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Expression changes of angiotensin II pathways and bioactive mediators during human preadipocytes-visceral differentiation

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Abstract

Obesity is a worldwide serious health problem; and it may result in a wide range of complications, such as hypertension and diabetes mellitus. As a consequence, molecular identification on the differentiation of preadipocytes and the generation of bioactive mediators is crucial in understanding the formation and development of obesity and obesity-associated health problems. In addition, exhaustive exhibition and purposeful control of adipocytes formation also play critical roles in the plastic and reconstructive surgical procedures. The primary purpose of this study was to exhibit the expression changes of angiotensin II (Ang II) pathways and 2 vital adipokines, leptin and resistin, during human preadipocytes-visceral differentiation by real-time quantitative reverse transcription–polymerase chain reaction. The present result indicated that the generation of Ang II during preadipocytes differentiation was achieved through both renin-angiotensin system pathway and non–renin-angiotensin system pathways, and the latter may be more important in this process. Gene expression of Ang II receptor type 1 and 2 increased in the initial phase of differentiation and then quickly decreased after 9 days. Moreover, the expression of both leptin and resistin increased significantly during preadipocyte-adipocyte conversion. The present work provided a fundamental understanding of human visceral preadipocytes differentiation molecularly. It may promote the understanding of obesity and obesity-associated diseases to some extent. However, there is still a long way to go to treat obesity and its complications effectively; and more efforts should be devoted urgently.

1. Introduction

Preadipocytes are fibroblast-like precursor cells of mature adipocytes. During the process of preadipocyte-adipocyte conversion or differentiation, preadipocytes accumulate massive triacylglycerols (TAG) and lipid vacuoles, and become rounded in shape [1]. In the present work, cellular morphology, content of intracytoplasmic lipids, and activity of glycerol-3-phosphate dehydrogenase (G3PDH) were detected during human preadipocytes-visceral (HPA-v) differentiation. The results demonstrated that the regularity of intracytoplasmic lipids accumulation was the same as the one of the increase of G3PDH activity during HPA-v differentiation.

Obesity, specifically visceral adiposity, is a serious health problem around the world; and it has close connections with a large number of terrible diseases, such as cardiovascular diseases, diabetes mellitus, and so on [2-4]. On the other hand, a large number of plastic and reconstructive surgical procedures are performed every year to repair adipose tissue defects that are caused by traumatic injury, tumor resection, and congenital defects with a large volume loss of adipose tissue typically [5]. Owing to their proliferative capability, preadipocytes are considered more advantageous than mature adipocytes in adipose tissue engineering application for restoring defect sites [6]. As a consequence, molecularly exhaustive exhibition and purposeful control of adipogenesis and preadipocytes differentiation may strongly promote the understanding and treatment of obesity and obesity-associated diseases, as well as the strategy establishment for adipose tissue engineering application.

The renin-angiotensin system (RAS) or renin-angiotensin-aldosterone system is a well-recognized hormone system that serves as a critical modulator in regulating systemic blood pressure and renal electrolyte homeostasis [7]. In addition to the systemic RAS, components of RAS, or rather local RAS or tissue RAS, also can be found in various

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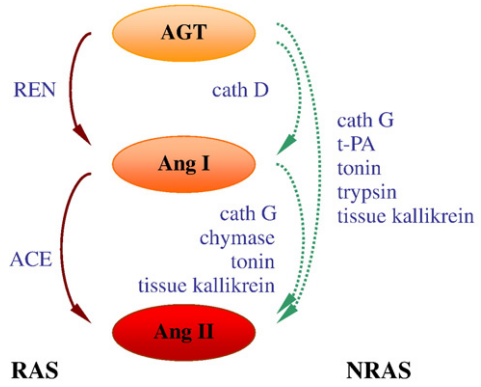

Fig. 1. Pathways for Ang II generation. The classic pathway (RAS) for Ang II generation is composed of sequential cleavages of AGT by the proteolytic enzymes REN and ACE (left, brown real line). Alternative pathways (NRAS) for Ang II production are shown on the right (green dotted line). Enzymes of alternative pathways include cath D, cath G, chymase, tissue plasminogen activator, tonin, and so on. t-PA indicates tissue plasminogen activator.

tissues, such as brain, heart, kidney, adipose tissue, and so on [8-11]. Previous researches have indicated that the local RAS was involved in the pathologic changes of organ development and function by influencing a series of biological processes. [12-14]. It was suggested that angiotensin II (Ang II), the final effector of RAS, is implicated in preadipocytes differentiation and obesity development for its contribution in increasing TAG content and lipogenic enzymes activity significantly [15-17]. Classically, the octapeptide Ang II is derived from angiotensinogen (AGT) through the sequential cleavage of renin (REN) and angiotensin-converting enzyme (ACE) with a decapeptide intermediate angiotensin I (Ang I) (Fig. 1). Finally, Ang II exerts its effects by binding to its receptor, angiotensin II receptor type 1 (AT_1R) or 2 (AT_2R). Moreover, the physiologically active component Ang II can also be generated by the enzymes of non–renin-angiotensin system (NRAS), such as cathepsin D (cath D), cathepsin G (cath G), tonin, and chymase [18-20] (Fig. 1).

In the present study, expression changes of AGT, enzymes required for the angiotensin conversion, and Ang II receptors were detected during HPA-v differentiation by real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR). The present data demonstrated that the expression of AGT was in direct proportion to the differentiation time and that the messenger RNA (mRNA) level of AGT in mature adipocytes (day 15) was more than 3 times that of preadipocytes (day 0). Among the enzymes of Ang II pathways, the expression of alternative pathways (NRAS) enzyme, cath D, increased significantly during HPA-v differentiation. However, the mRNA levels of classic pathway (RAS) enzymes, REN and ACE, of differentiating HPA-v were lower than that of preadipocytes on day 0. Consequently, it was suggested that NRAS may play a more important role in Ang II generation during HPA-v differentiation than RAS does. The results also indicated that the expression of both AT_1R and AT_2R increased in the initial stage (0-6 days) and decreased in later stage (>6 days) during preadipocyte-adipocyte conversion. It was worth noting that the mRNA levels of both these 2 receptors of mature adipocytes (day 15) were lower than that of the control group (day 0). Considering the results in the present work, we speculated that AT_1R mediated the negative feedback regulation of REN expression, whereas AT_2R presented a positive effect on lipogenesis by mediating the biological effects of Ang II on increasing the content of TAG and the activity of lipogenic enzymes.

Leptin (LEP), the 16-kd product of obese (*ob*) gene, is secreted mainly from adipocytes and regulates ingestive behavior as well as energy balance by acting on the central neural networks [21,22]. It is generally recognized that LEP takes its physiologic effects as decreasing food intake and increasing energy expenditure through the signal transduction between brain and adipose tissue, leading to the reduction of body weight finally [23-26]. Several researches, however, showed that LEP can promote the proliferation and differentiation of preadipocytes [27-29]. In addition to LEP, conflicting results of the effects on preadipocytes differentiation also reside in resistin (RES), another adipocyte-derived cysteine-rich secretory signaling molecule. Kim et al [30] showed that RES inhibited the differentiation of 3T3-L1 preadipocytes, whereas Gong et al [31] exhibited that the differentiation of 3T3-L1 preadipocytes was promoted by RES. For the interesting roles of these 2 adipokines in preadipocytes differentiation, mRNA levels of LEP and RES were also investigated during the process of HPA-v differentiation. The result here indicated that both the expression of these 2 adipokines increased markedly during preadipocyte-adipocyte conversion. Basing on the analysis of the present data, we hypothesized that both these 2 adipokines had a negative effect on the adipogenesis during HPA-v differentiation.

2. Materials and methods

2.1. Preadipocytes culture and differentiation

Human preadipocytes-visceral were purchased from ScienCell Research Laboratories (San Diego, CA) and incubated in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum at 37°C under a humidified atmosphere with 5% CO_2. Cells were stimulated to differentiate in differentiation medium (DMEM with 500 nmol/L insulin, 250 nmol/L dexamethasone, 1 nmol/L triiodothyronine, and 100 nmol/L hydrocortisone) for 24 hours. Under this condition, 70% of preadipocytes were able to differentiate. Subsequently, medium was changed with DMEM containing 10% fetal bovine serum; and cells were cultured for 0 to 15 days according to the requirement of investigation. All cells were cultured in 6-well plates (3.5-cm–diameter well) with a medium change every 2 days. Cells were observed using inverted phase contrast microscope (Leica DM IRB).

Table 1
Real-time quantitative PCR primers and templates

Gene products	Forward primers (5′-3′)	Reverse primers (5′-3′)	Annealing temperature (°C)	Size (base pairs)	GenBank accession numbers of templates
AGT[a]	CTTCACTGAGAGCGCCTGC	GAGACCCTCCACCTTGTCCA	60	73	NM_000029
REN	CTCCGTGATCCTCACCAACT	CTGTGTCACCGTGATTCCAC	57	295	NM_000537
ACE	CGGTCTCCACTCCTGAACAT	GCATCAAAGTGGGTTTCGTT	56	265	NM_000789
AT_1R	GGAAACAGCTTGGTGGTGAT	TGGGTGAACAATAGCCAGGT	57	267	S77410
AT_2R	AGAAGCTCCGCAGTGTGTTT	CAATGGGCAATTCCTGAAAGT	57	285	U20860
Cath D	GACACAGGCACTTCCCTCAT	TAGTAGCGGCCGATGAAGAC	59	299	NM_001909
Cath G	AAACACCCAGCAACACATCA	CTGCCTATCCCTCTGCACTC	57	265	NM_001911
LEP	GGCTTTGGCCCTATCTTTTC	CCAGGTCGTTGGATATTTGG	57	466	D63519
RES	CCATGGAAGAAGCCATCAAT	CTGGCAGTGACATGTGGTCT	55	209	AF323081
β-Actin	GATCATTGCTCCTCCTGAGC	CACCTTCACCGTTCCAGTTT	58	308	NM_001101

[a] Primers of AGT were designed as previous described [35].

2.2. Intracytoplasmic lipids measurement

Intracytoplasmic lipids were quantitated according to the method of Ramírez-Zacarías et al [32]. Cultures were washed directly in 6-well plates 3 times with phosphate buffer solution (PBS, pH 7.4) and then fixed with 10% formalin in isotonic PBS for at least 30 minutes. Subsequently, cells were washed with 500 μL 60% isopropanol. After about 5 minutes, isopropanol was evaporated; and adipocytes were stained with 300 μL working solution of oil red O for 2 hours. Afterward, the stain solution was removed; and cells were rinsed in 500 μL of 60% isopropanol for 5 seconds. To extract dye, 700 μL of 60% isopropanol was added per well; and the sealed plates were shaken for 2 hours. Finally, the absorbance of extracted dye was monitored spectrophotometrically at 510 nm. The working solution of oil red O was prepared as follows: 0.5 g oil red O was dissolved in 100 mL absolute isopropanol at 60°C for 1 hour, and then the solution was filtered using neutral filter paper. Finally, the working solution of oil red O was obtained by mixing 6 mL previous solution with 4 mL deionized water completely and undergoing a filtration subsequently.

2.3. G3PDH activity assay

After being washed with PBS (pH 7.4) 3 times, HPA-v cultured in 6-well plates were harvested by scraping into 200 mL of ice-cold 50 mmol/L Tris-HCl solution (pH 7.5) containing 1 mmol/L EDTA and 100 nmol/L β-mercaptoethanol; and then the mixture was transferred to prechilled microtube. Subsequently, cells were disrupted by sonication in an ice-water bath; and then the crude cell extract was centrifuged at 12 000*g* for 15 minutes at 4°C. Assay for G3PDH activity was performed as previous described [33]. The G3PDH activity of supernatant was assayed in a solution with a final concentration of 100 mmol/L triethanolamine-HCl (pH 7.5), 2.5 mmol/L EDTA, 0.12 mmol/L reduced form of nicotinamide-adenine dinucleotide, 100 nmol/L β-mercaptoethanol, and 0.2 mmol/L dihydroxy acetone phosphate (DHAP). Absorbance at 340 nm was monitored spectrophotometrically at 37°C with an interval of 30 seconds for 3 minutes. The protein concentration of supernatant was determined by Bradford protein assay using bovine serum albumin as protein standard [34]. Unit of measurement of G3PDH activity was milliunits per milligram of supernatant protein, and 1 mU of enzyme activity represented that 1 nmol reduced form of nicotinamide-adenine dinucleotide was completely oxidized in 1 minute.

2.4. Gene expression analysis

Cells exposed to differentiation medium in 6-well plates for 0, 3, 6, 9, 12, and 15 days were rinsed 3 times with ice-cold PBS (pH 7.4). After that, cells were lysed directly in 6-well plates (3.5-cm–diameter well) with 1 mL TRIzol Reagent (Invitrogen, Shanghai, China) accompanied with a mix by a pipette. After being incubated for 5 minutes at ambient temperature (15°C-30°C), the cell lysate was centrifuged at 12 000*g* for 10 minutes at 4°C. According to the manufacturer's instruction, the RNA of HPA-v was obtained after processing the supernatant with chloroform, isopropanol, and ethanol (75%) sequentially. The potential DNA contamination in RNA was eliminated by treating with deoxyribonuclease. Finally, the quality of RNA was checked by running on 1% agarose gel with ethidium bromide.

The RT reaction and real-time quantitative PCR for the quantitative analysis of gene expression were performed using PrimeScript RT-PCR Kit (Perfect Real Time) (TaKaRa) and SYBR PrimeScript RT-PCR Kit (Perfect Real Time) (TakaRa, Shanghai, China), respectively, following the manufacturer's protocol. Primers for RT reaction were oligo dT primer and random 6-mers. The real-time quantitative PCR was performed with the LightCycler1.5 (Roche Diagnostics, Mannheim, Germany), and the sequences of gene-specific primers as well as the GenBank accession numbers of templates are given in Table 1. Expression analysis was performed for the genes encoding AGT, REN, ACE, AT_1R, AT_2R, cath D, cath G, LEP, and RES, as well as for the housekeeping gene encoding β-actin as the endogenous control [36]. The data of real-time quantitative PCR were analyzed according to the method of Livak and Schmittgen [37]. The mRNA levels of target genes were normalized to that of β-actin, and the fold changes of mRNA levels were

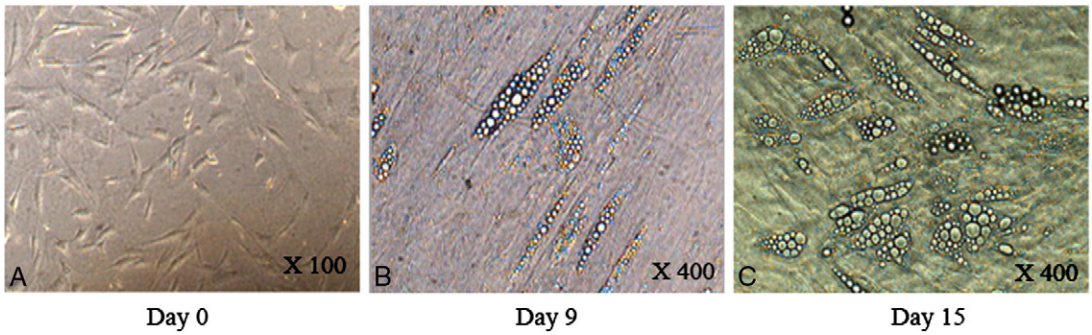

Fig. 2. Photomicrographs of HPA-v during differentiation. Cellular morphology of HPA-v has been observed during HPA-v differentiation. Morphologic character of HPA-v without differentiation is exhibited in photomicrograph A (magnification, ×100); and HPA-v that have been stimulated to differentiate for 9 and 15 days are exhibited in photomicrographs B and C, respectively (magnification, ×400). As shown in these photomicrographs, cells accumulate lipid vacuoles and become rounded in shape during the process of preadipocyte-adipocyte conversion.

presented as $2^{Ct(target) - Ct(\beta\text{-actin})}/2^{Ct(target,\ 0\ day) - Ct(\beta\text{-actin},\ 0\ day)}$. Single amplification of each PCR product was verified by melting curve analysis and agarose gel electrophoresis.

2.5. Statistical analysis

Data are presented as mean value ± standard deviation. Statistically significant differences between groups were assessed using Student *t* test and analysis of variance. *P* value less than .05 was defined as significant difference, whereas difference was considered extremely significant at *P* value less than .01.

3. Results

3.1. Cellular morphology of HPA-v

Cellular morphology of HPA-v in the process of differentiation is exhibited in Fig. 2. As shown in these photomicrographs, HPA-v accumulated lipid vacuoles and became rounded in shape during the process of preadipocyte-adipocyte conversion.

3.2. Intracytoplasmic lipids and G3PDH activity increase during cellular differentiation

Intracytoplasmic lipids and G3PDH activity have been detected during the process of HPA-v differentiation. The data indicated that a positive correlation yielded between the amount of intracytoplasmic lipids and the period of cellular differentiation, and the growth rate of lipids accumulation became slow after 12 days (Fig. 3). The lipids content of differentiating HPA-v reached extremely significant difference compared with that of the control group (day 0) ($P < .01$). The intracytoplasmic lipids of mature adipocytes (day 15) was about 20 times that of the control group (day 0) (0.20 ± 0.02 vs 0.01 ± 0.00, $P < .01$). A same regularity appeared between G3PDH activity and differentiation time during the

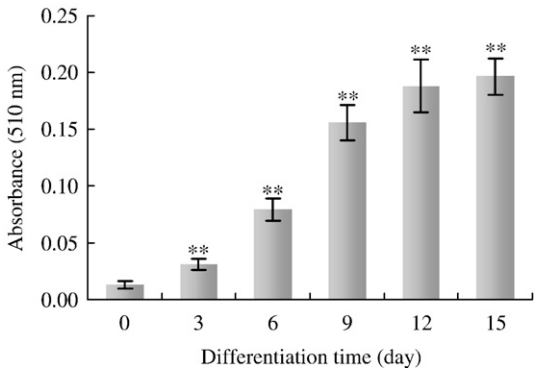

Fig. 3. Intracytoplasmic lipids accumulation during HPA-v differentiation. The HPA-v intracytoplasmic lipids content increased considerably in direct proportion to the differentiation time, and the growth rate became slow after 12 days. In sum, the present data demonstrated that the increased lipids content of differentiating cells reached extremely significant difference compared with that of the control group ($P < .01$). Results are presented as mean ± standard deviation of 3 independent experiments (columns, means of 3 independent experiments; error bars, standard deviations; $^{**}P < .01$, compared with the control group HPA-v on day 0).

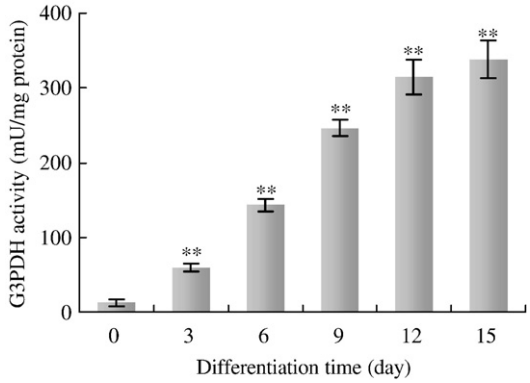

Fig. 4. The G3PDH activity assay during HPA-v differentiation. As exhibited, the G3PDH activity was in direct proportion to the differentiation time. The regularity that appeared here was the same as the one of intracytoplasmic lipids accumulation during HPA-v differentiation (Fig. 3). Extremely significant difference was found statistically between differentiating cells and the control group ($P < .01$). Assays were performed with 3 independent experiments, and results are expressed as mean ± standard deviation (columns, means of 3 independent experiments; error bars, standard deviations; $^{**}P < .01$, compared with the control group HPA-v on day 0).

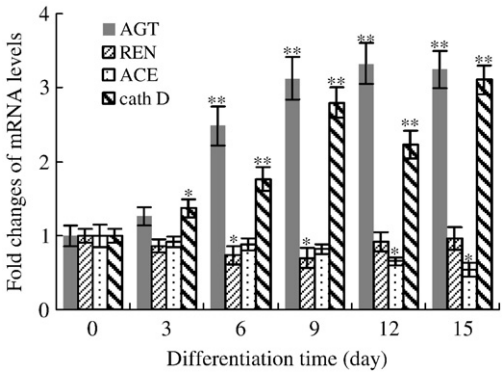

Fig. 5. Expression analysis of Ang II pathways components (AGT, REN, ACE, and cath D). The present data exhibited that mRNA level of AGT was directly proportional to the differentiation time. The AGT expression of HPA-v reached extremely significant difference statistically after differentiation for more than 6 days compared with that of the control group ($P < .01$). Unexpectedly, the expression of both REN and ACE was lower in differentiating HPA-v than that of the control group. Instead, the mRNA level of NRAS enzyme cath D increased significantly ($P < .05$) or even extremely significantly ($P < .01$) during HPA-v differentiation compared with the control group. The mRNA levels of target genes were normalized to that of β-actin, and the fold changes of mRNA levels were presented as $2^{Ct(target) - Ct(\beta\text{-actin})}/2^{Ct(target,\ 0\ day) - Ct(\beta\text{-actin},\ 0\ day)}$. Data are expressed as mean ± standard deviation of 3 independent experiments, and each error bar shows the corresponding standard deviation (*$P < .05$ and **$P < .01$, compared with the control group HPA-v on day 0).

process of adipogenesis of HPA-v (Fig. 4). G3PDH is the enzyme that catalyzes the formation of glycerol-3-phosphate (G3P) from DHAP. Because of the low activity of glycerokinase, G3PDH may play an important role in the generation of G3P for TAG accumulation in human adipose tissue [38,39]. During HPA-v differentiation, extremely significant difference was observed statistically between differentiating cells and the control group (day 0) ($P < .01$); and the G3PDH activity of mature adipocytes (day 15) was more than 27 times that of the control group (day 0) (338.21 ± 25.44 vs 12.33 ± 4.05, $P < .01$) (Fig. 4). On the whole, intracytoplasmic lipids accumulation was directly proportional to the activity of G3PDH.

3.3. Expression analysis of Ang II pathways and its receptors

Angiotensin II, the bioactive octapeptide, is generated from AGT directly or via intermediate Ang I by the cleavages of a series of enzymes. In the classic pathway (RAS), the generation of Ang II is achieved under the cleavages of REN and ACE sequentially, whereas in alternative pathways (NRAS), Ang II is produced by the catalysis of different enzymes, such as cath D, cath G, chymase, and so on. Physiologic functions of Ang II become effective through different signal transduction pathways by binding to the cell surface G protein–coupled receptors AT_1R and AT_2R [40].

In the present work, expression changes of the components of Ang II pathways and the Ang II receptors were detected by real-time quantitative RT-PCR during the process of human visceral preadipocytes differentiation. The results demonstrated that mRNA level of AGT increased in direct proportion to the differentiation time (Fig. 5). Angiotensinogen expression of HPA-v reached extremely significant difference statistically after differentiation for more than 6 days compared with that of the control group (day 0) ($P < .01$); the growth rate slowed down after 12 days. The mRNA level of AGT in mature adipocytes (day 15) was more than 3 times that of preadipocytes on day 0 (3.25 ± 0.25 vs 1.00 ± 0.14, $P < .01$). Surprisingly, among the enzymes in RAS, the expression of both REN and ACE of differentiating HPA-v was lower than that of the control group (day 0) (Fig. 5). Furthermore, significant difference of decreased REN expression was found on days 6 and 9 compared with that of the control group (day 6 vs day 0: 0.73 ± 0.13 vs 1.00 ± 0.10, $P < .05$; day 9 vs day 0: 0.69 ± 0.14 vs 1.00 ± 0.10, $P < .05$), whereas the decreased mRNA level of ACE reached significant difference statistically on days 12 and 15 vs that of the control group (day 12 vs day 0: 0.65 ± 0.05 vs 1.00 ± 0.15, $P < .05$; day 15 vs day 0: 0.54 ± 0.10 vs 1.00 ± 0.15, $P < .05$). On the contrary, the expression of NRAS enzyme cath D increased in direct proportion to the differentiation time (Fig. 5). Significant difference ($P < .05$) or extremely significant difference ($P < .01$) of cath D mRNA level was found statistically between differentiating cells and the control group (day 0). The expression of cath D on day 15 was more than twice higher than that of the control group (day 0) (3.10 ± 0.19 vs 1.00 ± 0.09, $P < .01$). However, we failed to detect the expression of another NRAS enzyme, cath G, in HPA-v differentiation. During the analysis of Ang

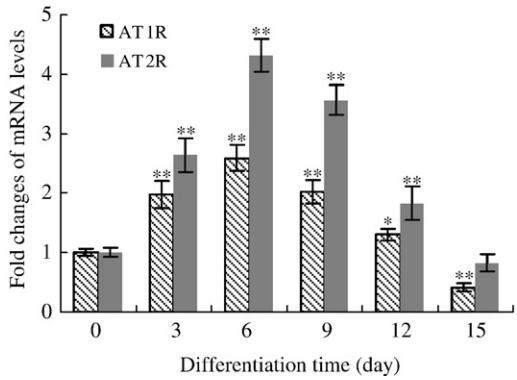

Fig. 6. Analysis of mRNA levels of the Ang II receptors (AT_1R and AT_2R). As shown, both the Ang II receptors AT_1R and AT_2R increased in the initial stage of differentiation (0-6 days) and then decreased in later stage (>6 days) during HPA-v differentiation. It was interesting that the expression of AT_1R and AT_2R of mature adipocytes (day 15) was lower than that of the control group; even the mRNA level of AT_1R decreased in extremely significant difference compared with that of the control group ($P < .01$). The mRNA levels of target genes were normalized to that of β-actin, and the fold changes of mRNA levels were presented as $2^{Ct(target) - Ct(\beta\text{-actin})}/2^{Ct(target,\ 0\ day) - Ct(\beta\text{-actin},\ 0\ day)}$. Data are expressed as mean ± standard deviation of 3 independent experiments, and each error bar shows the corresponding standard deviation (*$P < .05$ and **$P < .01$, compared with the control group HPA-v on day 0).

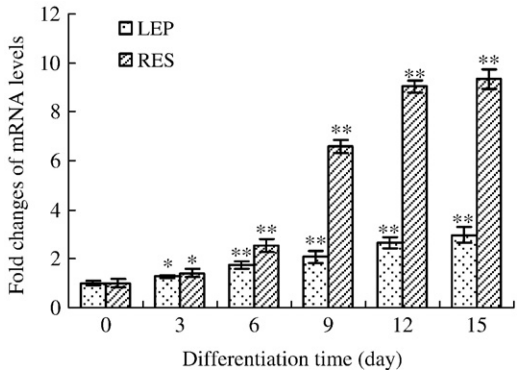

Fig. 7. Fold changes of LEP and RES mRNA levels. The present results indicated that in both the 2 adipokines, LEP and RES, mRNA levels increased along with the elapsing of differentiation time. Significant difference ($P < .05$) or extremely significant difference ($P < .01$) was observed on the mRNA levels of both LEP and RES between differentiating cells and preadipocytes on day 0. The mRNA levels of target genes were normalized to that of β-actin, and the fold changes of mRNA levels were presented as $2^{Ct(target) - Ct(\beta\text{-actin})}/2^{Ct(target,\ 0\ day) - Ct(\beta\text{-actin},\ 0\ day)}$. Results are presented as mean ± standard deviation of 3 independent experiments, and each error bar shows the corresponding standard deviation (*$P < .05$ and **$P < .01$, compared with the control group HPA-v on day 0).

II receptors (AT_1R and AT_2R) expression, mRNA levels of both AT_1R and AT_2R increased in the initial stage (0-6 days) and decreased in later stage (>6 days) in preadipocyte-adipocyte conversion (Fig. 6). The highest mRNA level of AT_1R (day 6) was more than twice that of the control group (day 0) (2.59 ± 0.22 vs 1.00 ± 0.06, $P < .01$), whereas the highest mRNA level of AT_2R (day 6) was more than 3 times higher than that of the control group (day 0) (4.31 ± 0.27 vs 1.00 ± 0.08, $P < .01$). Interestingly, the expression of both AT_1R and AT_2R of mature adipocytes (day 15) is lower than that of the control group (day 0); even extremely significant difference was observed between the mRNA level of AT_1R on day 15 and day 0 (0.42 ± 0.06 vs 1.00 ± 0.06, $P < .01$).

3.4. Gene expressions of LEP and RES during HPA-v differentiation

Adipocytes are endocrine cells that secrete a number of adipokines that are implicated in the regulation of various biological and physiologic processes. It is generally acknowledged that both the 2 adipocyte-derived hormones LEP and RES inhibited the proliferation and differentiation of preadipocytes as described in the "Introduction" section. However, contradictory opinions came up from several groups. In the present study, the expression of both LEP and RES was detected during the differentiation of HPA-v. The data showed that the mRNA levels of both LEP and RES increased in direct proportion to the differentiation time. The expression of LEP and RES of differentiating HPA-v reached significant difference ($P < .05$) or extremely significant difference ($P < .01$) after differentiation for more than 3 days compared with that of the control group (Fig. 7). The mRNA level of LEP in mature adipocytes (day 15) was about 3 times that of the control group (day 0) (2.97 ± 0.32 vs 1.00 ± 0.10, $P < .01$), whereas the mRNA level of RES in mature cells (day 15) was more than 8 times higher than that of the control group (day 0) (9.32 ± 0.40 vs 1.00 ± 0.17, $P < .01$).

4. Discussion

Owing to the high-calorie and high-fat diet as well as the decreased energy expenditure, obesity has become a high prevalence around the world. According to the announcement of Prof Philip James, the chair of the International Obesity Task Force, there were 1.7 billion people who were overweight or obese all over the world by estimate [41]. In obese individuals, adipose tissue secretes a great variety of bioactive mediators or adipokines that are involved in the regulation of a variety of biological and physiologic processes and influence the whole-body homeostasis finally [42]. It is generally believed that obesity, specifically the visceral adiposity, is a symptom that is associated with a considerable variety of serious medical conditions, including cardiovascular diseases, strokes, diabetes mellitus, asthma, cancer, and so on [2-4,43,44]. As a result, obesity is haunting a large number of individuals around the world, particularly in economically developed regions. Unfortunately, the mechanisms of the immoderate accumulation of TAG, the generation and function of bioactive mediators in adipocytes, as well as the development of obesity are still poorly understood. Consequently, molecular identification of adipogenesis and obesity development is urgent and crucial in treating the worldwide obesity and obesity-associated health problems. In addition, exhaustive display and purposeful control of adipocytes formation also play a critical role in the plastic and reconstructive surgical procedures.

Preadipocytes, the fibroblast-like precursor cells of mature adipocytes, accumulate TAG and lipid vacuoles massively, as well as become rounded in shape during the process of differentiation [1]. The cytoplasmic enzyme G3PDH plays a vital role in TAG synthesis as catalyzing the conversion of DHAP to G3P, an obligatory intermediary for TAG formation. Jones et al [15] demonstrated that increased activities of both G3PDH and fatty acid synthase (FAS) were observed along with the TAG accumulation in adipocytes, whereas Swierczynski et al [38] indicated that increased TAG accumulation was attributed to the elevated activity of G3PDH, not FAS, in the adipose tissue of obese individuals. In any case, a positive correlation was displayed between the G3PDH activity and the TAG accumulation. Congruously, this study indicated that the accumulation of intracytoplasmic lipids and the activity of G3PDH increased following the same regularity along with the elapsing of differentiation time (Figs. 3 and 4).

Generally, it is known that the critical regulatory system, circulating RAS or systemic RAS, plays a vital role in maintaining the homeostasis of electrolyte and blood pressure.

Local RAS, or tissue RAS, whose components present in several certain cells or tissues, operates independently or semi-independently of the systemic RAS. A number of studies have indicated that local RAS is implicated in the pathologic changes of organ development and function by its influences on a variety of biological processes, such as gene expression, growth, inflammatory response, and so on [13,14]. Considering the previous studies, it was suggested that adipose tissue hosts a local RAS [10,11,14]. Angiotensin II, the final bioactive octapeptide of RAS, is generated from AGT under the catalysis of enzymes of classic pathway (RAS) or alternative pathways (NRAS) (Fig. 1). It was suggested that Ang II is involved in preadipocytes differentiation and obesity development by increasing TAG content and lipogenic enzymes activity significantly [15-17]. Moreover, adipocytes RAS may contribute to the systemic RAS and was related to the obesity-associated health problems, such as hypertension and kidney disease [11,45].

Consistent with the previous observations [35], the present study indicated that the gene expression of AGT, the only known precursor of Ang II, was elevated significantly during the process of HPA-v differentiation (Fig. 5). In contrast, it was interesting that the mRNA levels of both RAS enzymes REN and ACE of differentiating HPA-v were lower than that of the preadipocytes on day 0. It was considered that the RAS is a classic endocrine feedback regulator system that regulates the formation and secretion of RAS components [46]. According to the previous researches, it was considered that the expression of REN was suppressed by the feedback regulation of Ang II mediated primarily by AT_1R [47,48]. In conformity with previous result that the differentiation of human mesenchymal stem cell to adipocytes was associated with a deceased expression of ACE [49], the mRNA level of ACE decreased continuously along with the increase of differentiation time (Fig. 5). Because of the decrease of ACE expression, a similar regulatory mechanism may underlie the expression of ACE in adipocytes. Previous investigation has showed that Ang II formation increased during the differentiation of human preadipocytes [50]. To achieve the generation of Ang II for the HPA-v differentiation, alternative pathways for Ang II formation are necessary. In the present study, the mRNA level of cath D, an enzyme of NRAS, increased markedly during the process of HPA-v differentiation (Fig. 5). It is generally known that cath D, a peptidase that belongs to the family of aspartic peptidases, exerts its physiologic effects as hormone and antigen processing [51]. It was suggested that adipose tissue produces cath D possessing the REN-like activity as cleaving the precursor AGT to produce Ang I [52,53]. Lavrentyev et al [54] indicated that cath D mRNA silencing caused a noticeable decrease of Ang I and Ang II in rat vascular smooth muscle cells. In sum, because of the decreased expression of both REN and ACE and significantly elevated expression of cath D, we speculated that Ang II generation during human visceral preadipocytes differentiation was achieved through both RAS and NRAS pathways, and NRAS pathways may be the dominant routes responsible for this process.

As described earlier, Ang II starts its biological effects on different signal transduction pathways by binding to the cell surface G protein–coupled receptors AT_1R and AT_2R [40]. During the analysis of Ang II receptors expression, the mRNA levels of both AT_1R and AT_2R increased in the initial stage of differentiation (0-6 days) and then decreased to a very low level gradually during HPA-v differentiation (Fig. 6). As mentioned previously, AT_1R mediates the feedback regulation of Ang II to suppress the expression of REN [47,48]. Considering the results in Figs. 5 and 6, an inverse correlation was exhibited between the expression of REN and AT_1R during HPA-v differentiation. Consequently, we speculated that decreased REN expression was attributed to the negative feedback regulation mediated by AT_1R. Jones et al [15] considered that AT_2R mediated the biological effects of Ang II leading to the increase of TAG and the activity of lipogenic enzymes (G3DPH and FAS) during preadipocytes differentiation. As observed (Figs. 3 and 4), intracytoplasmic lipids content and G3PDH activity increased significantly in the initial stage (0-12 days); and the growth rate became slow after 12 days. As shown in Fig. 6, the mRNA level of AT_2R increased firstly (0-6 days) and then decreased (>6 days) during HPA-v differentiation. In consideration of the reaction time delay, intracytoplasmic lipids content and G3PDH activity increased in direct proportion to the expression of AT_2R. Namely, the present results supported that AT_2R presented a positive effect on lipogenesis during HPA-v differentiation by mediating the biological effects of Ang II. Because of the importance of Ang II receptors in preadipocytes differentiation, the regulatory mechanism for the expression of Ang II receptors should be clarified in future studies.

Adipocytes are endocrine cells that secrete more than 50 different adipokines, so far as is known [11]. Adipokines are the collective designation of adipocyte-derived factors including LEP, RES, adiponectin, interleukin-6, and so on [55]. Generally, both the adipokines LEP and RES inhibited adipogenesis and preadipocytes differentiation [23-26,30]. However, opposite results were also displayed by several groups [27-29,31]. The results here indicated that the expression of both LEP and RES increased along with the elapsing of differentiation time and that the growth rate became even higher after 9 days. Considering that the growth rate of both intracytoplasmic lipids content and G3PDH activity is high at first (0-12 days) and became slow subsequently (>12 days), we hypothesized that both these 2 adipokines, specifically the RES, presented a negative influence on the adipogenesis during HPA-v differentiation. Furthermore, inhibition analysis should be preformed urgently in future studies for the unambiguous functions of LEP and RES in preadipocytes differentiation.

Because of the high energy–dense food and the lack of physical exercise, obesity has become a worldwide health

problem. It is worrying that more and more studies support that obesity has close relations with a wide range of terrible diseases. However, the mechanisms of the excessive accumulation of lipid in adipocytes and the development of obesity are still unclear and inconsistent. Besides, well-defined mechanism of preadipocytes differentiation will also play a critical role in adipose tissue engineering to meet the high demand of plastic and reconstructive surgical procedures nowadays. In this study, expression changes of Ang II pathways and 2 adipocyte-derived bioactive mediators were detected during human visceral preadipocytes differentiation. It provided a fundamental understanding of adipocytes generation and may promote the understanding of obesity and obesity-associated diseases to a certain degree. However, there is still a long way to go to understand the mechanisms of formation and development of obesity and its complications. More efforts should be devoted urgently to clarify the mechanisms of these diseases and to establish effective strategies to treat them.

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