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ORIGINAL ARTICLE

Activation of skin renin–angiotensin system in diabetic rats

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Abstract The renin–angiotensin system (RAS) is reportedly involved in chronic diabetic complications such as diabetic nephropathy, but changes of the RAS in diabetic skin remain unknown. The aim of this study was to investigate the expression of angiotensin (Ang) II and its type 1 (AT1) and type 2 (AT2) receptors in diabetic skin tissues, and explore the relationship between the local RAS and pathological changes of diabetic skin. Our results showed that thinning of epidermis, degeneration of collagen, fracture of dermal layer, and atrophy/disappearance of subcutaneous fat were observed in diabetic skin. The expression level of AngII was increased in diabetic skin tissues compared to that in controls. mRNA and protein expression of AT1 receptor were also increased while the level of AT2 receptor decreased; the relative expression of AT1 to AT2 receptors was approximately threefold higher in diabetes than in controls. Furthermore, in the culture medium of primary cultured fibroblasts from diabetic skin, the concentration of AngII was significantly higher than that of normal control. The mRNA and protein expression of AT1 receptor was also increased in fibroblasts of diabetic skin compared to controls, while the protein expression of AT2 receptor was decreased. Taken together, our results suggest that the local RAS system is activated in diabetic skin and AngII receptor is likely to mediate the pathological changes of diabetic skin.

Keywords Diabetes · Skin · Angiotensin II · Angiotensin II type 1 receptor · Angiotensin II type 2 receptor

Introduction

Components of the renin–angiotensin system (RAS) can be expressed by a variety of tissues and organs, which is called the local/tissue RAS, and plays an important role in the pathogenesis of wound repair, tissue reconstruction, embryonic development and tumor development [1, 2]. Some researches have confirmed that the activation of the local RAS was related to the pathological changes of some diabetic chronic complications such as diabetic nephropathy. It was found that the expressions of the components of the RAS such as angiotensin (Ang) II and its type 1(AT1) and type 2 (AT2) receptors were increased in heart, vascular endothelium, kidney, retina, and other tissues in diabetes.

Diabetic skin lesion is a chronic complication of diabetes and has become a global medical and public problem. In diabetic skin, a series of changes have been observed, including thinning of depth, decrease of tensile strength, atrophy of dermis, degeneration and disorder of collagen fibers, infiltration of chronic inflammatory cells. These changes have made the skin more susceptible to exogenous damage and easier to form ulcers [3]. Until now, the pathogenesis of diabetic skin lesions has not yet been clear. Besides common factors such as ischemia, neuropathy, and infection, there has been evidence to suggest that the changes of diabetic skin are related to the local RAS.

AngII affects proliferation, apoptosis, migration, collagen metabolism, and capillary formation of skin cells via its receptors [4]. The local expression of AngII is therefore likely to be important in pathological damage, wound

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healing, and scar formation of the skin [2, 5]. It was reported that some components of the RAS such as AngII and its receptors could be produced by skin tissues, and AngII receptor is also expressed in the skin. However, the changes of components of the local RAS in diabetic skin remain unclear and will be the subject of current investigation.

Methods

Animal experiment

Sixteen clean-grade Spreqne-Dawley rats each weighted about 220 g were randomized into control and experimental groups after fed adaptively for 1 week. Diabetes was induced by injection (i.p.) of streptozotocin (STZ, Sigma, USA) at 35 mg/kg prepared in 0.1 M citrate buffer (pH 4.2) for 3 consecutive days. Animals injected with buffer alone were used as normal controls ($n = 8$). 72 h after STZ injection, blood glucose (BG) was measured by a One-Touch II® Glucometer (LifeScan, USA) and animals were considered to be diabetic when the concentration of BG is equal to or higher than 16.7 mmol/l. STZ-treated rats were given daily injections of a small dose of protamine zinc insulin (0.5–3.0 units per day) as required to reduce mortality by maintaining blood glucose on the level above 16.7 mmol/l and avoiding ketosis occurence. Rats were further housed for 8 weeks before killed. 3 ml abdominal aortic blood was placed in frozen water to separate serum in 30 min and stored in −80°C refrigerator for radioimmunoassay.

HE staining and Masson-trichrome staining

At the end of the 8-week period, animals were intraperitoneally injected with 1% pentobarbital (40 mg/kg) and central dorsal skin tissues, 1–5 cm from the skull, were obtained. Skin tissues were fixed in 4% formaldehyde and then embedded in paraffin. The sections were stained with hematoxylin and eosin to examine their morphology. The collagen fibers were visualized using Masson-trichrome staining. The thickness of epidermis and dermis in diabetic and normal rat skin tissues were measured through microscope.

Transmission electron microscopy (TEM) assay of skin tissues

10 mm × 5 mm central dorsal skin tissues were excised and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide followed by 1% uranyl acetate, dehydrated through a graded series of ethanol and embedded in Epon resin. Ultrathin sections were cut on a MT-7000 ultramicrotome. Sections were stained with uranyl acetate and lead citrate, and examined under a Philips electron microscope.

Fibroblast Cell culture

The dorsal skin tissues of SD rats were cut into 2 mm × 5 mm stripes and placed in 0.2% dispase (Sigma, USA) at 4°C for digestion about 20 h. Later on, epidermis and dermis were separated, and dermis were cut into small pieces with addition of 0.25% trypsin/0.02% EDTA (Sigma), which were kept at 37°C for 15 min to digest into single cell suspension. Fibroblasts from diabetic and normal rats were resuspended, respectively, with high glucose or normal glucose DMEM (Invitrogen) containing 10% fetal calf serum (FCS). Cells were inoculated on the density of 1×10^5/ml, cultured in 37°C and 5% CO_2 condition, and culture medium were changed every 2 days. After reaching 80% confluence, the cells were digested with 0.25% trypsin/0.02% EDTA at 37°C and then subcultured. The second generation fibroblasts were used in this study. To identify fibroblasts, antibody to vimentin was used.

Cell viability analysis

The viability of fibroblasts from normal and diabetic rats was determined by 3-(4,5-dimethylthiazole-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay. Cells from normal and diabetic rats were seeded in 96-well plates, in 200 μl DMEM medium with normal glucose or high glucose. MTT assays were performed every 24 h until 9 days. At the time of the assay, 20 μl of a 5 mg/ml stock solution of MTT (Sigma–Aldrich) was added to each well. Four hours later, 150 μl DMSO was added to dissolve the dark blue formazan crystals that were formed by the living cells. The absorbance of each well at 490 nm (A490) was determined using a Bio-Rad model microplate reader (Bio-Rad Laboratories, Richmond, CA). The background A490 of the wells that did not contain cells was subtracted before the absorbance was calculated. All studies were performed at least four times independently.

Concentrations of AngII in skin tissues and supernatant of fibroblasts

The concentration of AngII in skin homogenate and fibroblasts from diabetic and normal rats was assayed with radioimmunoassay. Homogenate of skin tissues was centrifuged at 4°C, 3000 r/min for 15 min. Supernatant was stored at −80°C or below. The concentration of AngII in homogenate was assayed via radioimmunoassay (Beijing North Biotechnology Institute RIA kit), and total AngII

quantity was calculated in the homogenate according to its concentration and volume. AngII content in skin tissues was obtained as total AngII quantity divided by the weight of skin.

Primary fibroblasts from diabetic and normal rats were cultured with high glucose or normal glucose DMEM (Invitrogen) containing 10% FCS (Hyclone). After reaching 80% confluence, cells were shifted into DMEM medium containing 0.5% FCS for 24 h. Then culture medium was collected and centrifuged at 3000 rpm for 15 min. The concentration of AngII in supernatant was assayed with radioimmunoassay. The cells were digested and centrifuged and the cell numbers were counted.

Immunohistochemical staining of AngII, AT1, and AT2 receptors in skin tissues

Paraffin sections of skin tissues were labeled with primary antibodies such as anti-AT1 receptor antibody (1:200; Abcam); anti-AT2 receptor antibody (1:800; Abcam), and anti-AngII antibody (1: 100; Boster) and then kept at 4°C in a refrigerator overnight. The binding of the primary antibodies was revealed by biotin-labeled second antibody and detected with the diaminobenzidine tertrahydrochloride (DAB) staining. Controls for immunospecificity were included in all experiments and the primary antibody was replaced with PBS.

RT-PCR analysis of mRNA expression of AGT, AT1, and AT2 receptors

Total RNA in skin tissues was extracted (TRIzol, Invitrogen) and was then transcribed reversely by the reverse transcriptase (First Strand cDNA Synthesis Kit, Fermentas) into cDNA for PCR. Primers were designed and synthesized by Bio-engineering Techniques from Shanghai Public Health Services Ltd. PCR primer sequences and product sizes were shown in Table 1. Primers against rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal control. After electrophoresis, they were photographed and scanned for electrophoretic bands with AlphaImager 2200 software. The semi-quantitative test results of target amplified fragments were the gray-scale ratio of the specially amplified DNA fragments bands with the inner reference bands at the same time (optical density of the target gene/optical density of GAPDH).

When the fibroblasts from diabetic and normal rats reached 80% confluence, they were washed with D-Hanks solution and total RNA was extracted. The remaining steps were similar to that of assay of mRNA levels of AGT, AT1, and AT2 receptors in skin tissues.

Western blot of protein levels of AT1 and AT2 receptors

Total proteins in skin tissues and fibroblasts were extracted. Protein concentrations were determined using BCA protein concentration determination kit. 25 μg protein was separated by 10% SDS–PAGE gels and electrotransferred onto PVDF membranes. Then blots were blocked with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% dry milk for 1 h at room temperature and incubated overnight at 4°C with anti-AT1 antibody (1:500; Abcam) or anti-AT2 antibody(1:800; Abcam). After incubation with second antibody, immune complexes were detected using ECL method and immunoreactive bands were quantified using Alphaimager 2200. Values were corrected with the absorbency of the internal control (GAPDH).

Statistical analysis

All experiments were repeated at least three times. Values are given as mean ± SD. Data were analyzed using SPSS 11.0 software. Statistical significance was assessed by one-way ANOVA. $P < 0.05$ was considered statistically significant.

Table 1 Primer sequences used for RT-PCR

Gene	Primer	Annealing temperature	Product size (bp)
AGT	5′-CAGCATCCTCCTTGAACTCC-3′	54	236
	5′-CCCAGGATAGCTCTCTGACG-3′		
AT1	5′-AGGTATGTGGCCCATGCTAA-3′	54	354
	5′-CAGATGCGAAATAACGCAGA-3′		
AT2	5′-GCGCACGCTATTTAGTAAAACA-3′	56	393
	5′-CTCCCAAGAAGGCATCAAAC-3′		
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′	54	452
	5′-TCCACCACCCTGTTGCTGT-3′		

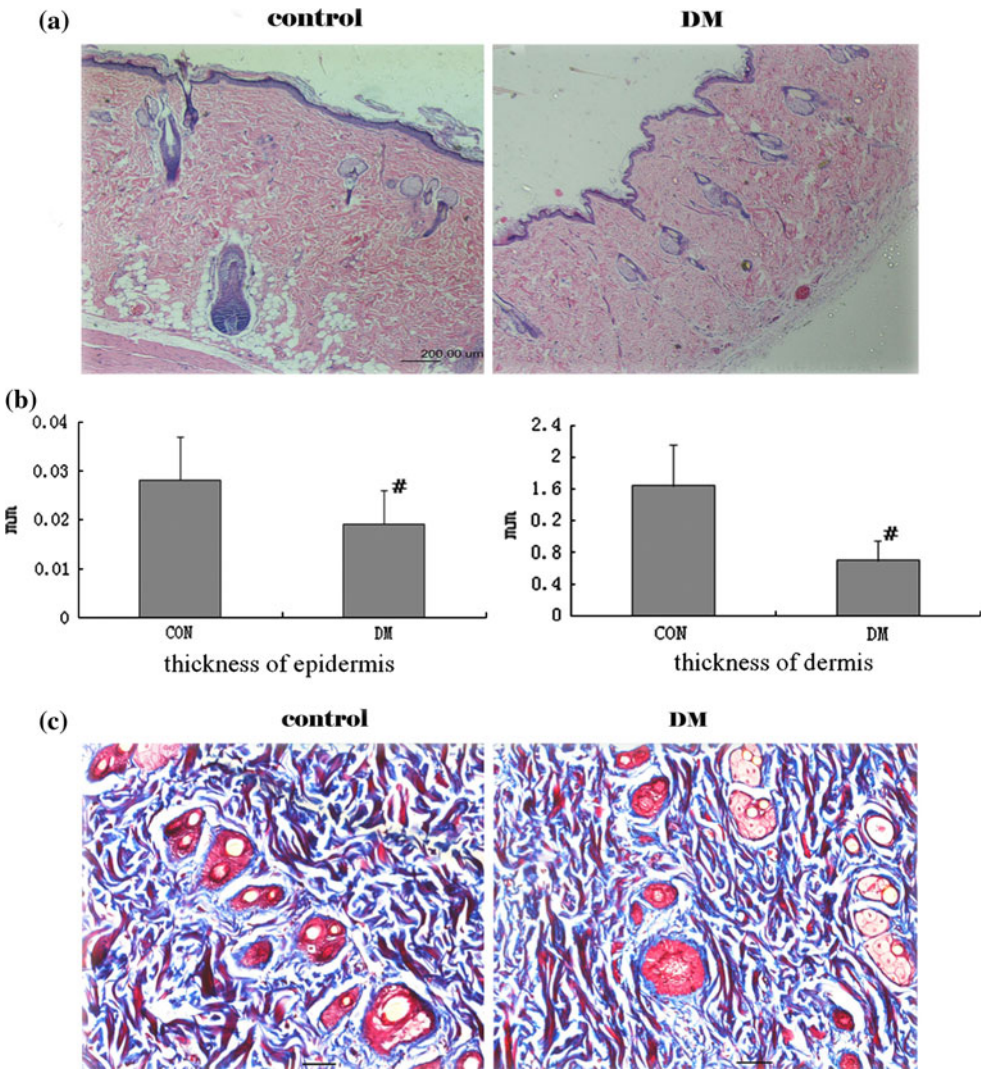

Fig. 1 Histological appearance of rat skin tissues. Tissues were fixed overnight in 4% polyformaldehyde, embedded in paraffin, and sectioned at 4 μm with a microtomb. Sections were then stained with hematoxylin–eosin or Masson's trichrome stained method. (**a**) HE staining showed histological appearance of skin tissue from control and DM group. (**b**) Histogram plots represented the thickness of epidermis and dermis of diabetic and normal skin tissues corresponding to the mean ± SD of three independent experiments. $^{\#}P < 0.05$ compared with control. (**c**) Masson staining showed collagen changes of control and diabetic skin. Magnification ×40 (**a**); ×200 (**b**)

Results

Histological changes of diabetic skin tissues

HE staining showed that in normal rat skin tissues, the structure of skin was complete with clear epidermal layers and rich collagens in dermis (Fig. 1a). In diabetic rat skin, however, the epidermal layers were not clear enough and the dermal layers were dramatically atrophic (Fig. 1a). Furthermore, in diabetic rat skin, the arrangement of fiber bundles in connective tissue was in disorder, with increased space between collagen fiber bundles. Some denatured and fractured collagens could be found, and chronic inflammatory cells were infiltrated in collagen degenerated regions. The thickness of epidermis and dermis in diabetic rats were 0.019 ± 0.007 and 0.695 ± 0.236 mm, significantly thinner compared to those of controls (0.028 ± 0.009 and 1.632 ± 0.521 mm, respectively) (Fig. 1b). Masson staining results also showed that collagens in dermis of diabetic rat skin were disorderedly arranged. The collagen fibers were thin, degenerated, and fractured (Fig. 1c).

Ultrastructure changes of diabetic skin tissues

Three kinds of ultrastructure changes were found between diabetic and normal skin. First, in normal skin, there were multilayer epidermal cells full of tension wires in the cytoplasm (Fig. 2a), while in diabetic skin, the layers of epidermal cells decreased, with sparse tension wire in the cytoplasm. Adjacent spines cells were connected by naive desmosomes and the organelle structure of cells was unclear. Second, in normal skin, the basement membrane between dermis and epidermis was wavy, which became thin and flat in diabetic skin (Fig. 2b). The collagen fibers in dermal of normal skin were dense and regularly arranged. Third, in diabetic skin, the collagen fibers were thin, sparse, and irregularly arranged, and vacuolar degeneration and swollen organelles were observed in the

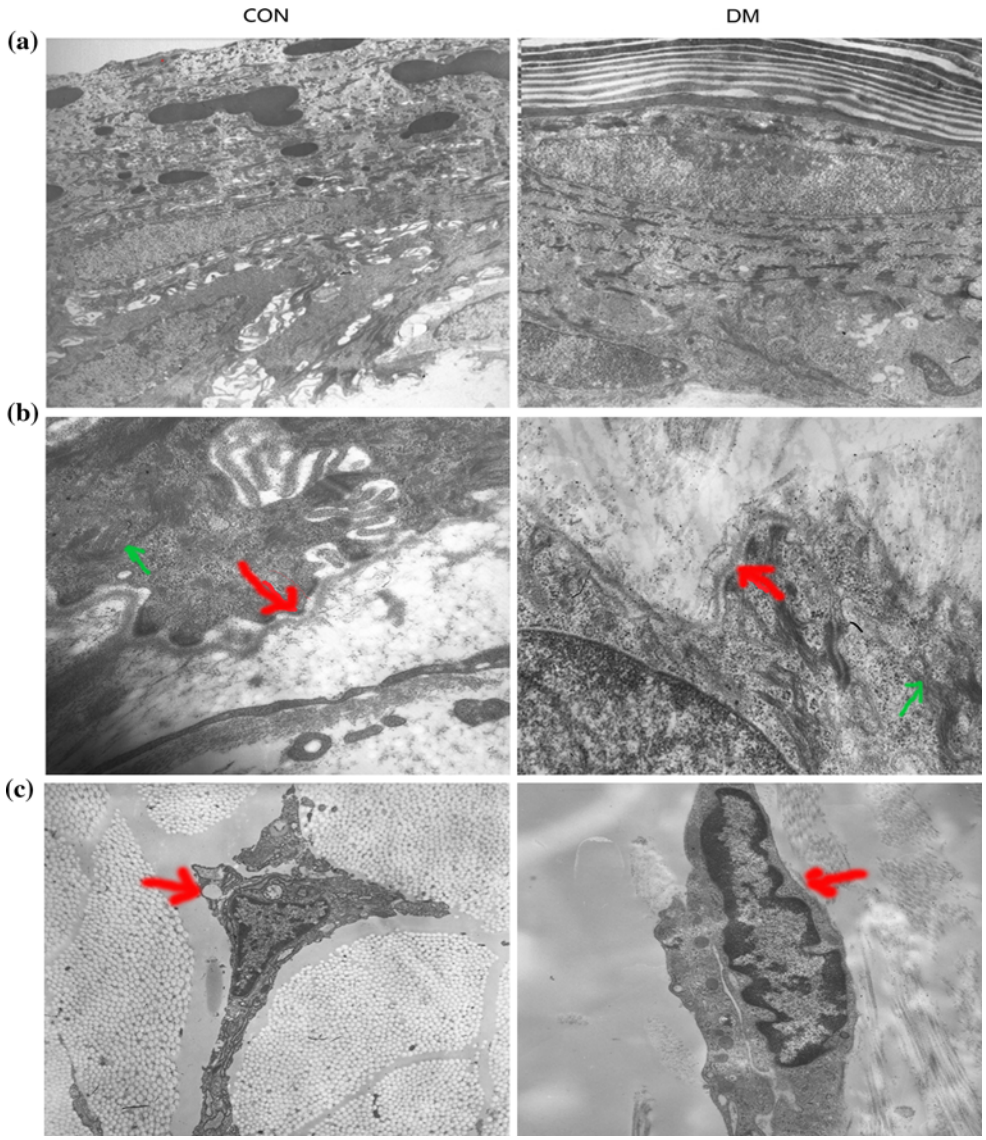

Fig. 2 Ultrastructure appearance of skin tissues from control and diabetic rats. Tissues were fixed with 2.5% glutaraldehyde then dehydrated, soaked, embedded, polymerized, sliced, stained, and examined under a Philips electron microscopy. (**a**) Epidermal cells. Magnification ×3,900 in CON, magnification ×6,400 in DM. (**b**) Basement membrane (*larger arrow*), tension wires (*smaller arrow*). Magnification ×15,000; (**c**) collagen fibers and skin capillary (*larger arrow*). Magnification ×6,600

cytoplasm of vascular endothelial cells in dermis (Fig. 2c).

Location of AngII, AT1, and AT2 receptors in skin tissues

Immunohistochemistry was performed to analyze the location of AngII, AT1, and AT2 receptors in skin tissues. AngII was positively expressed in full-thickness epidermis of normal and diabetic rats (Fig. 3a). Expression of AngII could also be detected in dermis, fibroblasts, vascular endothelial cells, hair follicles (inner hair root sheath, outer hair root sheath), sebaceous glands, and ducts.

AngII exerts its important physiological functions through two distinct receptor subtypes, AT1 and AT2 receptors. Immunohistochemistry staining results showed that there was no expression of AT1 receptor in epidermal cells of skin, yet positive expression could be observed in membrane of sebaceous glands, hair follicle cells (inner hair root sheath, outer hair root sheath) in dermis(Fig. 3b). In diabetic groups, both the membrane of inner and outer hair root sheaths had positive expression of AT1 receptor, while in normal group, AT1 receptor was expressed only in the membrane cells of inner hair root sheath (Fig. 3b). Expression of AT2 receptor could be found in epidermal cells, sebaceous glands, membrane

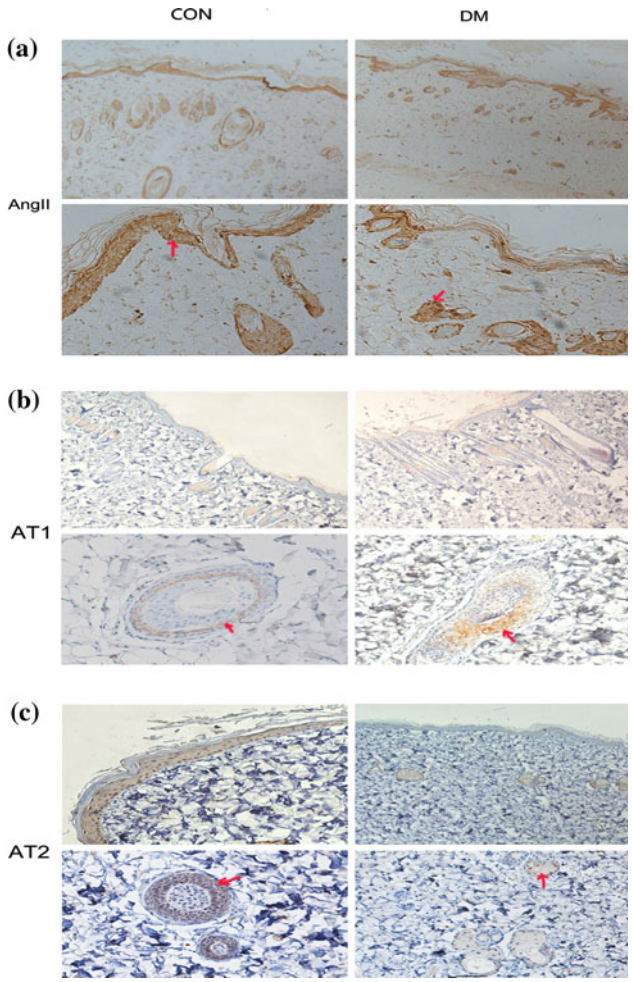

Fig. 3 Distribution of AngII, AT1, and AT2 receptors in rat skin tissues. Immunostaining of a paraffin section of control and diabetic rat skin tissues using the antibody directed against the rat AngII (**a**), AT1 receptor (**b**), and AT2 receptor(**c**). Positive staining was marked (*arrow*). The image represents one of five representative stainings. Magnification ×40 (*upper*); ×200 (*lower*)

and cytoplasm of hair follicle epithelial cells in dermal layer (Fig. 3c).

Expression of AGT, AT1, and AT2 receptors in skin tissues

AGT, the precursor of AngII, was detected through the method of RT-PCR. Results showed that mRNA expression of AGT could be detected in skin of both diabetic and control group. The expression level in diabetic rats was relatively higher than that of normal control, but with no statistically significant difference (Fig. 4a). Compared with controls, mRNA expression of AT1 receptor was increased in diabetic skin, with the expression level of AT1 receptor being approximately twofold higher than that of normal group. The mRNA expression of AT2 receptor showed no differences between normal and diabetic group (Fig. 4a).

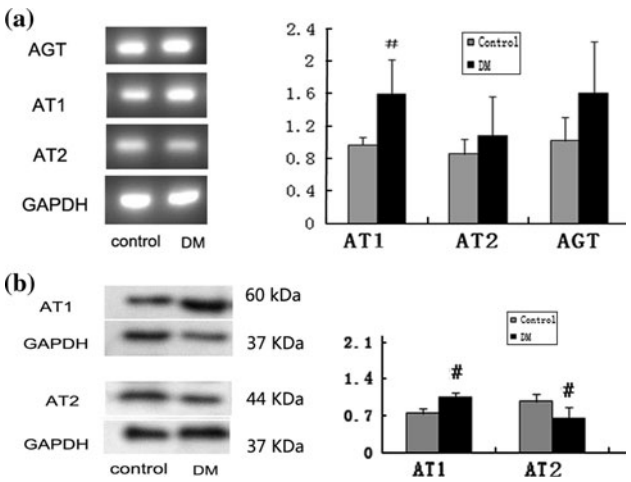

Fig. 4 mRNA and protein expression of AGT, AT1, and AT2 receptors in control and diabetic skin tissues. (**a**) mRNA expression of AGT, AT1, and AT2 receptors was analyzed by RT-PCR. GAPDH was used as an internal control to standardize the amount of total RNA used. (**b**) Protein expression of AT1 and AT2 receptors was analyzed by western blot. GAPDH was used as an internal control to standardize the amount of protein used. Histogram plots represented the densitometric analysis corresponding to the mean ± SD of three independent experiments. $^{\#}P < 0.05$ compared with control

Similar to mRNA expression, protein expressions of both AT1 and AT2 receptors could be detected with western blot in skin tissues of both normal and diabetic rats (Fig. 4b). Diabetic group demonstrated an increased AT1 receptor expression and a decreased AT2 receptor protein expression, with the AT1 to AT2 receptor protein expression ratio being threefold higher compared to controls.

Proliferation ability of fibroblasts from diabetic and normal group

We first analyzed the expression of vimentin in primary fibroblasts to determine their phenotype. As shown in Fig. 5a, fibroblasts from normal and diabetic rats were both strongly positive for vimentin.

The proliferation ability of isolated primary fibroblasts from diabetic and normal rats was also compared. The results of MTT assay revealed that high glucose had inhibitory effect on the proliferation of fibroblasts from diabetic rats, as compared to the cells derived from normal group (Fig. 5b).

Expression of AGT, AT1, and AT2 receptors in skin fibroblasts

In primary cultured fibroblasts, mRNA expression of AGT was also detected through RT-PCR. Results showed that AGT mRNA expression could be detected in primary skin fibroblasts from diabetic and control group. The expression

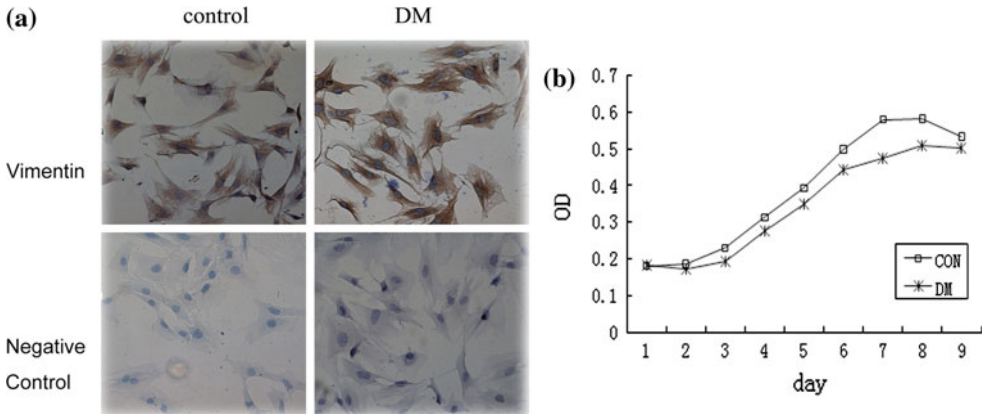

Fig. 5 Identification of isolated primary fibroblasts and the proliferation ability of fibroblasts from diabetic and control group. (**a**) Immunohistochemical staining of vimentin could be observed in cytoplasm in skin fibroblasts (*upper*). Negative control staining was performed without primary antibody (*lower*) (magnification ×200). (**b**) Proliferation ability of fibroblasts from diabetic and normal group was measured by MTT assay. The optical density was determined at 490 nm, and was expressed as mean ± SD for four independent experiments

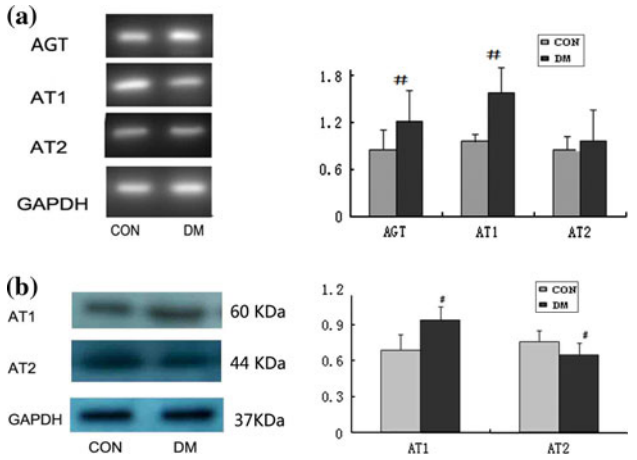

Fig. 6 mRNA and protein expression of AGT, AT1, and AT2 receptors in control and diabetic primary fibroblasts. (**a**) mRNA expression of AGT, AT1, and AT2 receptors in skin fibroblasts was analyzed by RT-PCR. GAPDH was used as an internal control to standardize the amount of total RNA used. (**b**) Protein expression of AT1 and AT2 receptors was analyzed by western blot. GAPDH was used as an internal control to standardize the amount of protein used. Histogram plots represented the densitometric analysis corresponding to the mean ± SD of three independent experiments. $^{\#}P < 0.05$ compared with control

level in cells from diabetic rats was significantly higher than that of normal control (Fig. 6a).

Further investigation of AT1 and AT2 receptor expression showed that AT1 and AT2 receptors were both expressed in skin fibroblasts. The mRNA and protein expression of AT1 receptor was increased in fibroblasts from diabetic rats, while the protein expression of AT2 receptor was decreased, with statistical differences compared to that of normal control (Fig. 6a, b).

Concentration of AngII in skin tissues and fibroblasts

The concentration of AngII in skin homogenate showed that there existed significant difference of the concentration of AngII between normal and diabetic rats. The concentration of AngII in skin tissue of diabetic group was 17.07 ± 2.5 pg/mg, which is significantly higher than that of normal control (13.14 ± 3.7 pg/mg).

The concentration of AngII in skin fibroblasts was also assayed with radioimmunoassay. In the culture medium of primary cultured fibroblasts from diabetic rats, the concentration of AngII was 7.3 ± 1.4 pg/10^6 cells, which is significantly higher than that of control group (5.2 ± 0.7 pg/10^6 cells).

Discussion

Our study shows that skin tissues not only express precursor of AngII (AGE), but also synthesizes AngII itself locally. AT1 and AT2 receptors of AngII were both expressed in skin, suggesting that skin is not just a source of AngII, but also its target organ [6]. Importantly, the local RAS system was activated in diabetic skin, with elevated AngII level and increased AT1 to AT2 receptor expression ratio.

Takeda [7, 8] reported that sweat glands and hair follicles in normal human skin only expressed AT1 receptor but not AT2 receptor, while other research [9] suggested that AT1 and AT2 receptors were both strongly expressed in hair follicles, sweat glands, sebaceous glands and other skin accessories. Here we found that regardless of normal rat skin or diabetic skin, the components of RAS, including AGT, AngII, AT1, and AT2 receptors were all expressed in

skin tissues. AT1 receptor could be found in sebaceous glands and ducts in dermis and membrane of hair follicle cells; and AT2 receptor could be found in sebaceous glands in epidermis and dermis, membrane and cytoplasm of hair follicle cells. All indicated that skin is not just a source of AngII but also its target organ, which would have made autocrine or paracrine regulatory mechanisms come into play independently of the level of AngII in circulation [6].

Many researches demonstrated that the local RAS directly linked to a variety of pathological changes. It has been found that activation of local RAS is in direct association with many chronic diabetic complications [10–16]. However, the effects of AngII and its receptors in diabetic skin, the largest organ in body, have not been fully characterized. In view of the potential physiological role of a cutaneous RAS, we hypothesized that it may link to mechanisms of tissue repair and remodeling. During the regulation of cell proliferation and differentiation, AT1 and AT2 receptors seem to counteract with each other [6, 10–12, 17]. Consequently, in skin tissues expressing both AT1 and AT2 receptors, AngII may establish its own regulatory system depending on different expression of these two receptor subtypes. Our study showed that local expression of AGT, AngII, and AT1 receptor was significantly increased in diabetic skin, while the expression of AT2 receptor was decreased, and the ratio of AT1 to AT2 receptor expression was about three times the level in normal rats. AT1 receptor has been shown to mediate the stimulation of proliferation, migration, and collagen synthesis in diverse cell types [11, 18, 19]. In AT1 receptor gene knock-out mouse, wound healing of skin was delayed [20, 21]. This proliferative effect is counteracted by the AT2 receptor, which can further initiate cell differentiation and apoptosis. Stimulating AT1 receptor could also cause increased collagen synthesis and reduced degradation, while the reverse could happen if stimulating AT2 receptor [4].

From these, one can speculate that in diabetic skin, proliferation of cells and increase of collagen synthesis would appear in the epidermis and dermis. However, this speculation was not consistent with what we actually observed in histological changes. In fact, the thickness of diabetic skin was significantly thinner, with unclear epidermal layers and atrophic dermal layers, and the arrangement of fiber bundles in connective tissues was also in disorder. The counterintuitive findings may be attributable to the following facts. Firstly, diabetic skin was of ischemia and hypoxia under the influence of peripheral neuropathy and peripheral vascular lesion [3, 22], so nutrients suitable for cell proliferation and collagen synthesis were deficient, leading to thinning of skin and decrease of collagen. In the present study, we also measured and compared the proliferation ability of isolated primary fibroblasts between diabetic and normal rats. The results showed that fibroblasts from diabetic rats had the decreased proliferation ability as compared with normal control, which may support our hypothesis that it is the ischemia and hypoxia environment interfere the proliferation of cells in diabetic skin but not the defect of local RAS of the cells. Secondly, infiltration of inflammatory cells, stimulation with high glucose and oxidative stress increased local "destructive" factors, which may antagonize the biological effect of the local RAS activation [23], and eventually caused formation of "invisible injury" in diabetic skin In addition, the local "invisible injury" and the disordered growth factors may play compensatory roles through stimulating more AT1 receptor expression. The pathological changes of "invisible injury" in diabetic skin may be conceived to be the results of decompensation. Nevertheless, the exact mechanism will require further investigation.

All taken together, skin is not just a source of AngII but also its target organ. In diabetes, the local RAS of skin was activated, which may play a part in the pathological changes of diabetic skin.

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