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**Mature Adipocytes and Perivascular Adipose Tissue Stimulate Vascular Smooth
Muscle Cell Proliferation: Effects of Aging and Obesity**

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Running head: Barandier et al, Adipocytes stimulate SMC growth

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Abstract

Adipocytes and perivascular adipose tissue are emerging to regulate vascular functions. Effects of adipocytes and perivascular adipose tissue on human smooth muscle cell (SMC) proliferation were investigated. Conditioned medium was prepared from cultured premature and differentiated 3T3-L1 adipocytes, from peri-aortic adipose tissue from young (3 months) and old (24 months) WKY rats, from lean and obese Zucker rats (3 months), and from WKY rats fed normal chow or a high fat diet for 3 months. Conditioned medium from differentiated (but not premature) adipocytes stimulated SMC proliferation, which was abolished by charcoal and proteinase K treatment, but resistant to heat, trypsin or phospholipase B (to hydrolyze lysophosphatidic acid). Further experiments demonstrated that the growth factor(s) is hydrosoluble, present in the fraction of molecular weight >100 kDa. Moreover, conditioned medium from peri-aortic adipose tissue stimulated SMC proliferation, which was significantly enhanced in aged rats and in rats fed high fat diet, but not in obese Zucker rats deficient in leptin receptor. In conclusion, mature adipocytes release a hydrosoluble protein growth factor(s) with molecular weight >100 kDa for SMC. Perivascular adipose tissue stimulates SMC proliferation, which is enhanced in aged WKY and in high fat diet-induced obesity but not in leptin receptor deficient obese Zucker rats. This adipocyte-derived growth factor(s) and the effect of perivascular adipose tissue may be involved in vascular disease associated with aging and obesity.

Keywords: Aging - cardiovascular disease - growth substances – obesity – risk factors

The pathogenesis of coronary artery disease and its complications is multifactorial, involving vasoconstriction, thromboembolism and vascular smooth muscle cell (SMC) proliferation (30). Risk factors such as aging, obesity, diabetes mellitus and dyslipidemia, adversely regulate the disease process (2; 33). Recent studies using co-culture or conditioned media techniques demonstrate that adipocytes are highly active in secretion of hormonal substances (6; 9; 14; 17) which play important roles in modulating vascular functions. In addition to angiotensin II (32) and plasminogen activator inhibitor-1 (25), potential factors in vasoconstriction and thromboembolism, adipocytes also produce hormones regulating SMC proliferation including growth promoters such as tumor necrosis factor- α (TNF α), leptin, fibroblast growth factor (FGF), insulin-like growth factor (IGF), transforming growth factor β (TGF β) and heparin binding-epidermal growth factor (HB-EGF)(23; 26; 31), and the growth inhibitor adiponectin (22). In addition, adipocytes also release the phospholipid growth promoter lysophosphatidic acid (LPA) (12; 27). Although the effects of the individual factors on SMC proliferation are well documented, direct effects of adipocytes on SMC proliferation have not been thoroughly investigated yet. The list of vasoactive factors released from adipocytes is growing (11). A novel yet to be identified vascular relaxing factor(s) released from peri-vascular fat tissue has been recently proposed (18; 36), suggesting that perivascular adipose tissue, whose function is usually ignored in vascular biology studies, may play a role in modulation of vascular functions. Therefore, the present study was designed to investigate the direct effects of adipocytes and perivascular adipose tissue on SMC proliferation and the influence of aging and obesity.

Materials and Methods

Materials: Bovine insulin, bovine serum albumin (BSA), indomethacin, phospholipase B, and all other chemicals were purchased from Sigma (Buchs, Switzerland). Proteinase K was from Roche (Rotkreuz, Switzerland); 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone were from Calbiochem (Lucerne, Switzerland); rabbit polyclonal anti-phospho-(Thr²⁰²/Tyr²⁰⁴) p42/44mapk and anti-p42/44mapk antibodies were purchased from Cell Signaling Technology (Allschwil, Switzerland); anti-rabbit IgG alkaline phosphatase (AP) conjugate and Western BlueTM stabilized substrate for AP were from Promega (Wallisellen, Switzerland); Fetal calf serum (FCS), Media for SMC culture were purchased from Bioconcept (Allschwil, Switzerland) and media for murine 3T3-L1 adipocytes (ATCC, LGC Promochem, Molsheim, France) were from Gibco (Basel, Switzerland).

Adipocyte culture and differentiation: Murine 3T3-L1 preadipocytes were cultured and differentiated in DMEM media with 10% FCS as described (19).

Preparation of adipocyte-conditioned medium (ACCM): Conditioned medium from preadipocytes and differentiated adipocytes were prepared as following: confluent cells were washed and kept in serum-free DMEM/F-12 with 0.2% BSA without any growth factors (110 µl medium/cm²) for 24 hours. The conditioned medium was collected and centrifuged (13'000 rpm, 10 minutes, and 4°C) to remove cell debris. To analyse whether prostaglandins are involved in stimulation of SMC growth, the adipocytes were treated with the cyclooxygenase inhibitor indomethacin (1 µmol/L) during the 24-hour conditioning. To characterize the chemical features of growth factor(s) released by the adipocytes, the adipocyte-conditioned medium was

treated with trypsin (2 µg/ml) at 37°C for 2 hours and/or heated at 65°C for 10 minutes, or treated with proteinase K (10 µg/ml, 37°C, 2 hours) and then heated at 90°C for 30 minutes, a condition which is required to inactivate the enzyme. The effect of heating at the temperature of 90°C on the biological activity of ACCM was also independently tested. In another series of experiments, ACCM was treated with activated charcoal (10%) for 1 hour. The charcoal was then removed by centrifugation at 13'000 rpm for 10 minutes followed by filtration through a filter paper with a pore size of 0.22 µm. To estimate molecular weight of the growth factor(s), trypsinized and heated ACCM was subjected to molecular size sieving using an Amicon ultrafiltration device fitted with a membrane with molecular weight cut-off at 100 kDa (Ultrafree-0.5 centrifugal filter device, Millipore). Both the concentrate and filtrate reconstituted to the initial volume with Ham/F12 culture medium were assayed for their biological activity. These experiments showed that the growth promoting effect of ACCM was mainly present in the concentrate. The trypsinized, heated and ultrafiltered concentrate was then subjected to saturated butanol to separate liposoluble and hydrosoluble fractions.

SMC culture: SMC were cultured from human saphenous veins (passages 6 to 14) or from human aorta (passages 9 to 11) as previously described (39).

SMC proliferation: SMC were distributed in 96 well plates at an initial density of 2×10^3 cells per well and allowed to attach overnight. The cells were rendered quiescent in serum-free DMEM with 0.2% BSA for 48 hours (38; 39) and then incubated with ACCM at different dilutions for 48 hours. Cell proliferation was measured using the Celltiter 96 Aqueous One Solution Proliferation Assay Kit

(Promega) according to the manufacturer's instructions, or by counting the cell number over 8 days. The cells were stimulated with conditioned medium (100 μ l/ml) every other day. Each measurement was performed in duplicate.

p42/44mapk activation: The quiescent SMC were stimulated with adipocyte conditioned medium (100 μ l/ml) for indicated times, and p42/44mapk activation (phosphorylation at Thr²⁰²/Tyr²⁰⁴) was measured as described(38). Protein concentration was determined by Bio-Rad D_C protein assay kit. Quantification of the signals was performed by NIH Image 1.62 software.

Animals and preparation of perivascular adipose tissue conditioned medium

(ATCM). Three young (3 months) and three old (24 months) male WKY rats, and three obese Zucker *fa/fa* and three lean Zucker *fa/+* rats (male, 3 month old) were fed normal chow. All rats were purchased from Harlan Netherlands. In addition, three male WKY rats (3 months) were fed a high fat diet (60 cal % of energy as fat, Provimi Kliba AG, Kaiseraugst, Switzerland) for a period of 3 months, and three age and sex matched WKY rats were fed normal chow during the same time period to serve as controls. All animals were maintained according to the local rules of animal experimentation. The animals were anesthetized with pentobarbital sodium (50 mg/kg body weight, i.p.) and sacrificed. The aortas were removed. Perivascular adipose tissue was dissected along the whole aortas and weighed. 400 mg of the fat tissue from each animal were collected and conditioned at 37°C in 1 ml of serum-free DMEM/F12 medium with 0.2% BSA for 4 or 24 hours. The adipose-tissue-conditioned medium (ATCM) was centrifuged, frozen and kept at -80°C until use. To

characterize the biological activities of ATCM, the medium was treated with trypsin or proteinase K and/or heated as described above for ACCM.

Statistics: Cell proliferation was expressed as % increase above the corresponding values of quiescent cells when assayed by the proliferation kit, or as number of cells per ml. Data were given as mean \pm SEM. In all experiments, n equals the number of experiments. Statistical analysis was performed by one-way ANOVA Tukey-Kramer multiple comparisons test or unpaired Student's t -test. Differences were considered statistically significant at values of $p < 0.05$.

Results

Conditioned medium from cultured adipocytes (ACCM) stimulates SMC proliferation.

Conditioned medium from differentiated murine 3T3-L1 adipocytes (ACCM, 2 to 100 $\mu\text{l/ml}$) stimulated human saphenous vein and aortic SMC proliferation in a concentration-dependent manner (**Fig. 1A** and **1B**, $206\pm 21\%$ increase in the vein and $145\pm 9\%$ increase in the aorta at the highest concentration used i.e. 100 $\mu\text{l/ml}$, $n=5-6$, $p<0.01$). Notice that the relative weaker response of the aortic SMC is due to an intrinsic weaker proliferative activity of the cell line in general compared to the venous SMC, since a weaker proliferative response to 5% fetal calf serum (FCS) was also observed in aortic ($299\pm 22\%$ increase) compared to venous SMC ($425\pm 29\%$) ($n=4$, $p<0.05$). Because the higher proliferative activity of venous SMC allows a more precise analysis of the functions of adipocytes on SMC proliferation, the following experiments were performed in the venous SMC. Our previous studies showed a similar growth property between human saphenous vein and coronary artery SMC (28; 37; 38).

Interestingly, conditioned medium from premature or undifferentiated adipocytes had no effects on SMC proliferation in both saphenous vein and aorta (**Fig. 1A** and **1B**, $n=6$). The SMC growth promoting effect of the conditioned medium from mature adipocytes was not affected by treating the cells with the cyclooxygenase inhibitor indomethacin (1 $\mu\text{mol/L}$) during the 24-hour conditioning (data not shown). Treatment of ACCM by charcoal fully eliminated the growth promoting effect (**Fig. 1C**).

Incubation of ACCM with phospholipase B (3 U/ml, 37°C) for 30 minutes to hydrolyse lysophosphatidic acid (LPA) released from adipocytes, caused only 33±3% inhibition of SMC proliferation ($p<0.05$, $n=14$), but a 93±12% inhibition of p42/44mapk activation stimulated by ACCM (100 µl/ml, 10 minutes, $p<0.05$, $n=3$). Furthermore, SMC proliferation stimulated by ACCM (100 µl/ml) at 48 hours or the increase in cell number stimulated by ACCM (100 µl/ml) over 8 days remained unaffected when the conditioned medium was heated (65°C, 10 minutes) or trypsinized (2 µg/ml, 37°C, 2 hours) ($n=3-4$; **Fig. 2A** and **2B**), although p42/44mapk activation by ACCM (100 µl/ml, 10 minutes) was inhibited by over 70% by the treatments (**Fig. 2C**).

Despite being resistant to trypsin, ACCM was sensitive to proteinase K treatment. The results in figure 2A and 2B showed that heating ACCM at 65°C for 10 minutes had no effects on its growth promoting activity. We further showed that heating ACCM at a higher temperature i.e. 90°C for 30 minutes (a condition which is required to inactivate proteinase K) only slightly reduced its growth promoting effect (22% reduction, $p<0.05$, **Fig. 3**) further confirming the heat-resistant feature of the growth factor(s) contained in the conditioned medium. This heat-resistant component of ACCM was, however, abolished by proteinase K treatment (10 µg/ml, 37°C, 2 hours, **Fig. 3**).

Ultrafiltration of heated and trypsinized ACCM with a membrane with molecular weight cut-off at 100 kDa showed that the growth promoting effect was retained in the fraction which has a molecular weight >100 kDa (**Fig. 4A**). The result indicates that the active substance(s) released from cultured adipocytes has a molecular weight higher than 100 kDa. Furthermore, lipid extraction of heated and trypsinized ACCM

with butanol showed that the growth stimulating activity of ACCM was retained in the hydrosoluble but not liposoluble fraction (**Fig. 4B**).

Conditioned medium from peri-vascular adipose tissue stimulates SMC

proliferation: effects of aging and obesity

The effect of perivascular adipose tissue on SMC growth and impact of aging and obesity were further investigated. We showed that adipose tissue conditioned medium (ATCM) prepared from peri-aortic fat of 3 month old WKY rats (4 hours conditioning) also stimulated SMC proliferation (**Fig. 5**). In contrast to ACCM, the growth stimulating effect of ATCM (100 μ l/ml) was partly sensitive to heat either at 65°C for 10 minutes ($p < 0.01$, **Fig. 5A**) or 90°C for 30 minutes ($p < 0.01$, **Fig. 5B**). It is resistant to trypsin (**Fig. 5A**). Indeed, trypsin treatment of ATCM alone or in combination with heat did not affect the growth promoting effect of ATCM (**Fig. 5A**). The growth promoting activity of ATCM was, however, further reduced by proteinase K treatment (**Fig. 5B**). Note that a significant portion of the growth promoting activity of ATCM was still retained after combined treatment of heat plus trypsin (41%, $p < 0.01$ vs. ATCM, **Fig. 5A**) or heat plus proteinase K (40%, $p < 0.05$ vs. ATCM, **Fig. 5B**).

To study aging- and obesity-dependent effect, young (3 months) and old WKY rats (24 months) and two obesity animal models namely Zucker *fa/fa* rats that are deficient of leptin receptor and high fat diet-induced obese WKY rats were used. The body weight of old (24 month old) and high fat diet fed obese (6 month old) WKY rats and that of obese Zucker *fa/fa* rats (3 month old) were comparable and significantly higher when compared to their respective controls (**table 1**). Although the old WKY rats had greater absolute amount of peri-aortic fat tissue than the young

rats, the ratio of peri-aortic fat / body weight, however, was comparable between the two groups (**table 1**). Interestingly, the absolute amount of peri-aortic fat tissue and the ratio of peri-aortic fat / body weight were higher in the two obesity animal models than in the lean littermates (**table 1**).

Conditioned medium of perivascular adipose tissue (ATCM, 100 μ l/ml) prepared from 4-hour conditioning concentration-dependently stimulated SMC proliferation (**Fig. 6A**). This effect was significantly enhanced in aged and high fat diet fed obese WKY rats as compared to the young and lean animals, respectively (**Fig. 6A and 6B**). In contrast, this effect of peri-aortic adipose tissue was, however, less pronounced in obese Zucker *fa/fa* rats as compared to the lean littermates (**Fig. 6C**).

Discussion

Recent research indicates that adipocytes release hormones regulating vascular functions including SMC growth (22; 23; 26; 29; 31). In the present study, we showed that conditioned medium from differentiated mature adipocytes but not that from premature or undifferentiated adipocytes stimulated SMC proliferation, suggesting that a growth factor(s) is specifically produced from differentiated adipocytes. In line with our results, a recent study by Manabe *et al.* demonstrated that mature primary adipocytes, but not preadipocytes, isolated from subcutaneous fat tissue of Wistar rats stimulate breast cancer cell proliferation (20). Our results, however, contrast with an early study which showed no growth promoting effect on rat and calf aortic SMC with conditioned medium from 3T3-F442A adipocytes (4). The discrepancy might be due to the different experimental conditions used such as different adipocytes or SMC lines or different dilutions of the preparation of the conditioned medium.

The chemical nature of the SMC growth factor(s) released from mature adipocytes shown in our present study was further investigated. The involvement of prostaglandins can be excluded, since conditioned medium from mature adipocytes treated with cyclooxygenase inhibitor indomethacin did not affect the growth promoting effect. Indomethacin has also been shown to activate PPAR γ in adipocytes(15). PPAR γ activation reduces the release of free fatty acids and adipokines mediating insulin resistance such as TNF α , leptin, and resistin, but increases SMC growth inhibitor adiponectin production, and PPAR γ activation in SMC has been reported to cause cell growth arrest (for details, see (21)). As a consequence of PPAR γ activation, the proliferation of SMC stimulated by adipocyte-conditioned medium should be inhibited by indomethacin treatment. However, this is not the case, suggesting that those factors that are regulated by PPAR γ could not be

responsible for the growth promoting effect of adipocyte-conditioned medium. The factor(s) can be adsorbed by charcoal, since charcoal fully eliminated the growth promoting effect of the adipocyte conditioned medium. It is known that adipocytes are able to produce oestrogen which promotes breast cancer cell growth (20; 35). However, oestrogen can not account for the SMC growth promoting effect, since oestrogen does not stimulate, but inhibits SMC proliferation as shown by our previous study(7). In addition, adipocytes also produce significant amount of LPA, a lipid which has been shown to stimulate SMC growth(12). In our experiments, treatment of adipocyte conditioned medium with phospholipase B, the enzyme that hydrolyses LPA (10), only partially reduced the growth stimulating effect (33% inhibition), but largely attenuated p42/44mapk activation by the conditioned medium (93% inhibition). These results suggest that LPA only partially contributes to the observed growth stimulating effect.

The most important finding of our present study is that the growth promoting effect of the conditioned medium from mature adipocytes is heat- and trypsin-resistant, but proteinase K-sensitive (Fig. 2 and Fig. 3), suggesting the proteinous nature of the growth factor(s). Further experiments with ultrafiltration demonstrated that the growth promoting effect of the adipocyte-conditioned medium was retained in the fraction which has a molecular weight >100 kDa (Fig. 4A). Moreover, lipid extraction of heated and trypsinized adipocyte-conditioned medium showed that the growth promoting effect is present in hydrosoluble fraction (Fig. 4B) suggesting the water soluble nature of the growth factor(s). The high molecular weight with the feature of trypsin- and heat-resistance might implicate a protein with a globular structure. Note that p42/44mapk activation by the conditioned medium was markedly inhibited by trypsin or heating, implicating that other factors which stimulate

p42/44mapk were inactivated, and the heat- and trypsin-resistant growth factor(s) stimulates SMC proliferation mainly independently of p42/44mapk. It is also to notice that heating, trypsin-digestion and phospholipase B treatment all independently reduced p42/44mapk activation by over 70%, which may be due to the synergistic effects of LPA and other growth factors(8).

Blood vessels are surrounded by adventitial perivascular fat tissue which is usually ignored by experiments investigating vascular functions. It is emerging that the perivascular adipose tissue may play a role in regulation of vascular functions as suggested most recently. Indeed, these studies showed that perivascular adipose tissue from rat aortas or mesenteric arteries produces a SMC relaxing factor(s)(18; 36). In the present study, we further demonstrated that peri-aortic adipose tissues also release growth factor(s) stimulating SMC proliferation (Fig. 5). The chemical features of the biological activity of ATCM were studied and compared to ACCM. The SMC growth promoting activity of ATCM was partly reduced by heat (Fig. 5). The remaining activity of ATCM after heating was resistant to trypsin but partly sensitive to proteinase K, suggesting that the proteinous component produced by cultured mature adipocytes is also present in ATCM. It is important to notice that about 40% of the biological activity of ATCM are still observed after heating and proteinase K treatment (Fig. 5B), suggesting a non-proteinous component produced from peri-vascular tissue. It is not surprising to observe a more complex feature of ATCM than that of ACCM, since peri-vascular adipose tissue contains heterogeneous cell types such as endothelial cells, fibroblasts, mesenchymal stromal cells, etc. It remains for future studies to analyze the individual components released by the different cells in the peri-vascular adipose tissues under physiological and pathological conditions.

Nonetheless, the sum effect of peri-vascular adipose tissue on SMC growth was investigated in aging and obesity animal models. The results showed that the growth promoting effect of peri-aortic adipose tissue was significantly enhanced in aged rats (Fig. 6A), suggesting that it might be involved in age-associated vascular intimal thickening or remodeling(16), although this effect appears modest under our *in vitro* experimental conditions. One could presume that during the chronic aging process which is associated with increased absolute fat mass deposition around blood vessels (**table 1**) and deterioration of vascular protective mechanisms such as endothelial functions(16), the growth promoting effect of perivascular adipose tissue could become dominant and promote negative vascular remodeling and vascular diseases.

The impact of obesity on perivascular adipose tissue-induced SMC growth was studied in two obesity models. In the genetic obese Zucker *fa/fa* rats deficient in leptin receptors, the growth promoting effect of perivascular adipose tissue, to our surprise, was less pronounced (**Fig. 6C**). In contrast, in the high fat diet induced obese WKY rats, the SMC growth promoting effect of peri-aortic fat was significantly increased as compared to the control group (**Fig. 6B**). In both obesity models, the amount of peri-aortic fat is almost twice as much in the obese than in the lean rats, the ratio of peri-aortic fat/body weight is higher in the obese than in the lean rats (i.e. perivascular fat mass increases proportionally more than body weight in the obesity, see **table 1**). It is presumable that the SMC growth stimulating effect of perivascular adipose tissue might become even more important in obesity, in particular, when adipocyte-derived growth promoters such as leptin and TNF α are increased and growth inhibitors such as adiponectin are decreased(13; 24).

The contrasting results between the two obesity models might be due to the fact that in the obese Zucker *fa/fa* rats, there is no effect of leptin due to leptin receptor deficiency. Leptin has been shown to be associated with increase in cardiovascular risk (5). It is also to notice that the genetic obese Zucker *fa/fa* rats are relatively resistant to atherosclerosis and changes in vascular functions(1; 3; 34). Also, the production of the newly suggested relaxing factor(s) released from peri-aortic adipose tissue in Zucker *fa/fa* rats remains preserved as demonstrated recently (18). Interestingly, in high fat diet-induced obese WKY rats, a more relevant obesity model, the SMC growth stimulating effect of perivascular adipose tissue conditioned medium was significantly enhanced as compared to the lean controls (**Fig. 6B**), implicating a more pronounced release of SMC growth factors from perivascular adipose tissue of the obese rats.

In conclusion, our study suggests that mature adipocytes release a hydrosoluble protein growth factor(s) with a molecular weight >100 kDa for SMC. Perivascular adipose tissue stimulates SMC proliferation, which is enhanced in aged WKY and in high fat diet-induced obesity. This adipocyte-derived growth factor(s) and the effect of perivascular adipose tissue may be involved in vascular disease associated with aging and obesity.

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Figure legends

Fig. 1: Adipocyte-conditioned medium (ACCM) stimulates SMC proliferation.

ACCM of differentiated (but not premature) 3T3-L1 adipocytes (24-hour conditioning) concentration-dependently stimulated SMC proliferation (48-hour stimulation) either from human saphenous vein (panel A) or aorta (panel B). The growth promoting effect of ACCM in the venous SMC was abolished by charcoal treatment (panel C). * $p < 0.05$ and ** $p < 0.01$ vs. control i.e. cells not treated with ACCM; § $p < 0.01$ vs. cells treated with ACCM alone.

Fig. 2: SMC proliferation stimulated by adipocyte-conditioned medium (ACCM) is heat- and trypsin-resistant.

Neither cell proliferation (panel A) nor increase in cell number (panel B) stimulated by ACCM (100 $\mu\text{l/ml}$, 24-hour conditioning) of mature adipocytes over 8 days was affected by treatment of ACCM by heating (65°C, 10 minutes) or trypsin (2 $\mu\text{g/ml}$, 37°C, 2 hours), whereas p42/44mapk activation (phosphorylation at Thr²⁰²/Tyr²⁰⁴) stimulated by ACCM (100 $\mu\text{l/ml}$, 10 minutes) was strongly attenuated by the treatments (panel C). * $p < 0.05$ and ** $p < 0.01$ vs. control; § $p < 0.05$ and §§ $p < 0.01$ vs. cells treated by ACCM alone.

Fig. 3: Treatment of adipocyte conditioned medium (ACCM) with proteinase K

(PK, 10 $\mu\text{g/ml}$, 37°C, 2 hours) abolishes its growth promoting effect. The SMC growth promoting effect of ACCM was only slightly reduced by heating at a higher temperature i.e. 90°C for 30 minutes, a condition which is required to inactivate PK. The effect of ACCM was, however, further abolished by PK treatment. ** $p < 0.01$ vs. control; # $p < 0.05$ and ## $p < 0.01$ vs. ACCM; § $p < 0.05$ vs. heated ACCM.

Fig. 4: The growth factor(s) released from cultured adipocytes is hydrosoluble and has a molecular weight higher than 100 kDa. (Panel A) Ultrafiltration of heated and trypsinized (h+t) ACCM with a membrane with molecular weight cut-off at 100 kDa showed that the growth promoting effect was retained in the fraction which has a molecular weight >100 kDa. (Panel B) Lipid extraction of heated and trypsinized (h+t) ACCM with butanol showed that the growth stimulating activity of ACCM was retained in the hydrosoluble fraction. *p<0.05 and **p<0.01 vs. control; #p<0.05 vs. heated and trypsinized (h+t) ACCM. Hydro = hydrosoluble and lipo = liposoluble.

Fig. 5: The growth promoting effect of periaortic adipose tissue conditioned medium (ATCM) is trypsin-resistant, but proteinase K- and heat-sensitive. (A) SMC proliferation stimulated by ATCM was slightly but significantly reduced by heating (65°C, 10 minutes) but not by trypsin treatment (2 µl/ml, 37°C, 2 hours). (B) SMC proliferation stimulated by ATCM was significantly reduced by heating (90°C, 30 minutes) and further by proteinase K treatment (PK, 10 µg/ml, 37°C, 2 hours). *p<0.05 and **p<0.01 vs. control; ##p<0.01 vs. cells treated by ATCM alone; §p<0.05 vs. heated ATCM-treated cells.

Fig. 6: Effects of peri-aortic adipose tissue conditioned medium (ATCM) on SMC growth in aging and obesity. ATCM stimulated SMC proliferation, which was significantly enhanced in aged (panel A) and obese (panel B) WKY rats, but decreased in obese Zucker *fa/fa* rats (panel C). *p<0.05 and **p<0.01 vs. control; §p<0.05 and §§p<0.01 young vs. old rats and lean vs. obese rats at the same concentration of ATCM.

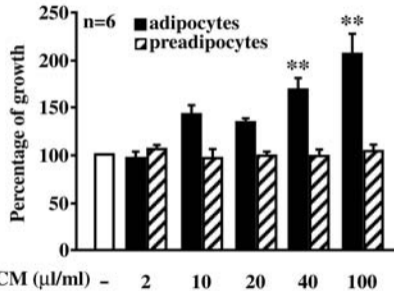
Table1. Body weight and perivascular fat in aging and obesity

	WKY rats				Zucker rats	
	young	old	lean	obese	lean	obese
Body weight (g)	339±12	469±13**	343±3	442±11 ^{††}	330±5	451±19 ^{††}
Perivascular fat weight (g)	0.4±0.1	0.6±0.1	0.5±0.3	0.8±0.1 ^{††}	0.4±0.05	0.8±0.01 ^{††}
Perivascular fat/body weight (x10 ⁻³)	1.1±0.1	1.2±0.1	1.3±0.1	1.9±0.2	1.3±0.1	1.8±0.06 [†]

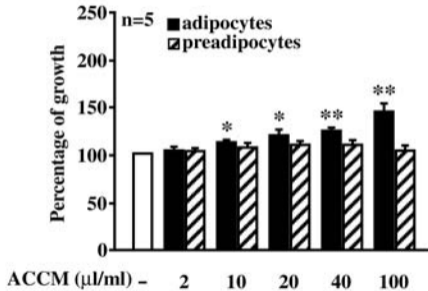
Values are means±SEM, n=3/group. **p<0.01 vs. young; ^{††}p<0.01 and [†]p<0.05 vs. lean.

Fig. 1

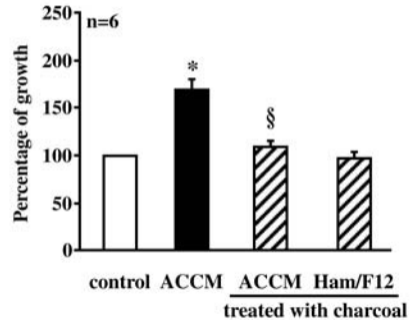
A.



B.



C.



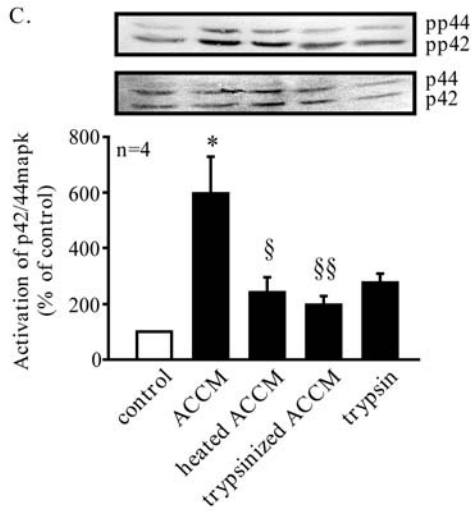
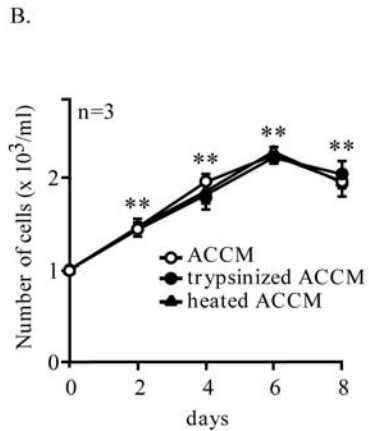
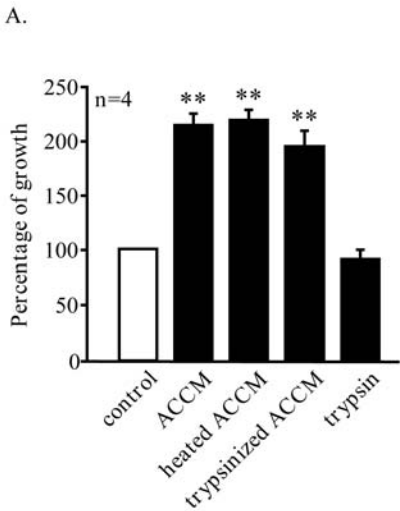


Fig. 2

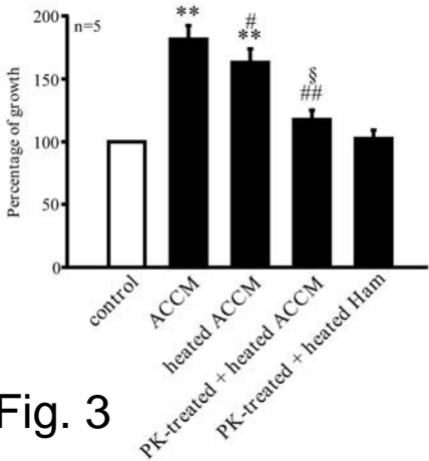
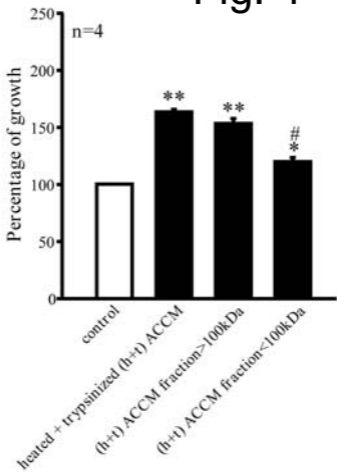


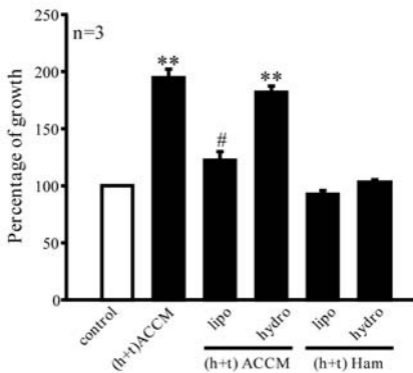
Fig. 3

A.

Fig. 4



B.



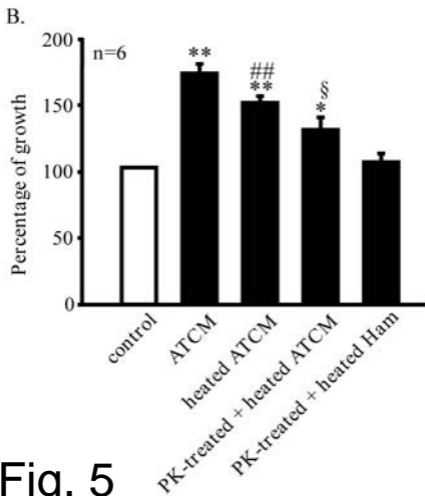
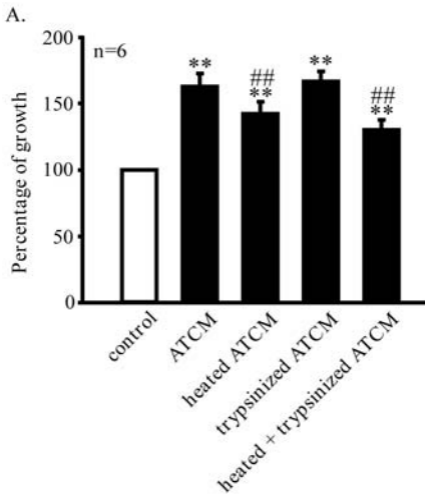


Fig. 5

