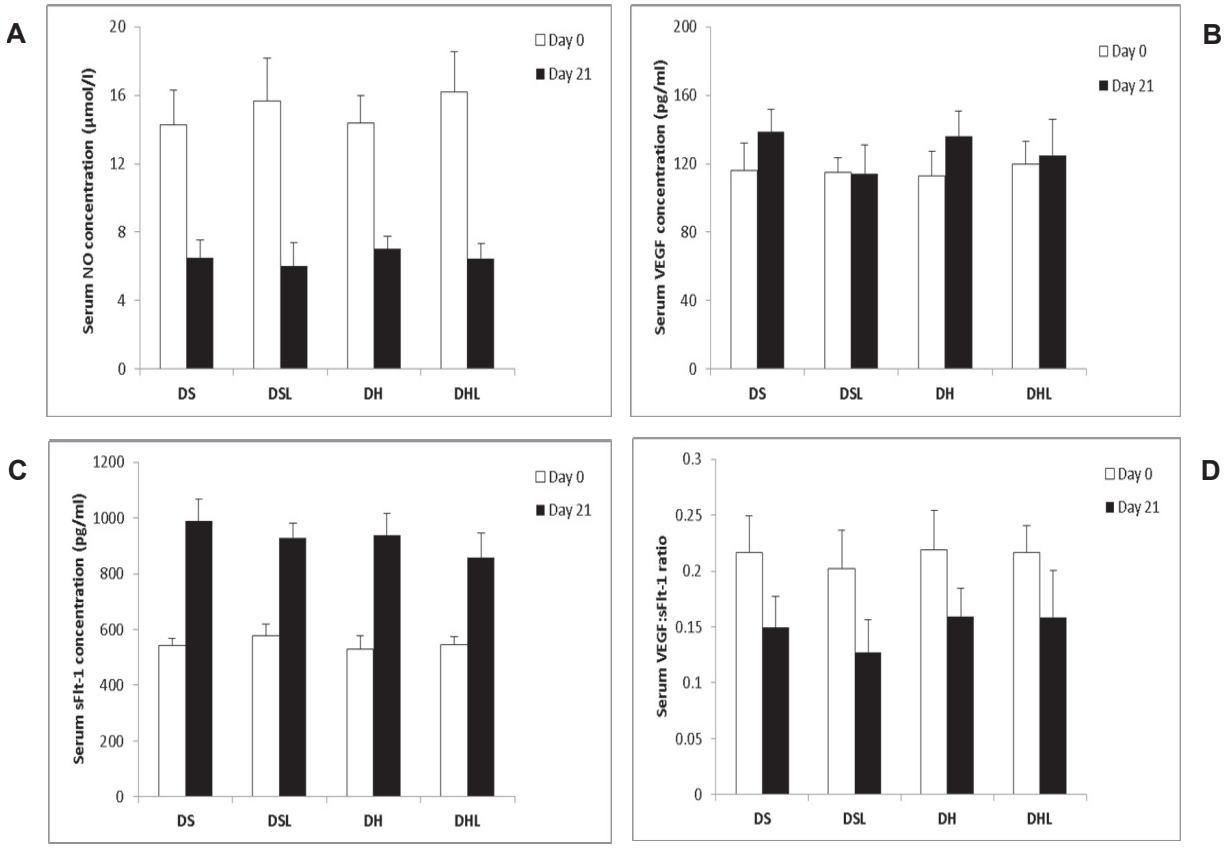

Fig. 1. Serum NO (A), VEGF (B), and sFlt-1 (C) concentrations and VEGF:sFlt-1 ratio (D) in experimental groups before (day 0) and after experiment (day 21). Values are expressed as mean ± SE, n=6 per group. * p<0.05 vs. day 0
DS – diabetic sham; DS – diabetic sham rats treated with losartan; DH – diabetic hind limb ischemia; DHL – diabetic hind limb ischemic rats treated with losartan

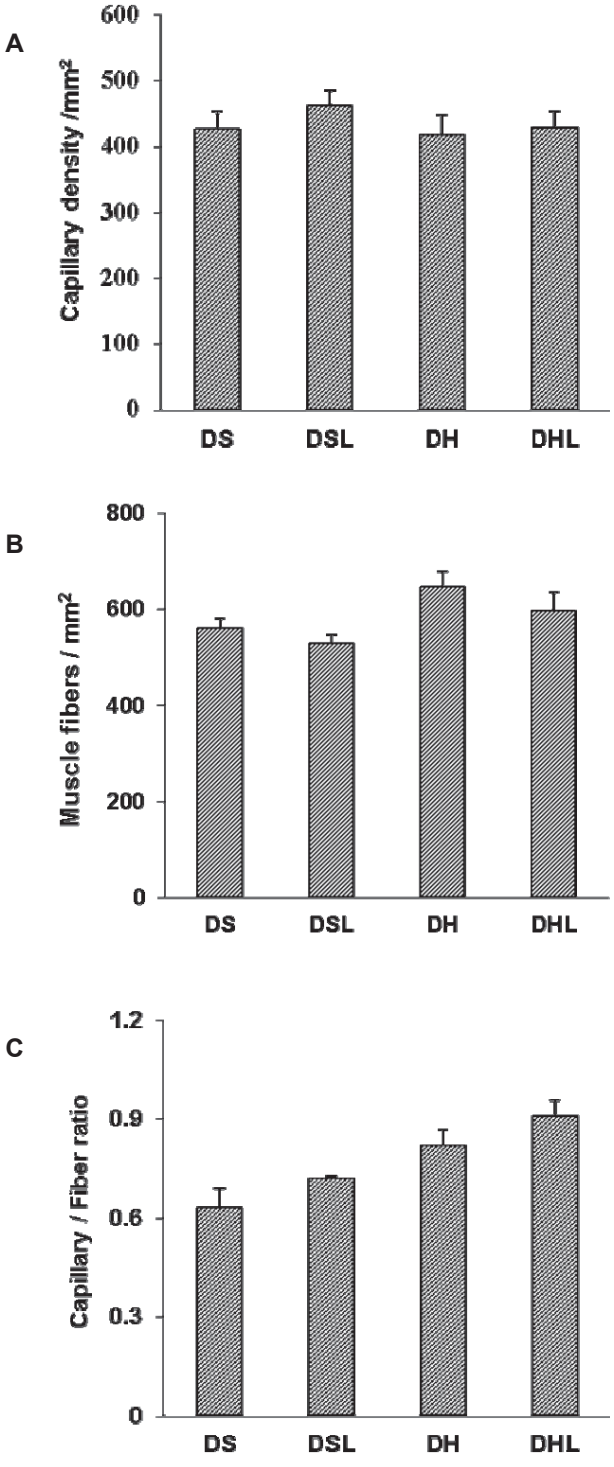

Fig. 2. Effects of losartan on capillary density (A), fiber density (B) and cap/fib ratio (C) in diabetic and diabetic hind limb of ischemic rats. Values are reported as mean ± SE, n=6 per group. * p<0.05 vs. DS group
DS – diabetic sham; DSL – diabetic sham rats treated with losartan; DH – diabetic hind limb ischemia; DHL – diabetic hind limb ischemic rats treated with losartan

the day 0 did not differ between the groups. After 21 days, the serum NO concentrations in all experimental groups were significantly lower than before experiment. Administration of losartan did not change serum NO concentration compared with non-treated groups ($p>0.05$). Fig. 1B and 1C illustrate serum VEGF and sFlt-1 concentrations before and after experiment. There were no significant changes in serum VEGF concentration in diabetic sham and hindlimb ischemia groups between before and after experiment, while serum sFlt-1 concentration was significantly increased after experiment. Administration of losartan for 21 days caused no significant changes in serum VEGF and sFlt-1 concentrations ($p>0.05$). VEGF:sFlt-1 ratio was reduced on day 21 in comparison to day 0 and losartan did not alter this ratio (Fig. 1D).

Capillary density analysis. Capillary density, fiber density, and cap/fib ratio were measured in ischemic gastrocnemius muscle. Treatment with losartan caused no significant changes in the capillary density (Fig. 2A), the muscular fiber density (Fig. 2B) and the cap/fib ratio (Fig. 2C) compared with non-treated group. Samples of tissue sections stained with immunohistochemistry in the study groups are illustrated in Fig. 3.

Discussion

Hyperglycemia and diabetes cause many cardiovascular complications. From the vascular point of view, diabetes is a paradoxical disease. On one hand, increased angiogenesis contributes to some diabetic complications including retinopathy (Wilkinson-Berka 2004; Simons 2005), while on the other hand, decreased angiogenesis in hind limb may impair wound healing and contributes to the formation of diabetic skin ulcer (Galiano et al. 2004; Ebrahimian et al. 2005). Angiogenesis is the growth of new vessels from preexisting ones which play a critical role in embryonic development and postembryonic life physiologically and pathologically (Ribatti 2007). Stimulating the formation of new vessels appears to be one of the method to prevent tissue ischemia in cardiovascular diseases.

The development of new vessels from preexisting ones under different conditions is affected by proangiogenic and antiangiogenic factors. NO is one of the most endothelium-derived releasing factors which is involved during the angiogenesis (Zhao et al. 2002). In this study, the serum NO concentration decreased during diabetes which supports the results of previous studies (Ding 2000; Bohlen 2001; Brodsky 2001; Bagi 2004).

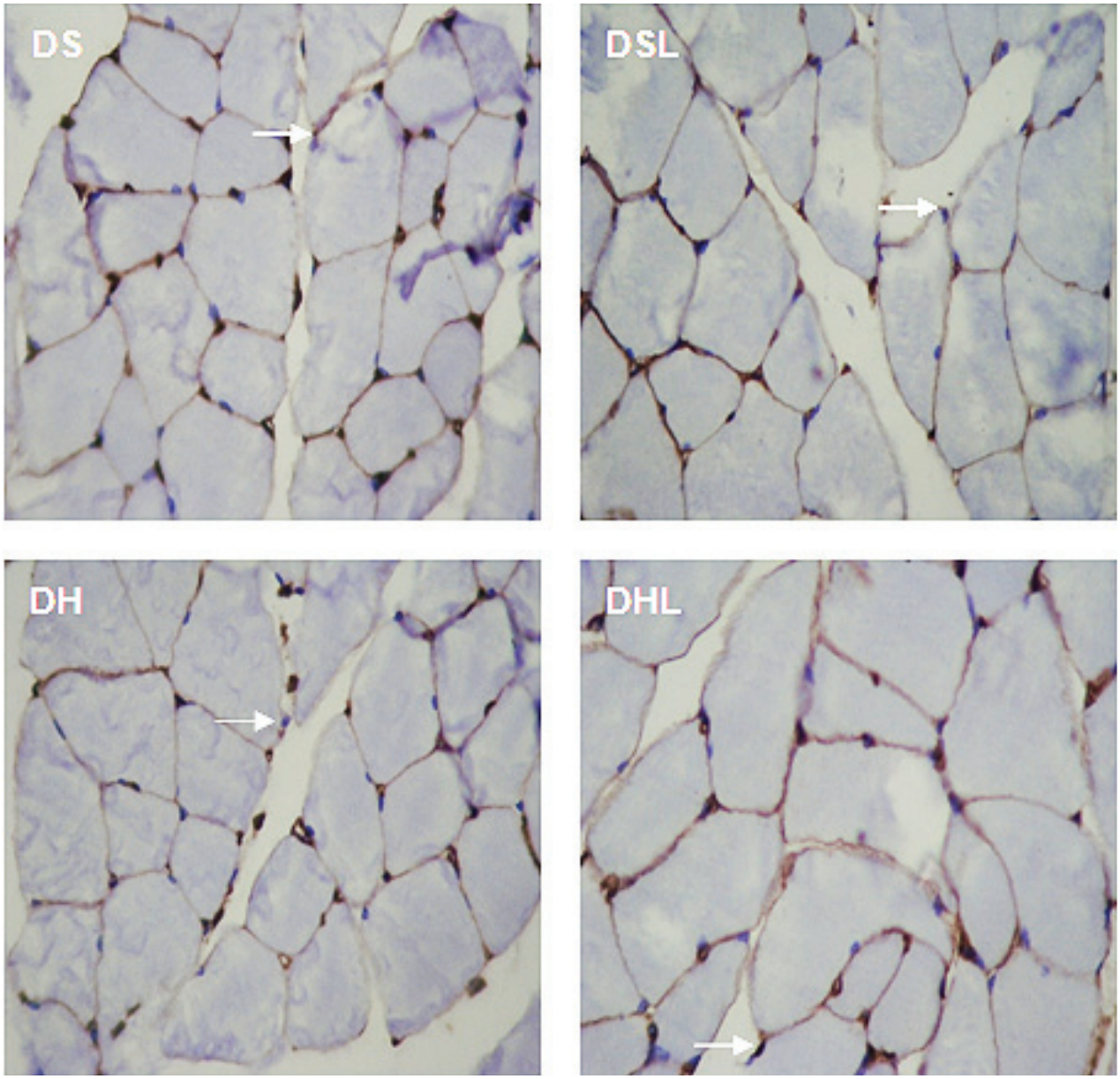

Fig. 3. Representative images of immunohistochemical staining with anti-CD31 monoclonal antibody of skeletal muscle tissues in experimental groups (×400).
DS – diabetic sham; DSL – diabetic sham rats treated with losartan; DH – diabetic hind limb ischemia; DHL – diabetic hind limb ischemic rats treated with losartan

Overproduction of superoxide (Bagi 2004), activation of protein kinase C (Bohlen 2001), and suppression of endothelial NO synthase expression (Ding 2000) are some possible mechanisms which may explain the reduced NO bioavailability in diabetes. VEGF is the major proangiogenic factor and has two receptors: VEGF-R1 (sFlt-1) and VEGF-R2. sFlt-1 is a soluble splice variant of Flt-1 and acts as an inhibitor of angiogenesis. This receptor binds to VEGF and reduces ability of VEGF to bind to VEGF-R2, and decreases angiogenesis (Roberts 2004). In the present study, serum VEGF concentration in diabetic sham and hind limb ischemic groups did not change during experiment, however, the serum sFlt-1 concentration was significantly enhanced during

diabetes. The effect of diabetes on VEGF is controversy. Increased (Sasso et al. 2005), decreased (Chua 1998) and not changed (Larger 2004) in expression of VEGF have been reported in diabetic subjects. In addition, defective VEGF signaling pathway, which considered as VEGF resistance, has been indicated (Waltenberger 2009).

We also found that losartan treatment could not restore angiogenesis in ischemic legs of diabetic animals. There are several controversial reports regarding the effect of AT1 receptor blocker on neovascularization and angiogenesis. AT1R blocker prevented retinal neovascularization (Moravski et al. 2000), angiogenic response to chronic intermittent hypoxia (Rakusan 2007), and VEGF mediated angiogenesis in male cardiomyopathic hamster (Shimizu et al. 2003). On the contrary, AT1R blockade restored myocardial capillary density after myocardial infarction (Schieffer 1994; Tamarat et al. 2002). It should be mentioned that most of the studies were conducted in non-diabetic animals and there are few studies about the ARBs effect on angiogenesis in diabetes and ischemia during diabetes. Discrepancy between the results might be somehow associated with differences in the studied organs and angiogenesis models.

Some studies have reported that VEGF/sFlt-1 ratio is a better indicator of angiogenesis status (Chang 2008). In our study, this ratio was reduced during diabetes which suggests impaired angiogenesis during diabetes and losartan could not increase this ratio.

In conclusion, losartan, an AT1R blocker, could not restore angiogenesis during hindlimb ischemia in diabetic animals and the present data indicate that it cannot be considered for treatment or prevention of peripheral artery disease in diabetic subjects.

Acknowledgment

This study was supported in part by a grant 188103 from Isfahan University of Medical Sciences. We also thank to Daroupakhsh Co. for sponsoring our research by losartan, in the form of a gift.

References

Bagi Z: Microvascular dysfunction after transient high glucose is caused by superoxide-dependent reduction in the bioavailability of NO and BH(4). Am J Physiol Heart Circ Physiol 287, H626-H633, 2004. http://dx.doi.org/10.1152/ajpheart.00074.2004

Baluchnejadmojarad T, Roghani M, Imani A: Protective effect of enalapril on vascular reactivity of the rat aorta. Vascul Pharmacol 40, 301-307, 2004. http://dx.doi.org/10.1016/j.vph.2004.02.003

Bohlen HG: Arteriolar nitric oxide concentration is decreased during hyperglycemia-induced betaII PKC activation. Am J Physiol Heart Circ Physiol 280, H621-H627, 2001.

Brem H, Tomic-Canic M: Cellular and molecular basis of wound healing in diabetes. J Clin Invest 117, 1219-1222, 2007. http://dx.doi.org/10.1172/JCI32169

Brodsky SV: Glucose scavenging of nitric oxide. Am J Physiol Renal Physiol 280, F480-F486, 2001.

Carmeliet P: Angiogenesis in health and disease. Nat Med 9, 653-660, 2003. http://dx.doi.org/10.1038/nm0603-653

Chang YT: Serum vascular endothelial growth factor/soluble vascular endothelial growth factor receptor 1 ratio is an independent prognostic marker in pancreatic cancer. Pancreas 37, 145-150, 2008. http://dx.doi.org/10.1097/MPA.0b013e318164548a

Chua CC: Upregulation of vascular endothelial growth factor by angiotensin II in rat heart endothelial cells. Biochim Biophys Acta 1401, 187-194, 1998. http://dx.doi.org/10.1016/S0167-4889(97)00129-8

Ding Y: Effects of simulated hyperglycemia, insulin, and glucagon on endothelial nitric oxide synthase expression. Am J Physiol Endocrinol Meta 279, E11-E17, 2000.

Ebrahimian TG, Tamarat R, Clergue M, Duriez M, Levy BI, Silvestre JS: Dual effect of angiotensin-converting enzyme inhibition on angiogenesis in type 1 diabetic mice. Arterioscler Thromb Vasc Biol 25, 65-70, 2005.

Emanueli C: Angiotensin AT(1) receptor signalling modulates reparative angiogenesis induced by limb ischaemia. Br. J Pharmacol 135, 87-92, 2002. http://dx.doi.org/10.1038/sj.bjp.0704461

Fallahzadeh AR, Khazaei M, Sharifi MR: Restoration of angiogenesis by enalapril in diabetic hindlimb ischemc rats. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 155, 137-142, 2011. http://dx.doi.org/10.5507/bp.2011.026

Galiano RD, Tepper OM, Pelo CR Bhatt KA, Callaghan M, Bastidas N, Bunting S, Steinmetz HG, Gurtner GC: Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by

mobilizing and recruiting bone marrow-derived cells. Am J Pathol 164, 1935-1947, 2004. http://dx.doi.org/10.1016/S0002-9440(10)63754-6

Larger E: Hyperglycemia-induced defects in angiogenesis in the chicken chorioallantoic membrane model. Diabetes 53, 752-761, 2004. http://dx.doi.org/10.2337/diabetes.53.3.752

Moosavi SM, Johns EJ: Effect of renal perfusion pressure on renal function, renin release and renin and angiotensinogen gene expression in rats. J Physiol 520 Pt 1, 261-269, 1999. http://dx.doi.org/10.1111/j.1469-7793.1999.t01-1-00261.x

Moravski CJ, Kelly DJ, Cooper ME Gilbert RE, Bertram JF, Shahinfar S, Skinner SL, Wilkinson-Berka JL: Retinal neovascularization is prevented by blockade of the renin-angiotensin system. Hypertension 36, 1099-1104, 2000. http://dx.doi.org/10.1161/01.HYP.36.6.1099

Munk VC, Sanchez de ML, Petrimpol M Butz N, Banfi A, Eriksson U, Hein L, Humar R, Battegay EJ: Angiotensin II induces angiogenesis in the hypoxic adult mouse heart in vitro through an AT2-B2 receptor pathway. Hypertension 49, 1178-1185, 2007. http://dx.doi.org/10.1161/HYPERTENSIONAHA.106.080242

Nematbakhsh M: Oestrogen promotes coronary angiogenesis even under normoxic conditions. Basic Clin Pharmacol Toxicol 103, 273-277, 2008. http://dx.doi.org/10.1111/j.1742-7843.2008.00286.x

Nematollahi S, Nematbakhsh M, Haghjooyjavanmard S, Khazaei M, Salehi M: Inducible nitric oxide synthase modulates angiogenesis in ischemic hindlimb of rat. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 153, 125-129, 2009. http://dx.doi.org/10.5507/bp.2009.021

Rakusan K: ANG II type 1 receptor antagonist irbesartan inhibits coronary angiogenesis stimulated by chronic intermittent hypoxia in neonatal rats. J Toxicol Environ Health B Crit Rev 3, 27-58, 2007.

Ribatti D: Nonclassic endogenous novel [corrected] regulators of angiogenesis. Pharmacol Rev 59, 185-205, 2007. http://dx.doi.org/10.1124/pr.59.2.3

Roberts DM: The vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation. Am J Pathol 164, 1531-1535, 2004. http://dx.doi.org/10.1016/S0002-9440(10)63711-X

Sasaki K: Evidence for the importance of angiotensin II type 1 receptor in ischemia-induced angiogenesis. J Clin Invest 109, 603-611, 2002.

Sasso FC, Torella D, Carbonara O, Ellison GM, Torella M, Scardone M, Marra C, Nasti R, Marfella R, Cozzolino D, Indolfi C, Cotrufo M, Torella R, Salvatore T: Increased vascular endothelial growth factor expression but impaired vascular endothelial growth factor receptor signaling in the myocardium of type 2 diabetic patients with chronic coronary heart disease. J Am Coll Cardiol 46, 827-834, 2005. http://dx.doi.org/10.1016/j.jacc.2005.06.007

Schieffer B: Comparative effects of chronic angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade on cardiac remodeling after myocardial infarction in the rat. Circulation 89, 2273-2282, 1994. http://dx.doi.org/10.1161/01.CIR.89.5.2273

Shimizu T, Okamoto H, Chiba S, Matsui Y, Sugawara T, Akino M, Nan J, Kumamoto H, Onozuka H, Mikami T, Kitabatake A: VEGF-mediated angiogenesis is impaired by angiotensin type 1 receptor blockade in cardiomyopathic hamster hearts. Cardiovasc Res 58, 203-212, 2003. http://dx.doi.org/10.1016/S0008-6363(02)00843-X

Silvestre JS, Levy BI: Molecular basis of angiopathy in diabetes mellitus. Circ Res 98, 4-6, 2006. http://dx.doi.org/10.1161/01.RES.0000200396.90220.41

Simons M: Angiogenesis, arteriogenesis, and diabetes: paradigm reassessed? J Am Coll Cardiol 46, 835-837, 2005. http://dx.doi.org/10.1016/j.jacc.2005.06.008

Szkudelski T: The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res 50, 537-546, 2001.

Tamarat R, Silvestre JS, Kubis N, Benessiano J, Duriez M, deGasparo M, Henrion D, Levy BI: Endothelial nitric oxide synthase lies downstream from angiotensin II-induced angiogenesis in ischemic hindlimb. Hypertension 39, 830-835, 2002. http://dx.doi.org/10.1161/hy0302.104671

Waltenberger J: VEGF resistance as a molecular basis to explain the angiogenesis paradox in diabetes mellitus. Biochem Soc Trans 37, 1167-1170, 2009. http://dx.doi.org/10.1042/BST0371167

Walther T: Differential regulation of in vivo angiogenesis by angiotensin II receptors. FASEB 17, 2061-2067, 2003. http://dx.doi.org/10.1096/fj.03-0129com

Watanabe T, Barker TA, Berk BC: Angiotensin II and the endothelium: diverse signals and effects. Hypertension 45, 163-169, 2005. http://dx.doi.org/10.1161/01.HYP.0000153321.13792.b9

Wilkinson-Berka JL: Vasoactive factors and diabetic retinopathy: vascular endothelial growth factor, cycoloxygenase-2 and nitric oxide. Curr Pharm Des 10, 3331-3348, 2004. http://dx.doi.org/10.2174/1381612043383142

Zhao X, Lu X, Feng Q: Deficiency in endothelial nitric oxide synthase impairs myocardial angiogenesis. Am J Physiol Heart Circ Physiol 283, H2371-H2378, 2002.