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**DYSFUNCTION OF HUMAN SUBCUTANEOUS FAT ARTERIOLES IN OBESITY  
ASSOCIATED OR NOT WITH TYPE 2 DIABETES**

running title: **arterioles dysfunction in obese and type 2 diabetic - obese patients**

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## Abstract

*Objective:* The present study aimed to examine the effects of obesity and obesity associated with type 2 diabetes on the structure, vascular reactivity, and response to insulin of isolated human subcutaneous fat arterioles; these effects were correlated with the expression of insulin signaling proteins. *Research Design and Methods:* Periumbilical subcutaneous adipose tissue was explanted at surgery, small arterioles (internal diameter  $220 \pm 40 \mu\text{m}$ ) were dissected out and investigated by electron microscopy, myography and immunoblotting. *Results:* Compared to the subcutaneous arterioles of lean subjects, obesity activates the endothelium, enhances the accumulation of collagen within vascular wall and increases the sensitivity of adrenergic response; obesity also diminishes eNOS protein expression, NO production, endothelium-dependent and insulin-induced vasodilatation, as well as the protein expression of both insulin receptor substrates IRS-1 and IRS-2, and of the downstream molecules in insulin pathway, such as PI3-kinase, p-AKT and AKT. When obesity is joined with type 2 diabetes these changes are severely augmented. *Conclusions:* Obesity associated or not with type 2 diabetes alters human peri-umbilical adipose tissue arterioles in terms of structure, function and biochemistry, including diminished eNOS expression and reduced levels of IRS-1, IRS-2, PI3-kinase and AKT in insulin-signaling pathway.

Key words: arterioles, obesity, type 2 diabetes, insulin signaling, endothelial dysfunction.

## Introduction

Obesity, insulin resistance, and endothelial dysfunction closely coexist throughout the natural history of type 2 diabetes. Insulin resistance contributes to endothelial dysfunction through a complex cascade of events that are not fully understood. Also, obesity, known as a well established risk factor, generates adverse health effects, including cardiovascular disease, diabetes and cancer [1]. On the other hand, obesity, particularly “central or abdominal” obesity, usually involving increased visceral fat, leads to an imbalanced production of several metabolic products, hormones and cytokines (adipocytokines), which favor decreased insulin sensitivity in liver and skeletal muscle, and impair endothelial function through direct and/or indirect mechanisms [2]. In humans, the subcutaneous adipose tissue has a greater volume and is more strongly associated with insulin resistance than the visceral one [3]. Type 2 diabetes mellitus significantly alters the adipose tissue distribution with less influence on the subcutaneous one and with more influence on the visceral and intermuscular ones, both fat depots known to exacerbate insulin resistance [4]. The interest in studying the subcutaneous adipose tissue arterioles comes not only from the need to understand the interplay between the adipocytes and vasculature within this fat depot, but also from the therapeutic potential of adipose tissue-derived stroma cells in physiological and pathological wound healing [5].

Considering the rather disparate studies on the subcutaneous adipose tissue arterioles, our objective was to understand the structural-functional correlates and cellular mechanisms by which obesity and obesity aggravated by type 2 diabetes induce local effects in this particular microvascular bed, with adverse consequences on vascular wall-adipocyte crosstalk.

## Methods

### *Reagents*

Noradrenaline, KCl, acetylcholine, insulin, N $\epsilon$ -Nitro-L-Arginine Methyl Ester and sodium orthovanadate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The primary antibodies to IRS-1, IRS-2, PI3-kinase, AKT, p-AKT, eNOS, p-eNOS were purchased from Santa Cruz (USA), rabbit polyclonal to  $\beta$ -actin was from Abcam (<http://www.abcam.com>, USA) and the secondary antibody, goat anti-rabbit IgG coupled with peroxidase (HRP) was from Sigma (St. Louis, MO, USA). All others reagents used were of analytical grade.

### *Study groups and samples*

In collaboration with “CFR 2” University Hospital Bucharest, the study was performed on 60 middle-aged subjects ( $44.32 \pm 4.83$  years old): 35 men and 25 women. The subjects were divided into three equal groups: healthy as control subjects (C group), obese patients, and obese patients with type 2 diabetes. At hospital, patients evaluation consisted of physical, electrocardiographic and echocardiographic examination, establishment of body mass index (BMI), measurement of waist circumference (WC) and of systolic and diastolic blood pressure; the blood was collected by venipuncture and subsequently used for the assay of glucose and cholesterol concentrations. Small pieces of subcutaneous adipose tissue were removed during surgery from the periumbilical area, at 1.5-2.0 cm deep from the skin surface. These specimens

(otherwise discarded) were subsequently transferred to the lab, where the arterioles (internal diameter  $220 \pm 40 \mu\text{m}$ ) were dissected out, and used in physiological and biochemical assays.

Exclusion criteria for the three experimental groups were considered: the current tobacco use, established cardiovascular disease, medications for hypertension, and hyperlipidaemia. All the subjects had blood pressure of  $\leq 140/90\text{mmHg}$ , fasting total cholesterol  $\leq 200\text{mg/dl}$ , and no family history of cardiovascular disease. The controls were healthy volunteers, all in good physical health and did not take any medication at the time the experiments were performed. None had a family history of diabetes or obesity among first-degree relatives. In addition, they had BMI, WC and glucose concentrations in normal range. For obese patients, we included subjects with evidence of increased values of BMI and WC, but with normal baseline blood tests and without another medication. Obese type 2 diabetic patients were maintained in a good metabolic control with standard oral anti-diabetic agents (see Table 1). They discontinued all drug treatment 3 days before the study. The small pieces of subcutaneous adipose tissue were removed during the esthetic surgery for C group and during the necessary surgery for obese patients and obese patients with type 2 diabetes.

The experiments associated with this study have been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and have been approved by the Ethic Committee of the Institute of Cellular Biology and Pathology "N. Simionescu", Bucharest, Romania. Also, each patient or subject taken in consideration in our study gave their consent after full explanation of the purpose, nature and risk of all procedures used.

### ***Electron microscopy***

Tissue processing for electron microscopy was performed essentially as in Popov et al., [6]. Briefly, the dissected arterioles were fixed in a mixture made of 2.5% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate - HCl buffer, supplemented with 2.5 mmol/l  $\text{CaCl}_2$ , pH 7.4, postfixed in 1%  $\text{OsO}_4$ , dehydrated in graded concentrations of ethanol, and embedded in Epon 812. The thick sections stained with 0.25% toluidine blue were examined by light microscopy, and used to locate the position where thin sections are to be cut. The thin sections were stained with 7.6% uranyl acetate (in distilled water) and 0.4% lead citrate (in 0.1 N NaOH), and were finally examined by electron microscopy (FEI Tecnai F20 field emission gun TEM, Philips Electron Optics, Eindhoven, The Netherlands).

### ***The isometric myograph technique***

One mm long segments of subcutaneous arterioles were dissected out from peri-umbilical fat, and the small amount of connective tissue surrounding their thin wall was removed. Two stainless steel wires ( $\phi = 40 \mu\text{m}$ ) were threaded through the arteriole lumen and the preparation was mounted in a small vessel myograph (Model 410A; J.P. Trading, Aarhus, Denmark) as described by Mulvany and Halpern [7]. The myograph chamber was filled with HEPES salt solution maintained at  $37^\circ\text{C}$  and continuously gassed with  $\text{O}_2$  [8, 9]. Following an equilibration period of 20 min, the arterioles were set to a normalized internal circumference at which they gave the maximum of the isometric response. The mean internal diameter of arterioles used in this study was  $220 \pm 40 \mu\text{m}$ . Then, for each vessel a routine "run-up" procedure consisting of consecutive contractions in  $5 \times 10^{-6}$  M noradrenaline (NA),  $125 \times 10^{-3}$  M  $\text{K}^+$ , and  $5 \times 10^{-6}$  M NA in  $125 \times 10^{-3}$  M  $\text{K}^+$  was performed. To measure arteriolar wall contractility,  $10^{-8}$  -  $10^{-4}$  M NA was

cumulatively added (at 2 min intervals) in the organ bath of myograph, and the active wall tension recorded; the contractile force of arteries was expressed in  $\text{mN mm}^{-1}$ . To measure arteriolar wall vasodilation, NA contracted vessels (as above) were exposed to  $10^{-8}$ - $10^{-4}$  M acetylcholine (ACh) cumulatively added at 2 min intervals in the organ bath of myograph; relaxation induced by ACh was expressed as percentage of NA-induced contraction.

In other experiments, the effect of insulin on the vascular reactivity of subcutaneous fat arterioles was investigated. To this purpose, arterioles were first precontracted in NA ( $10^{-8}$ - $10^{-4}$  M), and then exposed to various concentrations of insulin (0.4 IU/ml – 6.4 IU/ml) in the presence or absence of eNOS inhibitor L-NAME ( $10^{-4}$  M). The force developed by vascular wall was measured after 15 minutes of incubation, and relaxation was expressed as percentage of NA-induced contraction.

Time control experiments were not performed, since the aim of study was to compare the responses of wiremounted arterioles from the three equal groups subjected to identical protocols. It should be noted, however, that for subcutaneous arterioles dissected out from peri-umbilical fat, agonist concentration-response relations were repeatable for many hours.

### ***The measurement of endothelial NO production***

We measured NO concentration at the luminal side of longitudinally opened arterioles using the NO sensor technique (amiNO-700; Innovative Instruments, Inc., Tampa, FL, USA) as previously described [10]. With the isolated arteriole bathed in 2 ml HEPES salt solution [11], NO sensor was placed perpendicularly to the luminal arteriole side (upwards), in the close vicinity of endothelial cells layer, in order to minimize diffusion path of NO, and to obtain a high signal. The continuous monitoring of current (in pA) or of NO production (in nmoles/l) by intact endothelium of the arterioles was also performed in presence of  $3 \times 10^{-5}$  M ACh or 6.4 IU/ml insulin.

### ***The immunoblotting technique***

Lysates of isolated arterioles explanted from human peri-umbilical fat were subjected to SDS-PAGE and transferred on nitrocellulose membranes, as previously described [12]. The latter were washed in 0.05% Tween-20 in PBS, blocked in 5% BSA in PBS-Tween-20, and incubated with the first antibody (chosen function of the molecules studied, see Reagents) at 4°C. After 17 hrs, membranes were washed again, and incubated for 1 h with the secondary antibody, goat anti-rabbit IgG coupled with HRP. The bound antibody was detected by ECL technique. The density of immunoreactive bands was measured using a microcomputer imaging system (Scion Image). The densitometric evaluation of protein expression of studied molecules in obese patients with and without type 2 diabetes was compared to C group (considered as 100%) and was calculated as percentage (%).

### ***Data analysis***

All values were expressed as mean  $\pm$  SE of the mean. One-way analysis of variance (ANOVA) was employed to quantify the results. The data were considered significant when  $P < 0.05$ .



## Results

### *Clinical characterization of the study subjects*

The clinical parameters of middle-age subjects in healthy (C), obese and obese type 2 diabetic groups are presented in Table 1. In C group, plasma glucose concentration was in the range 70 - 105 mg/dl, cholesterol concentration was between 120 -190 mg/dl, BMI < 25 kg/m<sup>2</sup> and WC was less than 100 cm in men and less than 87.5 cm in women. In the obese group, plasma glucose concentration exceeded 105 mg/dl, cholesterol concentration was ~ 10 % augmented (versus C group), BMI was over 31 kg/m<sup>2</sup> and WC over 100 cm in men and over 87.5 cm in women. The obese patients with type 2 diabetes displayed ~ 2.4 fold augmented glucose concentration and ~ 15 % increase in cholesterol concentration (versus C group), BMI was in the range 37-42.6 kg/m<sup>2</sup> and WC ~ 122 cm. All middle-age subjects in this study had normal systolic and diastolic blood pressure (Table 1).

### *Morphological examination of the human subcutaneous adipose tissue arterioles*

We questioned whether obesity and obesity associated with type 2 diabetes influence the morphology of subcutaneous adipose tissue arterioles. Light microscopy examination of cross sections of arteriolar wall showed that at obese patients with or without type 2 diabetes the media layer was thickened and the lumen diameter was reduced, compared to arteries in C group (Figure 1A). In these samples the endothelial cells (ECs) appeared as secretor, containing abundant rough endoplasmic reticulum, and an expanded subendothelial extracellular matrix (Figure 1B, b). The two-three layers of smooth muscle cells (SMCs) displayed abundant collagen deposits that separated nearby SMCs (Figure 1B, c). In contradistinction, the subcutaneous arterioles in C group showed a rather normal appearance, with a thin basal lamina separating endothelium from the SMCs layer (Figure 1B, a). These data suggest that, in the human subcutaneous adipose tissue arterioles, obesity associated or not with type 2 diabetes, activates the endothelium, and enhances collagen accumulation within vascular wall.

### *The vascular reactivity of human subcutaneous adipose tissue arterioles*

The isometric myograph technique was used to assess the effect of obesity and obesity associated with type 2 diabetes on arterioles contractile and relaxant properties, in an attempt to correlate their vascular reactivity with the morphological structure. To this purpose, the small amount of connective tissue surrounding their thin wall was removed, and the genuine vascular reactivity of wall was measured, without contribution of anti-contractile effect of perivascular adipose tissue. The results showed that in response to  $3 \times 10^{-5}$  M NA the subcutaneous arterioles explanted from lean subjects (C group) developed a maximal contractile force of  $0.044 \pm 0.015$  mN/ mm; at an identical NA concentration, arterioles in obese group contracted to  $0.11 \pm 0.02$  mN/mm, while in obesity associated with type 2 diabetes the force of contractile response was even higher, measuring  $0.24 \pm 0.027$  mN/mm (Figure 2). Analysis of the concentration-dependent curves of contractile response to NA ( $10^{-8}$ - $10^{-4}$ M) showed that half-maximal NA concentrations (EC50) were significantly different between the three groups, i.e.  $5.15 \pm 0.02$  in C group,  $6.45 \pm 0.02$  in the obese group, and  $5.99 \pm 0.03$  in the obese-diabetics group (Figure 2). These data suggest that obesity without or with type 2 diabetes is associated with an increased sensitivity (-log EC50) of

the contractile response of subcutaneous arterioles. In another set of experiments, NA-contracted arterioles were exposed to  $10^{-8}$ -  $10^{-4}$  M ACh. The results showed that arterioles in group C relaxed to ACh (EC50:  $6.30 \pm 0.05$ ), and the maximal vasodilatation was equivalent to  $87.19 \pm 4.03$  % of NA -induced contraction (at  $3 \times 10^{-5}$  M ACh) (Figure 3a). Pre-contracted arterioles in the obese groups without or with type 2 diabetes relaxed very little ( $2.72 \pm 1.56$  % and  $1.99 \pm 0.95$  %, respectively) in the presence of ACh (Figure 3a). Taken together, these data suggest that obesity associated or not with type 2 diabetes alters the vascular reactivity of subcutaneous adipose tissue arterioles by enhancing the sensitivity and adrenergic hyperresponsiveness, and by desensitization to ACh induced endothelium-dependent vasodilation.

### ***The effect of insulin on vascular reactivity of human subcutaneous adipose tissue arterioles***

As metabolic syndrome is associated with elevated levels of insulin, we tested the effect of insulin (0.4 IU/ml – 6.4 IU/ml) on vascular reactivity of subcutaneous adipose fat arterioles explanted from the peri-umbilical fat of obese and obese with type 2 diabetes patients (versus arterioles from lean, C group). The results showed that arterioles in group C relaxed to insulin (EC50:  $0.72 \pm 0.07$ ), and the maximal vasodilatation measured  $21.2 \pm 1.7$  % of NA -induced contraction (at 6.4 IU/ml insulin) (Figure 3b). Pre-contracted arterioles in the obese groups without or with type 2 diabetes had a significantly diminished relaxation ( $1.4 \pm 0.6$  % and  $1.2 \pm 0.4$  %, respectively) in the presence of insulin (Figure 3b). To investigate whether NO contributes to insulin-induced vasorelaxation, we supplemented the organ bath of myograph with  $10^{-4}$  M L-NAME and the responses of wire-mounted arterioles were recorded. In C group vascular relaxation was significantly diminished ( $P < 0.05$ ), measuring only  $6.32 \pm 1.3$  % of NA -induced contraction (Figure 3c). These data suggest that in physiological condition the isolated subcutaneous adipose tissue arterioles respond to insulin in a process NO-dependent, while in obesity and obesity associated with type 2 diabetes, the arterioles respond very little to insulin.

### ***The concentration of NO released by the endothelial cells of human subcutaneous fat arterioles***

These experiments examined if the suppression of vessels relaxation, induced by obesity, can be related to a reduced NO production by ECs layer. To this purpose, the endothelial NO production was continuously recorded at the lumen of human subcutaneous fat arterioles exposed to  $3 \times 10^{-5}$  M ACh or 6.4 IU/ml insulin, applied for 10 min. The results in Table 2 showed that in presence of ACh, the arterioles explanted from obese and obese-diabetic patients produced  $\sim 5.7$  and  $\sim 11.6$  fold less NO, respectively, compared to C group. In presence of insulin, the arterioles explanted from obese and obese-diabetic patients produced  $\sim 8.3$  and  $\sim 13$  fold less NO, respectively, compared to C group (Table 2). These results demonstrate that obesity diminishes the endothelial NO production of subcutaneous adipose tissue arterioles, and that type 2 diabetes associated with obesity induces even more severe NO diminishment.

### ***The protein expression of eNOS, and of insulin signaling intermediates in human subcutaneous adipose tissue arterioles***

To examine whether the diminished endothelial NO production and the abolished vasodilatation (endothelium-dependent and insulin-induced) observed in obesity and obesity associated with



type 2 diabetes is related to a defect in eNOS expression, immunoblotting experiments were performed. The densitometry evaluation of both phosphorylated and non-phosphorylated eNOS show that in obesity these were ~2.51 and ~5.20 fold reduced, respectively compared to C group. Moreover, association between obesity and type 2 diabetes produced the most attenuated p-eNOS and eNOS protein expression, that was diminished by ~13.55 and ~17.48 fold, respectively (Figure 4A,a and 4B).

To have a deeper insight into insulin effects on human adipose tissue arterioles, the protein expression of signaling molecules in insulin pathway was investigated (Figure 4A,b). Densitometry of the bands assessed that, in the subcutaneous arterioles of obese patients both insulin receptor substrates were reduced, i.e. IRS-1 by ~2.61 fold, and IRS-2 by ~3.35 fold (compared to proteins expression in C group) (Figure 4B). Obesity impeded also the expression of downstream molecules in insulin pathway, such as PI3-kinase (by ~1.43 fold), activated AKT (by ~2.14 fold) and AKT (by ~1.44 fold). Furthermore, obesity joined by type 2 diabetes reduced protein expression of all examined molecules (Figure 4A, b and 4B). Thus, IRS-1 was reduced by ~3.64 fold, IRS-2 by ~4.73 fold, PI3-kinase by ~4.30 fold, and both phosphorylated and non-phosphorylated AKT by ~2 fold. These data suggest that in the subcutaneous adipose tissue arterioles obesity diminishes the protein expression of insulin signaling molecules, a process more severely impeded when obesity is joined by type 2 diabetes.

## Discussion

This study reports novel findings on the effect of obesity and obesity associated with type 2 diabetes on arterioles isolated from human peri-umbilical subcutaneous adipose tissue. Firstly, we emphasize the structural-functional correlates in endothelial vascular dysfunction; increased contractility and drastically reduced endothelium-dependent vasorelaxation. Secondly, we provide evidence on the diminishment of insulin-induced vasorelaxation associated with obesity, and the molecular defects in its signaling pathway. Thirdly, we show that type 2 diabetes associated with obesity induces even more severe alterations, impeding NO release within arteriolar lumen, and lowering the expression of insulin signaling molecules, such as IRS-1, IRS-2, PI3-kinase, AKT and eNOS.

This study was conducted on subcutaneous fat arterioles explanted from middle-aged patients, allowing comparisons between groups in the absence of significant comorbidities. As expected, the middle-aged obese and obese-diabetics used as source for subcutaneous fat arterioles had higher BMI and WC compared to C group. In our experiments, the small amount of fat and connective tissue surrounding the thin wall of fat arterioles was removed allowing the direct measurement of endothelium and SMCs layer response. In a different vascular bed (skeletal muscle) it was reported that the fat depot localised around arterioles origin may be involved in local TNF- $\alpha$  signaling, resulting in impaired tissue perfusion and insulin resistance [13,14].

Here we provide morphological and functional evidence on the effect of obesity and obesity joined with type 2 diabetes in human peri-umbilical subcutaneous fat arterioles. The ultrastructural examination of arterioles showed that obesity and obesity aggravated by type 2 diabetes induced similar structural effects, i.e. activate endothelium, thickened the media layer, and apparently reduced the arterioles lumen diameter; the enhanced extracellular matrix accumulation within vascular wall may account for media layer thickening (Figure 1). These structural modifications suggest the installment of a remodeling process, and a possible

restriction of blood perfusion of subcutaneous fat in obesity. In another subcutaneous region, such as the gluteal fat, the hypertrophic remodeling of small arteries wall was found to be associated with type 1 diabetes [15]. Then, it is generally admitted that impaired perfusion may underline much of tissue and organ dysfunction associated with hypertension, obesity and diabetes mellitus [16].

At the functional level, our data show that obesity associated or not with type 2 diabetes produced an enhanced contractile response to NA of human peri-umbilical subcutaneous fat arterioles, and the suppression of endothelium-dependent vasodilation to ACh (Figure 2). We correlate the enhanced vasoconstriction, the thicker media layer, and the drastic diminishment of relaxation to: (i) extracellular matrix accumulation within vascular wall, (ii) decreased eNOS protein expression, and (iii) reduced NO production (Figure 4 and Table 2). Recent data underline obesity-enhanced vasoconstriction of human gluteal subcutaneous small arteries, and the fact that in obesity are lost both the anticontractile property of perivascular fat and the increase in NO bioavailability caused by adiponectin secreted by nearby adipocytes [17]. Then, in the subcutaneous microvascular endothelial cells of type 2 diabetic patients endothelin -1 mRNA levels were found to be significantly higher [18]. As for the vasorelaxation, previous reports show that in type 1 diabetes, the vasodilatation of subcutaneous gluteal small arteries induced by exposure to ACh or BK was reduced, and ~50% diminished in presence of eNOS inhibitors [15]. It was reported that NO contributes to reduced BK-mediated dilation of subcutaneous fat microvessels especially in male subjects [19]. Recently, the reports assess that in diabetes the abnormalities of microvascular blood flow are evident, and become more marked with the development of microvascular complications [20].

Although it is known that insulin stimulates the production and the release of NO [21], we show here that this applies also to subcutaneous adipose tissue arterioles in physiological condition; these display a robust protein expression of phosphorylated and total eNOS (Figure 3b and Figure 4). However, in subcutaneous adipose tissue arterioles, obesity significantly reduced insulin-induced vasorelaxation by diminishment of eNOS protein expression and NO production, and all these processes were aggravated in obesity joined by type 2 diabetes (Figure 4 and Table 2). An explanation for these results came from the studies which showed that, in obesity, the enhanced production of circulating tumor necrosis factor (TNF) may reduce insulin-mediated effects on the microvascular function by impairing the balance between endothelial-derived vasodilator and vasoconstrictor substances. Also, there are evidences which demonstrate that TNF down-regulates the expression of eNOS and up-regulates ET-1 expression in the human endothelial cells [22].

Examination of insulin signaling proteins in arterioles of lean subjects show the strong expression of insulin receptor substrates IRS-1 and IRS-2, as well as of the downstream molecules PI3-kinase, and AKT (serine phosphorylated AKT included) (Figure 4). In accordance with our data, it was established that: (i) the metabolic action of insulin to stimulate the glucose uptake in skeletal muscle and adipose tissue is mediated through the stimulation of insulin receptor, insulin receptor substrate IRS-1, PI3- kinase, phosphoinositide-dependent kinase 1 (PDK-1), and AKT [23], (ii) insulin increased directly eNOS activity leading to augmented NO production [23, 24]. Beside its vasodilator action, insulin has also vasoconstrictor effects. These vasoconstrictor effects are mainly mediated by vasoconstrictor peptide endothelin-1 (ET-1) [23]. ET-1 is produced in the vascular endothelium through stimulation of intracellular MAP-kinase signaling pathway and extracellular signal-regulated kinase-1/2 (ERK1/2) [25]. Thus, insulin has

opposing vasodilator and vasoconstrictor effects, the net effect being dependent on the balance between these two, and its imbalance causes the development of microvascular dysfunction.

In addition, our results revealed that obesity associated or not with type 2 diabetes disrupts the molecules involved in signaling pathway mediated by insulin (IRS-1, IRS-2, PI3-kinase, p-AKT, AKT, p-eNOS, e-NOS), impairs endothelial NO production, and generates insulin resistance condition. In insulin signaling pathway, eNOS is activated through AKT-mediated phosphorylation of serine residue 1179 (p-eNOS serine 1179). The mechanisms regulating eNOS activity and endothelial NO production are complex and sometimes overlapping. For example eNOS contains multiple phosphorylation sites in addition to serine 1179, and increasing evidence has implicated the participation of a variety of kinase and phosphatase activities in the regulation of eNOS activity. The phosphorylation events also participate in controlling the interactions between eNOS and other regulatory proteins [26]. Therefore, we report here that obesity and obesity associated with type 2 diabetes impede the protein expression of molecules associated with insulin signaling, and also blunt arterioles vasodilation.

### **Conclusion**

The results of this study indicate that obesity associated or not with type 2 diabetes alters human peri-umbilical adipose tissue arterioles in terms of structure, function and biochemistry, including diminished eNOS expression and reduced levels of IRS-1, IRS-2, PI3-kinase and AKT in insulin-signaling pathway.

### **Limitations of the study**

This study was carried out on specimens to be discarded from peri-umbilical subcutaneous adipose tissue; although we know the clinical data of the patients (Table 1), they underwent surgery for multiple reasons, and were likely taking a variety of medications. Therefore, one can not exclude the interference of the latter in the results presented. However, in the three groups studied the intact arterioles originate allways from the same body area (i.e. peri-umbilical) and from the deep compartment of the subcutaneous fat (i.e. at 1.5-2.0 cm from the skin surface).

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### **Author contribution**

All authors of this manuscript had the same contribution to the scientific results.

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**Table 1.** Clinical characteristics of the study subjects

Clinical characteristics	Control group (n=20)	Obese group (n=20)	Obese + type 2 diabetes (n=20)	P
BMI (kg/m <sup>2</sup> )	23.5 ± 1.9	35.7 ± 4.8	39.8 ± 2.8	<0.0001†
WC (cm)	81.25 ± 1.7	110.5 ± 1.9	122.25 ± 2.5	<0.002†
Glucose (mg/dl)	92.8 ± 2.6	98.95±6.9	217.03 ± 9.86	< 0.001*
Cholesterol (mg/dl)	151 ± 10.2	165.45 ± 9.04	173.6 ± 13.7	0.12
Systolic blood pressure (mm Hg)	127 ± 2.5	129 ± 1.9	131 ± 2.3	0.18
Diastolic blood pressure (mm Hg)	78 ± 1.2	79 ± 1.6	80 ± 1.8	0.21

Data are means ± SE. *P* values represent the statistical significance of comparisons. Symbols indicate which post hoc comparisons were significant: \* differs from others; †all three groups differ.

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**Table 2.** The NO concentration (nmol/l) delivered at the luminal side of human subcutaneous fat arterioles explanted from lean subjects (control group), obese patients, and obese-type 2 diabetic patients.

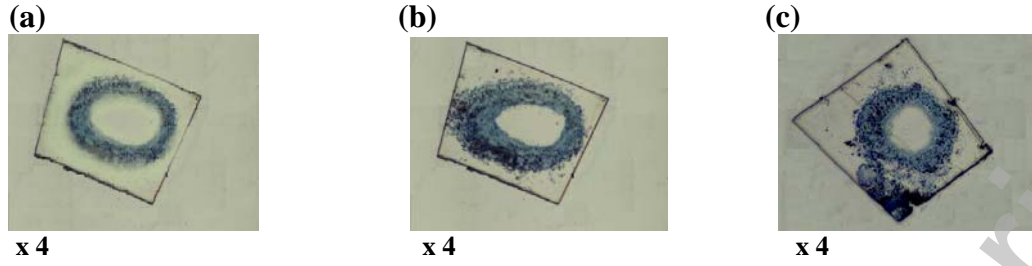
NO (nM)	Control group (n=10)	Obese group (n=10)	Obese + type 2 diabetes (n=10)	P
<b>in the presence of <math>3 \times 10^{-5}</math> M ACh</b>	34.68 ± 1.98	6.11 ± 1.54	2.98 ± 0.7	< 0.0005*
<b>in the presence of 6.4 IU/ml insulin</b>	21.06 ± 1.89	2.55 ± 0.6	1.62 ± 0.5	< 0.0001*

Data are means ± SE. P values represent the statistical significance of comparisons. \* differs from others.

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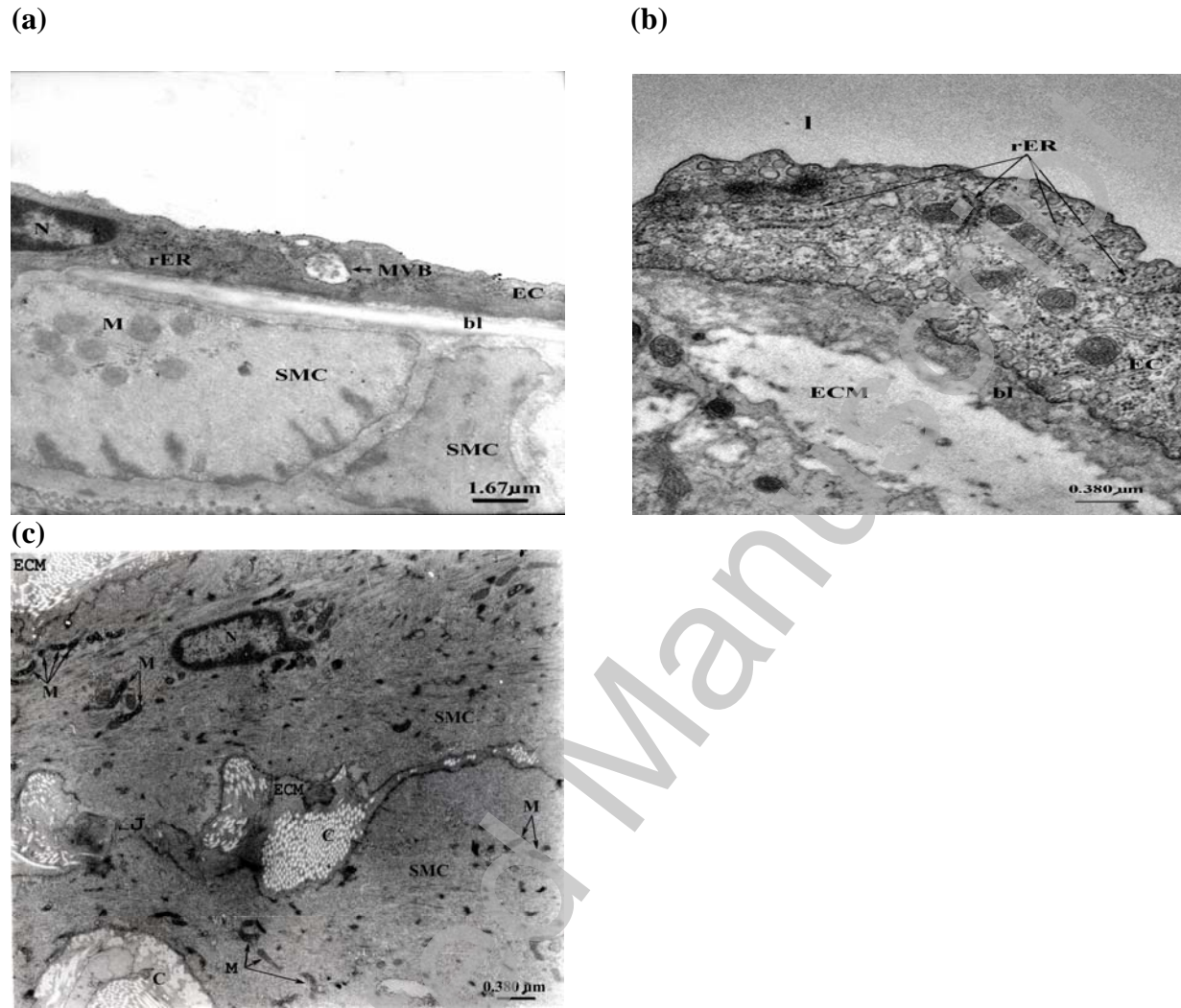
Figure 1A.



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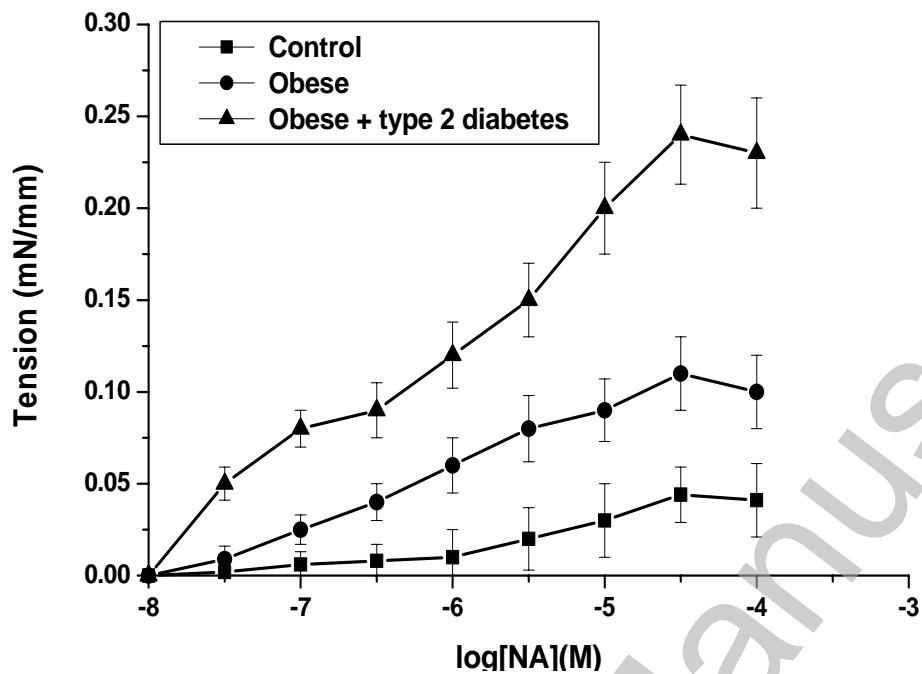
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Figure 2.



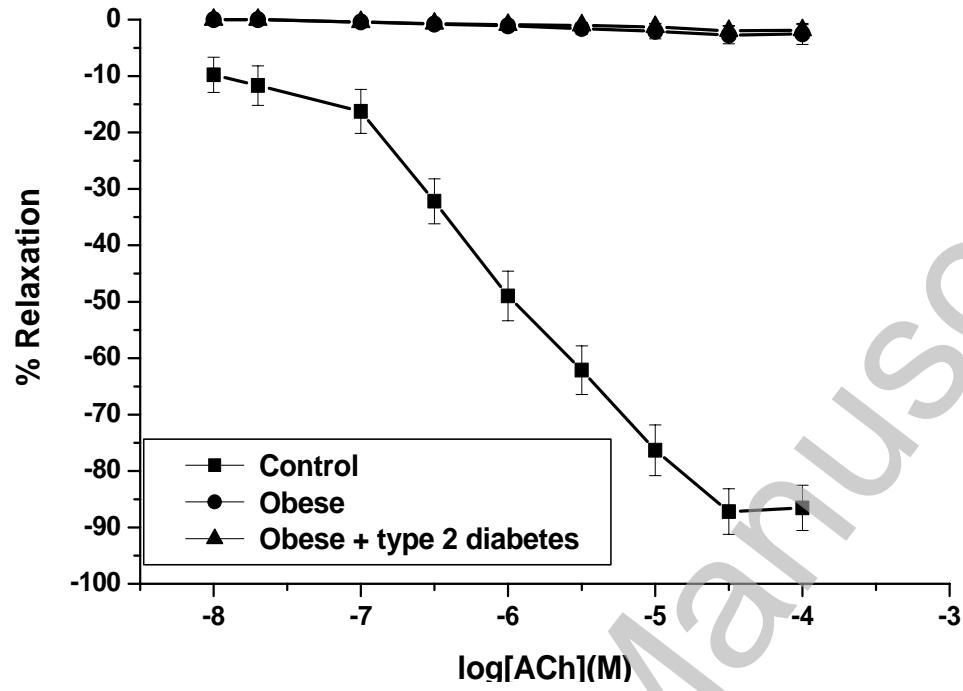
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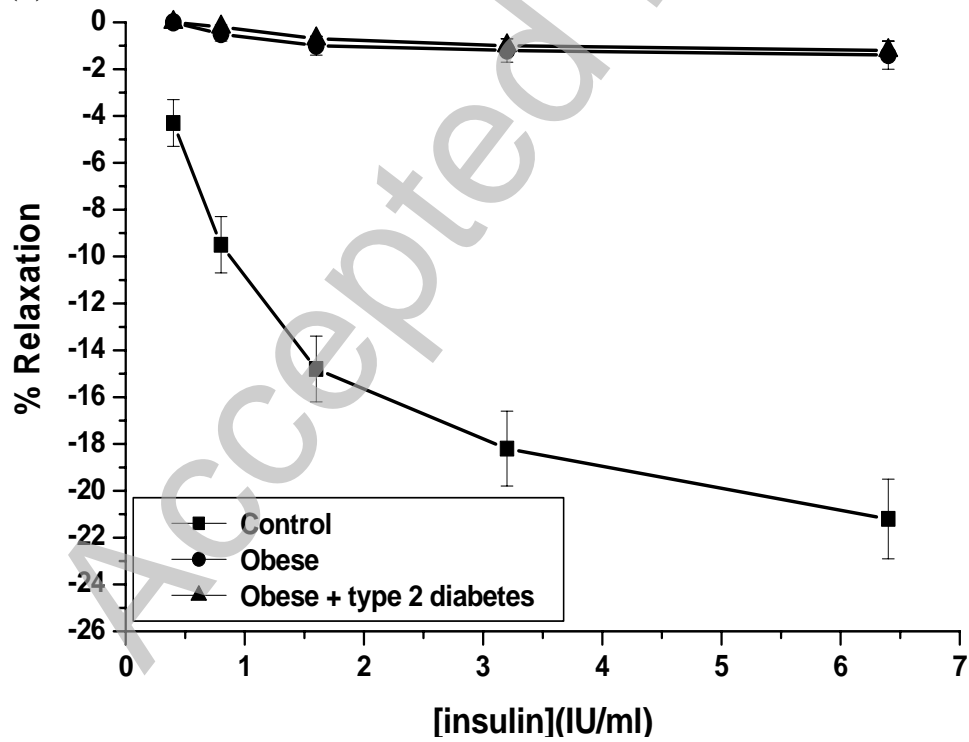


Figure 3.

(a)

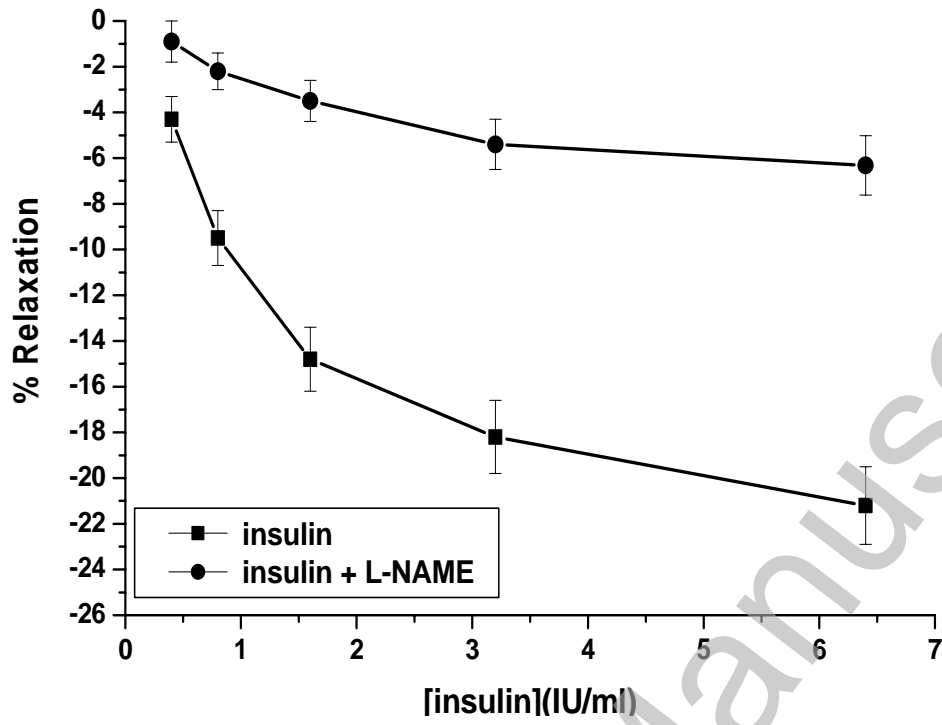


(b)



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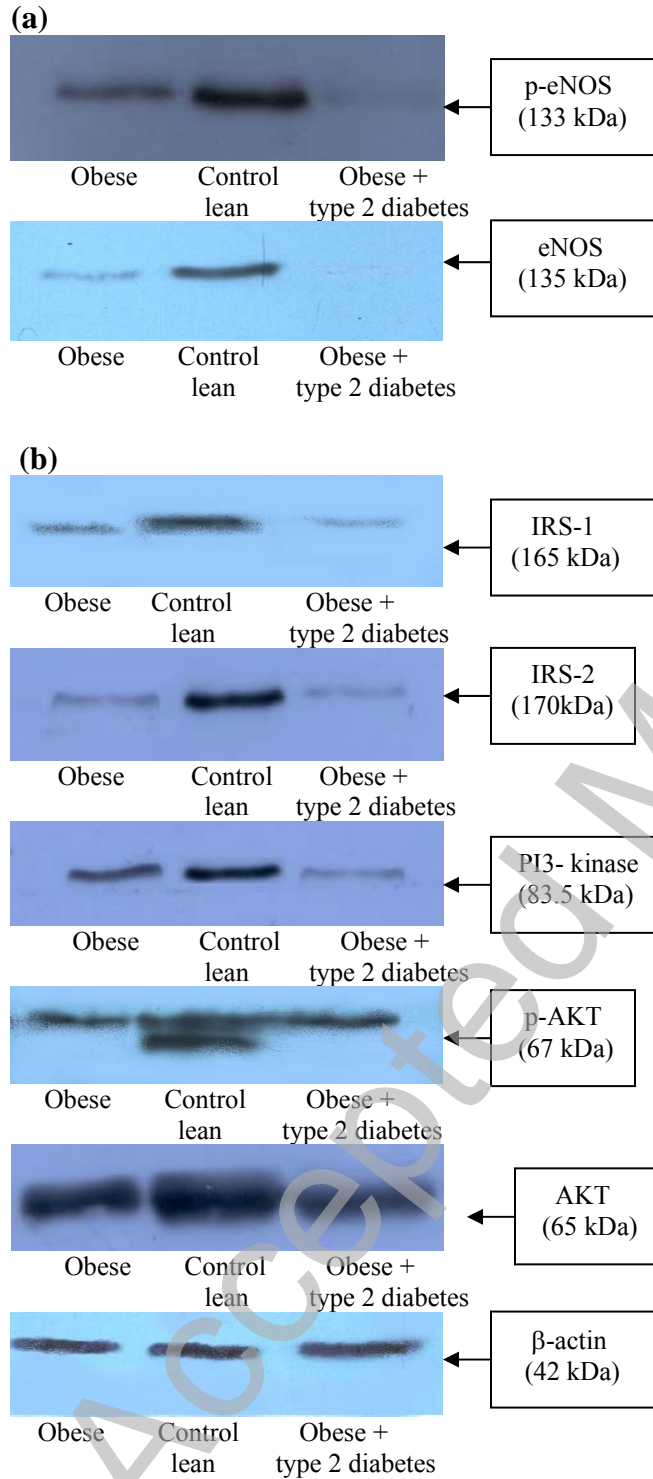
(c)



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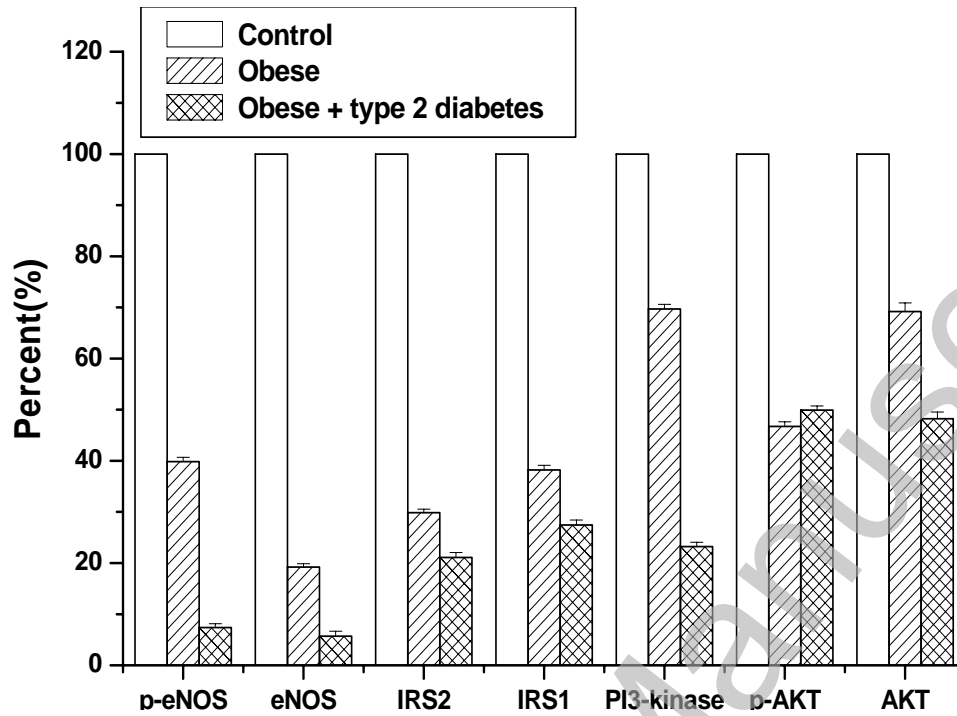
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**Figure 4A.**



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Figure 4B.



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## Figure legends

**Figure 1. A.** Light microscopy aspect of the human peri-umbilical subcutaneous adipose tissue arterioles. (a) C group, (b) obese, and (c) obese with type 2 diabetes. Sections have been stained with 0.25% toluidine blue, and the images are from a representative experiment done twice; **B.** The ultrastructure of the human peri-umbilical subcutaneous adipose tissue arterioles. (a) C group, (b) endothelial cell layer in arterioles explanted from obese patients, and (c) the smooth muscle cell layer in arterioles explanted from obese patients. Note that that arteriolar wall structure was similar in obese and obese with type 2 diabetes groups. For representation, we gave the representative images from the single subject in each experimental group. l: vascular lumen; EC: endothelial cell, M: mitochondria, rER: rough endoplasmic reticulum, SMC: smooth muscle cell, bl: basal lamina, ECM: extracellular matrix, C: collagen fibers, N: nucleus.

**Figure 2.** Dose-dependent contractile response to NA ( $10^{-8}$ - $10^{-4}$ M) of human peri-umbilical subcutaneous adipose tissue arterioles.

**Figure 3.** The vasorelaxation of human peri-umbilical subcutaneous adipose tissue arterioles. (a) dose-dependent vasorelaxation to ACh ( $10^{-8}$  –  $10^{-4}$  M); (b) dose-dependent vasorelaxation to insulin (0.4 IU/ml – 6.4 IU/ml); (c) the vasorelaxant effect of insulin (0.4 IU/ml – 6.4 IU/ml) in the presence and absence of L-NAME ( $10^{-4}$  M).

**Figure 4. A.** Representative Western blot of the arteries explanted from white adipose tissue of obese patients with and without type 2 diabetes compared to C group. The expression of IRS-1, IRS-2, PI3-kinase, AKT, p-AKT, eNOS, p-eNOS; the presence of  $\beta$ - actin on the same transfers was used to prove equal loading of the samples; **B.** The diagram of densitometric measurements: n=10 individual experiments.

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