Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 972–977
Published online 28 March 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.1035

JOURNAL OF PEPTIDE SCIENCE

Differences in the amount of lipolysis induced by atrial natriuretic peptide in small and large adipocytes

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Received 7 December 2007; *Revised 6 February 2008*; *Accepted 14 February 2008*

Abstract: Atrial natriuretic peptide (ANP) is a 28-amino acid polypeptide that is primarily secreted by the heart. ANP is believed to be a hormone that regulates cardiovascular dynamics and renal functions; however, studies conducted in the past few decades revealed that ANP is also a potent lipolytic agent in human adipocytes that functions through the cGMP-dependent pathway. In this study, we separated human adipocytes within the same fat depot into small and large fractions using their floating properties and nylon filters of different pore sizes. Real-time PCR revealed that large adipocytes expressed higher mRNA levels of natriuretic peptide receptor (NPR)-A and hormone sensitive lipase, and binding studies showed that large adipocytes expressed more NPR-A on the membrane than small adipocytes. This finding was confirmed by the increase in the amount of glycerol that was released from adipocytes as the cell size increased. Taken together, these results clearly suggest that adipocyte size is an important determinant of ANP-stimulated lipolysis. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: ANP; lipolysis; adipocyte size; NPR-A; HSL

INTRODUCTION

ANP is a 28-amino acid polypeptide with a Cys^7–Cys^{23} bridge that was first isolated from human atrium in 1984 [1]. This peptide is primarily synthesized as a preprohormone in the atrium, and then stored as a prohormone until it is released into the plasma after cardiomyocyte stretch [2]. ANP is believed to be a hormone that directly regulates salt excretion, induces vasorelaxation, inhibits the renin–angiotensin–aldosterone system, and consequently participates in blood pressure–volume homeostasis [3]. In addition, ANP has receptors classified as NPR-A and NPR-B, which activate guanylyl cyclase activity, as well as an NPR-C receptor, which participates in the clearance of natriuretic peptides from the extracellular environment [4,5]. The physiological actions of ANP, which are mediated primarily via cGMP production, occur due to the activation of NPR-A or NPR-B. The elevated intracellular cGMP in turn activates the downstream effector, PKG [6].

Studies conducted in the past few decades have advanced the understanding of the homeostatic role of natriuretic peptides. One interesting finding is that ANP acts as a potent lipolytic agent in humans. This occurs as a result of ANP binding with a high affinity to specific NPR on the membranes of human adipocytes [7,8]. The adipose tissue then releases energy in the form of free fatty acids through the lipolysis of triglyceride. Several signaling pathways are involved in adipocyte lipolysis. The major lipolytic regulators in human adipocytes such as insulin and catecholamines are known to exert their effects by regulating the intracellular cAMP concentration. In this process, stimulation of the G_s-coupled receptors by catecholamines causes activation of adenylyl cyclase, and the subsequent increase in intracellular cAMP levels leads to the activation of PKA. PKA and PKG are activated by catecholamines and ANP, respectively, and then phosphorylate two main targets, HSL and perilipin A, the coating protein of lipid droplets [4]. In unstimulated adipocytes, perilipin A functions as a barrier to lipolysis because of its location on the surface of lipid droplets, thereby preventing the interaction of HSL with the lipid droplet [9]. However, activation of the β-adrenergic receptor (AR) induces a conformational change in phosphorylated perilipin A, which is essential for proper translocation of HSL from the cytosol to the surface of lipid droplets and the subsequent attachment to triglycerides to occur. The attachment to triglycerides then leads to the initiation of triglyceride hydrolysis [10,11].

Abbreviations: ANP, atrial natriuretic peptide; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; NPR, natriuretic peptide receptor; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; HSL, hormone sensitive lipase; PDE, phosphodiesterase.

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The size of an adipocyte is an important determinant of its function. In general, large adipocytes synthesize more triglycerides and release more fatty acid than small adipocytes [12,13]. In addition, large adipocytes are less responsive to insulin [14,15]. However, it is unknown to what extent the lipolytic activity changes in adipocytes of different sizes. To date, the only studies conducted to evaluate the difference in lipolytic activity that occurs as a result of size have compared small adipocytes from young animals with large adipocytes from old animals [16,17]. Therefore, to investigate the exclusive effect of adipocyte size on lipolysis, we separated human adipocytes within the same fat depot into small and large fractions by using nylon filters of different pore sizes and then determined the difference in the amount of ANP-stimulated lipolysis.

MATERIALS AND METHODS

Subjects

Human omental adipose tissues were obtained from 15 subjects (eight men and seven women) who underwent abdominal surgery at Chonbuk National University Hospital. The physical characteristics of the subjects, who were all Korean, are presented in Table 1. The study protocol was approved by the ethical committee of the Chonbuk National University Hospital and written consent was obtained from all subjects.

Adipocyte Isolation and Fractionation

Isolated adipocytes were obtained following the method described by Rodell [18], with some modification. Adipose tissue samples were cut into small pieces and then digested in Krebs-Ringer bicarbonate buffer (119 mM NaCl, 4.74 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 20 mM $NaHCO_3$, 10 mM HEPES, 2.54 mM $CaCl_2$, pH 7.4) containing 3.5% BSA, 6 mM glucose (KRBA), and 1 mg/ml type II collagenase (Sigma, St. Louis, MO) under gentle shaking at 70 rpm in a 37 °C water bath for 1 h. Next, samples were filtered through a 200-μm nylon mesh (Spectrum Laboratories Inc, Rancho Dominguez, CA) to remove the undigested tissue; the isolated adipocytes were washed three times to eliminate collagenase and then re-suspended in KRBA buffer. The cells were then separated into small and large adipocytes using the method described by Skurk *et al.* [19], with slight modification. Briefly, isolated adipocytes were transferred into a 50-ml tube containing 40-ml KRBA buffer. After gently shaking the suspension, cells were allowed to float for 45 s, and then 20 ml of KRBA containing the small adipocytes were collected from the tube bottom. Next, the 20 ml of KRBA buffer was replaced and this procedure was repeated to obtain a second small adipocytes fraction. The first and second fractions were then combined and filtered through a 55-μm nylon mesh and cells that passed through the mesh were defined as the small adipocytes fraction. The remaining 20-ml suspension was filtered through a 70-μm nylon mesh, and the cells that did not pass through the mesh were re-suspended in KRBA buffer and defined as the large adipocytes fraction. Pictures of the adipocyte suspensions were then taken using a microscope attached to a digital camera (Nikon, Japan). Scion Image software from the National Institute of Health (Alpha 4.0.3.2) was used to measure the size (diameter) of 100 adipocytes obtained from each sample.

Table 1 General characteristics of the study population

	Mean ± SEM	Min–Max
Age (year)	54.3 ± 3.3	25–73
BMI (kg/m^2)	21.9 ± 0.6	17.3–25.6
Waist circumference (cm)	76.8 ± 2.0	62–84
Waist/hip ratio	0.873 ± 0.015	0.74–1.01
Blood pressure (mmHg)		
Systolic pressure	118 ± 2	110–140
Diastolic pressure	72 ± 2	60–90

Lipolysis Measurements

Adipocyte suspensions were incubated in KRBA at 37 °C for 2 h with or without 10-μM isoproterenol (Sigma), a nonselective β-agonist or 1 μM ANP (Sigma). The tubes were then placed on ice for 15 min to stop the incubation and then centrifuged. The amount of glycerol released into the incubation buffer, which was used as an index of the lipolysis, was then measured by a colorimetic method using glycerol assay kits (Sigma), as described previously [20].

Determination of the cGMP Concentrations

Adipocytes were incubated for 10 min at 37 °C with 0.1 μM ANP. The reaction was stopped by adding 300 μl TCA to give a final concentration of 6% and then centrifuged at 4 °C. Next, 100 μl of the supernatant was transferred to a polypropylene tube, extracted with water-saturated ether three times, and then dried using a SpeedVac concentrator (Savant, Farmingdale, NY). The dried samples were then re-suspended in 100 μl of 50 mM sodium acetate buffer (pH 4.8) containing 8 mM theophylline. The production of cGMP was then measured using an equilibrated RIA, as described previously [21]. Briefly, standards or samples were incubated with antiserum for cGMP (Calbiochem-Novabiochem, San Diego, CA) and iodinated cGMP (guanosine 3′,5′-cyclic phosphoric acid, 2′-*O*-succinyl [^{125}I]iodotyrosine methyl ester, NEN Life Science Products, Boston, MA). The bound form was then separated from the free form using a charcoal suspension. The nonspecific binding was less than 2.5% and the 50% intercept was at 0.39 ± 0.03 pmol/tube (n = 15). The intra- and interassay coefficients of variation were 6.7% (n = 12) and 8.6% (n = 9), respectively.

RNA Isolation and Real-time PCR

Total RNA was extracted from isolated adipocytes using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was then precipitated with isopropanol and dissolved in diethylpyrocarbonate (DEPC) treated distilled water. Total RNA (2 μg) was then treated with RNase-free DNase (Invitrogen) and first-strand cDNA was generated with the random primers by reverse transcriptase (TaKaRa, Japan). Specific primers for each gene

DOI: 10.1002/psc

(Table 2) were designed using primer express software (Applied Biosystems, Foster City, CA). The sequence for the control 18S ribosomal RNA was purchased from Applied Biosystems and used as the invariant control. The real-time PCR reaction, which was contained in a final volume of 10 μl, 10 ng of reverse transcribed total RNA, 200 nM of forward and reverse primers and 2 × PCR master mixtures. The PCR reaction was carried out in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). All reactions were conducted in triplicate.

Radioligand Binding Experiments

Small and large isolated adipocytes were incubated with ^{125}I-ANP (specific activity 2000 Ci/mmol) in a total volume of 200 μl KRBA for 45 min at 25 °C with gentle shaking. The nonspecific binding was defined using 1 μM ANP that was preincubated for 15 min at 25 °C. Saturation binding was determined in the presence of varying concentrations of ^{125}I-ANP. Bound ^{125}I-ANP was separated from free ligands by rapid filtration through GF/B filters (Whatman, England) that were presoaked in 0.05% polyethyleneimine using a 48-sample harvester (Inotech, Switzerland), followed by three washes with ice-cold 50 mM Tris-HCl (pH 7.4). The activity of each sample was determined using a γ counter (Packard BioScience, Meriden, CT). All experiments were performed in triplicate.

Statistical Analysis

Values are expressed as mean ± SEM. Statistical analyses were performed using Student's *t*-tests for comparisons between small and large adipocytes. Differences with a $p < 0.05$ were considered statistically significant. The kinetic parameters (B_{max} and K_d) and Scatchard transformation were analyzed by nonlinear regression using the Prism 4 software (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Separation of Small and Large Adipocytes

In addition to being increased by the amount and location of adipose tissue, the risk of metabolic complication also increases as the size of the adipocytes increases [15]. Adipocytes are the only cells whose sizes vary dramatically under physiological conditions, and regional growth of adipose tissue is primarily dependent on the metabolism of mature adipocytes, which is determined by the capacity of the adipocytes to accumulate and mobilize triacylglycerol. Hypertrophy of adipocytes is a characteristic of obesity, and may contribute to insulin resistance and proinflammation [15,19]. In this study, the size of the two populations of adipocytes obtained from visceral adipose tissue was found to vary significantly.

Table 2 Sequences and accession numbers for primers (forward, FOR and reverse, REV) used in real-time RT-PCR

Gene	Sequences for primers	Accession No.
NPR-A	FOR: CGTGTGAACCGTAAACGCATT	NM_000906
	REV: TGCACATCCCGCATATGCT	
NPR-C	FOR: TCCAGGTGGCTTACGAGGAT	NM_000908
	REV: CGGTCCACCAAGCTGAAGAG	
HSL	FOR: ACCTGCGCACAATGACACA	NM_005357
	REV: TGGCTCGAGAAGAAGGCTATG	
Perilipin	FOR: CACATTTCCATTTGCATCATTACTG	NM_002666
	REV: TGGTTCCCCAGCATCAAAAG	

After isolation of the human adipocytes by collagenase digestion, the adipocytes were further separated into small and large adipocytes based on their floating properties and by filtering them through nylon filters with different pore sizes (Figure 1(A)). The average diameter of the cells obtained from each patient was then determined by measuring the diameter of 100 cells obtained from each fraction in each patient. After separation, the small and large adipocytes showed a distinguishing distribution of cell diameters, which ranged from 20 to 100 μm (with mean value of 63.6 ± 1.71 μm) for the small fraction and from 60 to 170 μm (with mean value of 100.6 ± 1.84 μm) for the large fraction of one representative separation (Figure 1(B)). The diameter and volume of the two fractions differed significantly (Figure 1(C)). The methods used in this study were reproducible and would facilitate the study of different metabolic activities, as well as the alternation of signal pathway and gene expression between small and large adipocytes.

ANP-stimulated Lipolysis in Small and Large Adipocytes

ANP elevates the intracellular levels of cGMP, which in turn activates PKG. PKG-dependent phosphorylations of HSL and perilipin stimulate lipolysis in human adipocytes [6,7], therefore, an increase in the intracellular level of cGMP in large adipocytes suggests an increase in the level of lipolysis in these cells. We therefore investigated the responses of guanylyl cyclase activity to ANP stimulation in small and large adipocytes in the presence of 0.1 μM ANP for 10 min (Figure 2(A)). Although ANP induced a potent increase in the cGMP level in small and large adipocytes, large adipocytes showed a higher cGMP level (1153.5 ± 184.7 pmol/10^6 cells/10 min, $p < 0.01$, $n = 10$) than small adipocytes (378.4 ± 59.0 pmol/10^6 cells/10 min).

We next measured the amount of extracellular glycerol release that was induced by ANP and isoproterenol (a positive control) to evaluate the lipolytic responses of different sized adipocytes (Figure 2(B) and (C)). When small and large adipocytes were incubated with either 1 μM ANP or 10 μM isoproterenol for 2 h, more glycerol release from triacylglycerol hydrolysis was observed in large adipocytes (2.5 ± 0.32 μmol/10^6

DOI: 10.1002/psc

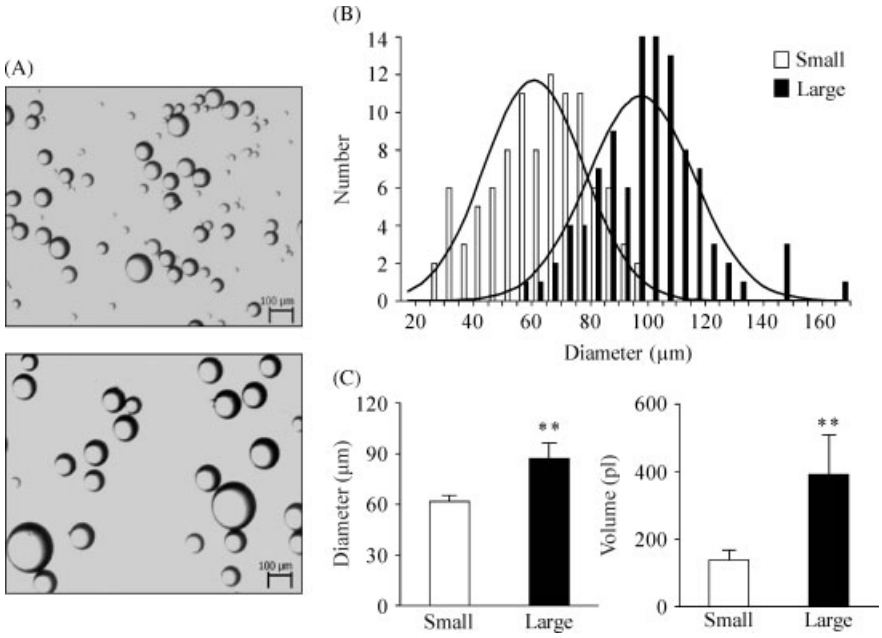

Figure 1 Separation of human adipocytes according to cell size and the measurement of cell diameter. Adipocytes were separated into small and large fractions as described in the Materials and Methods section. (A) Representative photos of the population of small (upper panel) and large (lower panel) human adipocytes after separation. (B) The histogram shows the diameter distribution of adipocytes of one representative experiment. (C) The mean diameter (left panel) and volume (right panel) of small and large adipocytes from ten individual subjects. Values are given as means ± SEM, $n = 10$. $^{**}p < 0.01$ small *vs* large adipocytes.

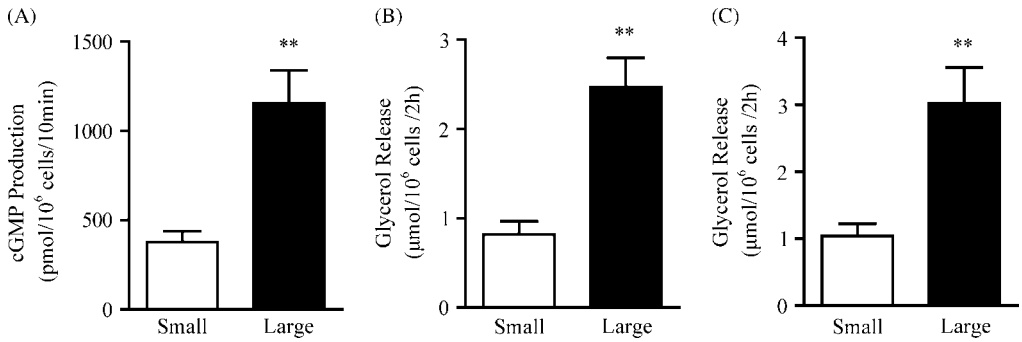

Figure 2 Different responses of human small and large adipocytes to ANP. (A) Adipocytes were incubated with 0.1 μM ANP at 37 °C for 10 min and the cGMP production was determined. (B) The amount of extracellular glycerol released by human small and large adipocytes at 37 °C for 2 h in the presence of 1 μM ANP (B) or 10 μM isoproterenol at 37 °C for 2 h (C) was determined. All experiments were performed in triplicate. Values are given as means ± SEM, $n = 10$. Basal cGMP production (3.93 ± 0.40 pmol/10^6 cells/10 min for small adipocytes and 4.81 ± 0.55 pmol/10^6 cells/10 min for large adipocytes) or glycerol release (0.62 ± 0.031 μmol/10^6 cells/2 h for small adipocytes and 0.91 ± 0.037 μmol/10^6 cells/2 h for large adipocytes) was subtracted from the values. $^{**}p < 0.01$ small *vs* large adipocytes.

cells/2 h for ANP, $p < 0.01$, $n = 10$, 3.0 ± 0.54 μmol/10^6 cells/2 h for isoproterenol, $p < 0.01$, $n = 10$) than in small adipocytes (0.82 ± 0.14 μmol/10^6 cells/2 h for ANP, 1.00 ± 0.19 μmol/10^6 cells/2 h for isoproterenol), which suggests that large adipocytes are more sensitive to ANP-stimulated lipolysis.

Expression of NPR-A, NPR-C, HSL and Perilipin in Isolated Human Small and Large Adipocytes

The different lipolytic responses to ANP observed in small and large adipocytes may have occurred as a result of the differences of the receptor level or the lipolytic cascade located at a post-receptor level. ANP stimulates plasma membrane receptors located in adipocytes bearing an intrinsic guanylyl cyclase activity and increases cGMP levels, and then phosphorylates HSL and perilipin A through PKG [6]. Therefore, we examined the mRNA expression profiles of NPR-A, NPR-C, HSL, and perilipin in small and large adipocytes by real-time PCR to investigate the possible mechanisms of different lipolytic responses. As shown in Figure 3, the mRNA levels of NPR-A and HSL were found to be higher in large adipocytes than in small adipocytes, but

DOI: 10.1002/psc

not those of NPR-C and perilipin. These data suggest that the different expression levels of NPR-A and HSL in adipocytes of different sizes may lead to different rates of lipolysis.

Comparison of ^{125}I-ANP Binding to Human Small and Large Adipocytes

Next, we used a radioligand binding assay to further examine the different levels of membrane NPR-A in small and large adipocytes. Total binding was determined in the presence of increasing concentrations of ^{125}I-ANP, whereas nonspecific binding was determined in the presence of 1 μM ANP. Specific binding of ^{125}I-ANP was saturable in both small and large adipocytes (Figure 4(A)). In addition, the equilibrium dissociation constants (K_d) of the large and small adipocytes were similar, being 2.611 ± 0.538 pmol/l and 2.507 ± 0.370 pmol/l, respectively (Figure 4(B)). The membrane receptor densities (B_{max} value) were 0.153 ± 0.00726 fmol/5×10^4 cells and 0.0959 ± 0.00303 fmol/5×10^4 cells for large and small adipocytes, respectively; however, the number of ^{125}I-ANP binding sites was significantly lower in small adipocytes. The results of the ^{125}I-ANP binding assay suggest that the NPR type in the two fractions is identical and a different number of NPR-A present in the membrane of small and large adipocytes leads to their dissimilar lipolytic effect in response to ANP.

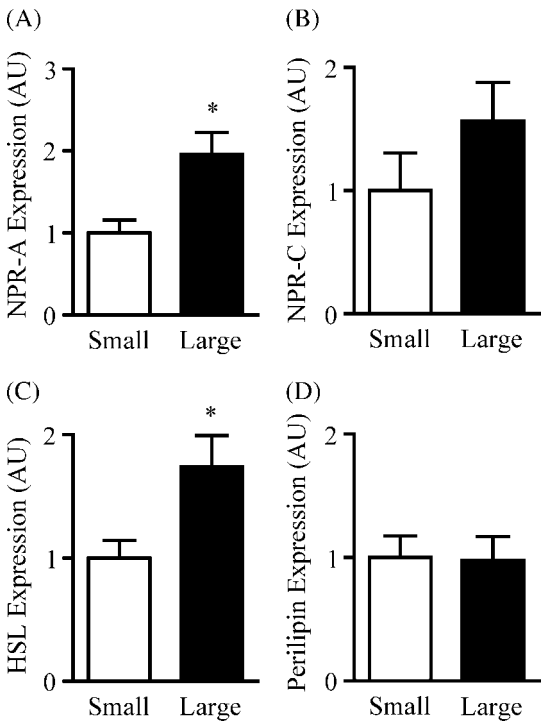

Figure 3 The mRNA expression in human small and large adipocytes. Total RNA were extracted from adipocytes as described in the Materials and Methods section. The NPR-A (A), NPR-C (B), HSL (C) and perilipin (D) mRNA level was determined by real-time PCR. As a reference, 18S ribosomal RNA was used to normalize the expression levels. Results are expressed as a ratio of large *vs* small adipocytes. AU, arbitrary units. $^*p < 0.05$ small *vs* large adipocytes.

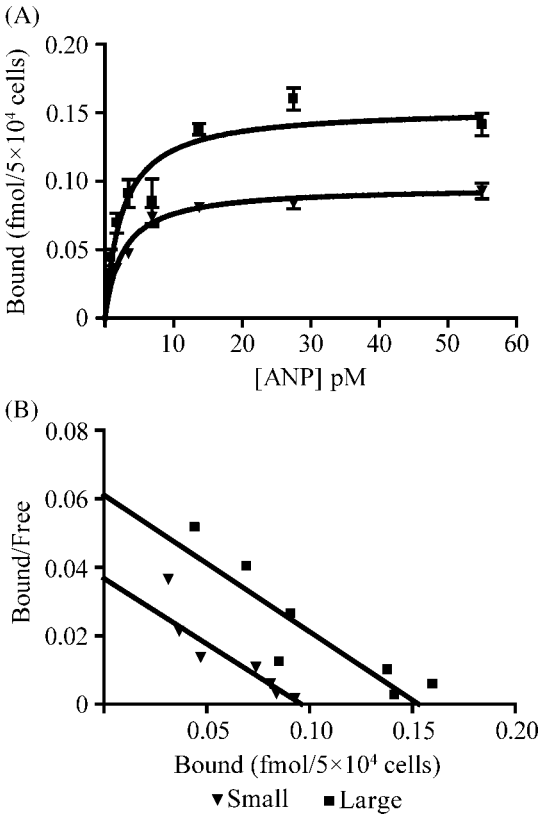

Figure 4 Comparison of ^{125}I-ANP binding to human small and large adipocytes. (A) Saturation curve of ^{125}I-ANP binding to adipocytes. Isolated adipocytes were incubated with ^{125}I-ANP at 25 °C for 45 min. (B) Scatchard analysis of the binding assay. B_{max} is 0.0959 ± 0.00303 fmol/5×10^4 cells for small adipocytes and 0.153 ± 0.00726 fmol/5×10^4 cells for large adipocytes ($p < 0.01$ small vs large adipocytes), K_d is 2.611 ± 0.538 pmol/l for small adipocytes and 2.507 ± 0.370 pmol/l for large adipocytes ($p = 0.22$ small *vs* large adipocytes).

Taken together, this study demonstrates that the ANP-stimulated lipolysis rates are modified by adipocyte size. The large adipocyte fraction was found to have a high mRNA expression levels of NPR-A and HSL compared with the small adipocyte fraction. Radioligand binding studies showed that the large adipocytes had more ANP binding sites on the membrane. Since NPR-A is the predominant NPR subtype in human adipocytes [7], there are probably more NPR-A in the large adipocytes. ANP-stimulated lipolytic rates on a per cell basis showed higher rates with increased cell size. These results are in agreement with previous reports [16,17,22]. However, we could not find a correlation between ANP-stimulated lipolysis and cell size when we analyzed them on a cell surface area or volume basis. For example, the cGMP production,

DOI: 10.1002/psc

ANP-stimulated lipolysis, and isoproterenol-stimulated lipolysis of large adipocytes were increased about 3-fold on a per cell basis and 1-fold per volume compared to small adipocytes. The NPR-A mRNA was increased about 1.96-fold on a per cell basis and 0.98-fold per surface area, and ANP binding sites were increased about 1.66-fold per cell and 0.83-fold per surface area.

When large adipocytes were compared to small adipocytes, the augmentation patterns of glycerol release were almost identical between ANP-stimulated and isoproterenol-stimulated lipolysis. Since the lipolytic pathway of isoproterenol is not dependent on NPR-A/cGMP/PKG system, the same downstream effectors of PKA or PKG such as HSL and perilipin may contribute to the different lipolytic responses. Real-time PCR analysis revealed that large adipocytes expressed a higher mRNA level of HSL compared to small adipocytes, indicating modification at a post-receptor level in addition to NPR-A contributes to the different lipolytic responses in large adipocytes.

PDEs, which play an important role in the regulation of intracellular cGMP levels, might be another factor related to the different lipolysis rate between two fractions. However, recent evidence shows that PDEs are not involved in the ANP-stimulated lipolysis, although functional PDEs are present in human adipocyte [23].

In conclusion, this study suggests that the increased ANP-stimulated lipolysis rates in large adipocyte might be due to the different number of NPR-A, as well as the alterations in lipolytic cascade at a post-receptor level.

Acknowledgements

This work was supported by the Korea Research Foundation Grant (KRF-2005-042-E00005).

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