Vascular Insulin/Insulin-Like Growth Factor-1 Resistance in Female Obese Zucker Rats

M.F. Walsh, S.S. Ali, and J.R. Sowers

Because insulin resistance/diabetes may cause inordinate vascular complications in females, we have investigated the effects of insulin and insulin-like growth factor (IGF-1) on vascular reactivity in 12-week-old female Zucker obese (Ob) rats, a rodent model of insulin resistance and its lean (Ln) age-matched counterpart. Endothelium intact aortic rings from Ob animals and their Ln littermates (12 weeks of age) were subjected to contractile concentration responses to phenylephrine (PE) followed by relaxation to isoproterenol (Iso), with and without preincubation for 2 hours with cholera toxin (CTX; 1 μg/mL) or pertussis toxin (PTX; 2 μg/mL) and before and after incubation with either insulin or IGF-1 (100 nmol/L) for 1 hour. Systolic blood pressure was higher (138 ± 3 *v* 109 ± 4 mm Hg; $P < .0001$) in the 12-week-old Ob rats. Contractile responses to PE were similar in both groups; however, both insulin and IGF-1 induced a paradoxical increase ($P < .001$) in contraction in Ob vasculature (929 ± 92 *v* 679 ± 25 mg, respectively). CTX alone decreased contraction in the Ob ($P < .02$) and PTX in the Ln ($P < .02$), but there were no interactions between either IGF-1 or insulin and the toxins. Marked impairment of relaxation to Iso was seen in aortic rings of these female Ob rats (ED_{50} = 2.6 μmol/L *v* 418 nmol/L, $P = .0002$), an effect exacerbated by preincubation with either insulin or IGF-1 ($P = .0001$). Again, no role for G-proteins could be demonstrated. Insulin-dependent glucose uptake was severely impaired ($P < .05$) in aortic segments of the Ob insulin-resistant rats. Insulin receptor binding, tyrosine kinase activity (TKA), and abundance of several G-protein α subunits (inhibitory and stimulatory) in solubilized arterial membrane preparations (assessed by Western blot) were comparable in the 2 groups. These results indicate that resistance to the vascular actions of insulin/IGF-1 in female Ob rats is a postreceptor event that parallels glucose uptake resistance and is independent of G-proteins.

HEART DISEASE REMAINS the leading cause of death for women in the United States and, in premenopausal women, diabetes mellitus negates the gender-related benefit women exhibit in terms of prevalence of coronary heart disease.[1,2] Although abnormalities in the vascular actions of insulin may be especially pronounced in female models of hypertension and insulin resistance,[3] gender-related differences are rarely studied.[2,4] Parallel resistance to insulin and insulin-like growth factor-1 (IGF-1) has been observed in male Zucker obese (Ob) rats, as well as other hypertensive rodent models.[5,6] A number of contractile and endothelium-dependent relaxation abnormalities, including resistance to the vasodilatory actions of insulin, as well as altered insulin/IGF-1–mediated glucose uptake kinetics in aortic vascular smooth muscle cells (VSMC), have been documented in male, but not female, Ob rats.[7-11]

A growing body of data supports the existence of cross-talk, or cross-regulation, between tyrosine kinase (eg, insulin/IGF-1) and G-protein coupled (eg, adrenergic) receptors.[12-15] In most tissues, insulin action has been related to activity of inhibitory α subunits ($G\alpha_i$).[16,17] The notion has been advanced that insulin resistance is due, in part, to a decreased ability of this peptide to activate $G\alpha_i$ subunits. Recent studies specifically implicate $G\alpha_{i2}$, as transgenic mice deficient in this subunit exhibit hyperinsulinemia and impaired glucose tolerance in vivo,[18] while specific overexpression of this subunit mimics insulin action in liver and adipose tissue.[19] Because human studies suggest that the vasodilatory effects of insulin may be mediated, in part, via α-adrenergic mechanisms,[20,21] we investigated the role of G-proteins in insulin/IGF-1 modulation of adrenergically induced-vasoconstriction/dilation in female Ob rats. Accordingly, we examined the effects of pertussis toxin (PTX) and cholera toxin (CTX) on insulin/IGF-1 modulation of α-adrenergic (phenylephine [PE])-induced contraction and β-adrenergic (isoproterenol [ISO])-mediated relaxation of endothelium-intact aortic rings of female Ob rats and their lean (Ln) littermates. Ribosylation of $G\alpha_i$ subunits by PTX inhibits its action. In contrast, ribosylation of $G\alpha_s$ by CTX activates this subunit. Thus, both PTX and CTX should increase vascular cyclic adenosine monophasphate (cAMP) production attenuating contraction and increasing relaxation in normal vasculature,[21] but their interactions with insulin/IGF-1 are unknown. Accordingly, we have assessed a possible role of abnormalities, G protein subunit activation in the genesis of vascular resistance to insulin/IGF-1 in the female Ob rat. In addition, we investigated possible relationships between vasomotor responses to insulin/IGF-1, insulin receptor concentration, and tyrosine kinase activity (TKA), as well as insulin-mediated glucose transport in the vasculature of Ob and Ln rats.

MATERIALS AND METHODS

Methods

Female Ob rats, and their Ln littermates (n = 30 of each), were purchased at 12 weeks of age from Harlan (Indianapolis, IN). The animals were housed 2 to a cage and fed and watered ad libidum. On the day of sacrifice, animals were weighed, and their blood pressure

From the Division of Endocrinology, Diabetes, and Hypertension, SUNY Health Science Center at Brooklyn, and Veterans Administration Medical Center (VAMC) Brooklyn, Brooklyn, NY and VAMC Detroit, MI.

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Address reprint requests to J.R. Sowers, MD, Professor of Medicine and Cell Biology, Director, Division of Endocrinology, Diabetes, and Hypertension, SUNY Health Science Center at Brooklyn, 450 Clarkson Ave, Box 1205, Brooklyn, NY 11203.

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was assessed via tailcuff measurement (NarcoSystems, Portland, OR). Animals were anesthetized with sodium pentobarbital (35 mg/kg; intraperitoneal [IP]) and a blood sample removed by cardiac puncture for glucose determination (Yellow Springs Instruments, Yellow Springs, OH). Aortae were dissected free for immediate vascular reactivity and glucose transport studies. Mesenteric arteries were also excised, cleaned of fat, snap frozen, and stored at −70°C for future preparation of insulin receptors and measurement of G-protein subunit abundance by Western blot techniques. All procedures were approved by the institutional animal committee.

Vascular reactivity. Aortae obtained from Ln and Ob rats were immediately placed in ice-chilled buffer containing (mmol/L): 130 NaCl, 15 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.5 $CaCl_2$, 1.2 M_gSO_4, 0.03 EDTA, and 5.6 glucose. Aortic rings (3 mm) were suspended in a muscle bath (Gould Instruments, Cleveland, OH) in aerated buffer (95% 02/5% CO_2) maintained at 37°C.[22,23] Rings were stretched to 1.5 g of tension and equilibrated for 30 minutes before cumulative additions of PE (10^{-9} to 10^{-5} mol/L) followed by relaxation to graded concentrations of Iso (10^{-9} to 10^{-5} mol/L). Rings were then washed and incubated for an additional hour with either insulin or IGF-I (100 nmol/L) and the contraction/relaxation studies repeated.[22] Integrity of the endothelium was ascertained with acetylcholine (10^{-5} mol/L).[22] For the toxin experiments, rings were preincubated for 2 hours in Dulbecco's Modified Eagle's Medium (GIBCO, Rockville, MD) with either 1 μg/mL CTX or 2 μg/mL PTX before contractility/relaxation studies before and after exposure to insulin or IGF-1. To further assess cAMP-dependent relaxation, rings were precontracted with PE and challenged with increasing concentrations of forskolin (all chemicals were purchased from Sigma, St Louis, MO).

Insulin receptor preparation, binding, and TKA. Frozen mesenteric arteries were homogenized in solubilization buffer: 50 mmol/L Hepes (pH 7.6) supplemented with 1% Triton-X, 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.2 g/mL aprotinin, 2 mmol/L $NaVO_4$, 20 μg/mL leupeptin, 25 μg/mL soybean trypsin inhibitor, and 5 mmol/L iodoacetamide. After centrifugation, the receptors were eluted from the supernatant with 50 mmol/L Hepes, 150 mmol/L NaCl (pH 7.6) containing 0.1% Triton-X, 0.45 mol/L N-acetylglucosamine, 10% glycerol, and 0.2 g/mL aprotinin as previously described by us.[23] Protein content was measured with a commercially available kit (Biorad, Hercules, PA). To determine maximal receptor binding, equal protein aliquots were incubated overnight at 4°C with ^{125}I Insulin (Amersham, Chicago, IL) in the presence and absence of excess cold porcine insulin. Separation was accomplished by centrifugation (21,000 × *g*) after sequential addition of 0.3 gamma globulin and 22% and 11% polyethylene glycol. The pellet was counted in a gamma counter (Packard AutoGamma, Houston, TX) and total binding corrected for nonspecific binding.[11,23] Basal receptor TKA was measured using a colorimetric method (Oncogene Products, Boston, MA) and results presented in arbitrary densitometric units.

Aortic glucose uptake. Rats were fasted overnight before removal of the aorta and a blood sample. Vessel segments were preincubated in basic bath buffer (as for vascular reactivity) with aeration for 30 minutes. ^{3}H-2-deoxyglucose (Amersham) was added before addition of insulin (100 nmol/L). After a 20-minute exposure, rings were flash frozen in liquid nitrogen, dried in a 58°C oven, weighed, and solubilized for counting (Packard Tricarb). ^{14}C-mannitol was used as a control.[11,23] Uptake was corrected for dry weight and is expressed as percent over baseline.

G-alpha subunit immunoblotting. $G_{s,\ il\text{-}2}$ and $_{i3}$ were quantitated following sodium dodecyl sulfate-polyacry lamide gel electrophoresis (SDS-PAGE) separation on a 15% gel as follows. Frozen mesenteric arteries were homogenized in 250 mmol/L sucrose and centrifuged at 24,000 rpm for 20 minutes to obtain the crude membrane pellet. This was solubilized in a buffer containing 1 mmol/L dithiothreitol (DTT), 1 (in mmol/L) EDTA and 1% Na cholate in 20 mmol/L Tris/100 mmol/L NaCl (pH 8.0). Equal amounts of lysate proteins were separated by electrophoresis, transferred onto nitrocellulose, and blotted with specific antibodies for the various G-protein subunits (Upstate Biologicals, Lake Placid, NY). Detection was via ECL (Amersham) and quantitation of the resulting bands was done via Phosphol Imager.

Statistics. All data are presented as mean ± SEM. Statistics for animal weights, blood pressures, blood glucose, insulin binding, and TKA were calculated by Student's *t* test. Vascular reactivity was assessed by 2-way analysis of variance (ANOVA) (with Fisher's protected least significant difference) and ED_{50} were calculated utilizing the Pharm C Program (Springer-Verlag, New York, NY).

RESULTS

As shown in Table 1, at time of sacrifice, the female Ob animals were heavier than their Ln littermates (n = 30). Systolic blood pressure was also higher in the Ob, even at this young age (12 weeks). Although nonfasting plasma glucose levels were similar in both groups, fasting glucose concentrations were significantly lower in the Ln, suggesting the Ob animals already manifested some degree of insulin resistance.

Vascular Reactivity

Insulin/IGF-1 modulation of contractility. Contractile responses to graded concentrations of PE were similar in aortic rings obtained from each group of female rats (Fig 1). Maximal contractility was 620 ± 65 mg of developed tension with an ED_{50} of 153 nmol/L in the Ln (n = 10) and 679 ± 75 mg with an ED_{50} of 126 nmol/L in the Ob (n = 10). A 60-minute preincubation with 100 nmol/L insulin did not change contraction in the Ln, but strikingly enhanced it in the Ob (to 929 ± 92 mg; $P = .0001$). Preincubation with the same dose of IGF-1 attenuated the contractile response to PE in the Ln ($P = .024$; data not shown), but augmented contractility in the Ob vasculature ($P = .03$) (Fig 1). Thus, aortic rings of Ob animals showed a paradoxical increase in PE-mediated contractility after exposure to insulin/IGF-1.

Relaxation to graded concentrations of Iso was strikingly impaired in aortic rings of Ob rats when compared with Ln controls; ED_{50} increased from 418 nmol/L to 2.6 μmmol/L ($P = .0002$) (n = 10). Preincubation with both insulin and IGF-1 further attenuated relaxation in Ob vasculature ($P = .0001$) (Fig 2).

Role of G-proteins. To examine the role of G-proteins in these actions of insulin/IGF-1, the experiments were repeated in aortic rings preincubated for 2 hours with either toxin[16,17] (n = 10). CTX alone had no effect on PE-induced contraction in the Ln, but lowered contraction in Ob aortic rings ($P = .021$) (Fig 3), suggesting that Gs may already be maximally stimulated in Ln, but not in Ob. The opposite was true for PTX; the toxin inhibited contractile responses only in the Ln, increasing

Table 1. General Characteristics of Female Zucker Ln and Ob Rats (n = 30)

	Ln	Ob	*P*
Weight (gm)	293 ± 4	357 ± 16	.001
SBP (mm Hg)	109 ± 4	138 ± 3	.0001
Fasted plasma glucose (mg/dL)	105 ± 10	157 ± 9	.005

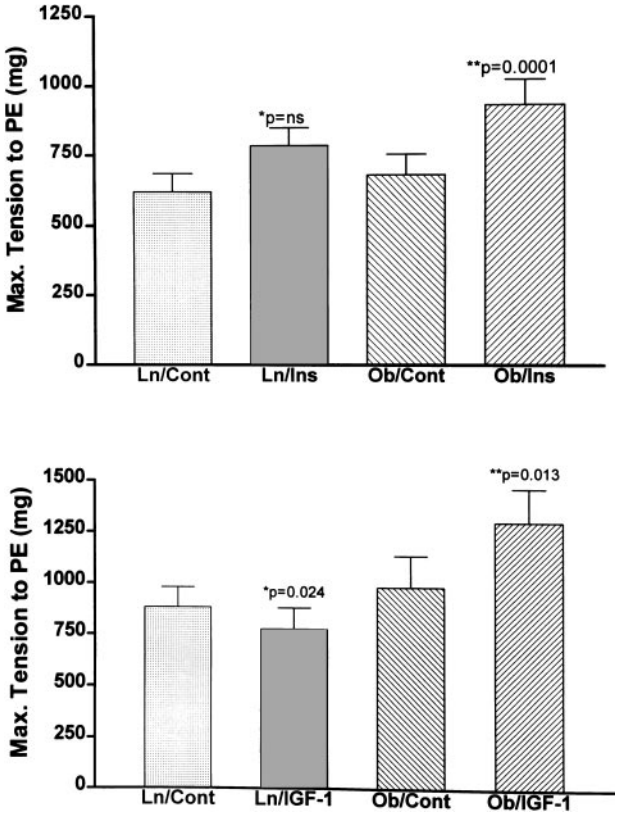

Fig 1. Maximal developed tension to PE (10^{-9} to 10^{-5} mol/L) in endothelium-intact aortic rings of female Ln Zucker rats and their Ob littermates (n = 10). Contraction was similar in the Ln and Ob; both insulin and IGF-1 (100 nmol/L for 60 minutes) paradoxically enhanced contraction in Ob. (P = .0001, **P = .013 *v* respective controls). In the Ln group, IGF-1 attenuated the contractile response to PE (*P < .02). Statistics calculated by 2-way ANOVA over the entire dose response.**

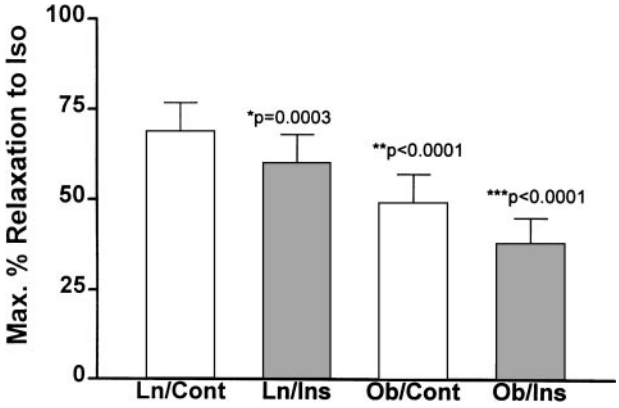

Fig 2. Maximal relaxation to Iso (10^{-9} to 10^{-5} mol/L), expressed as percent of submaximal precontraction to PE, in Ob and Ln aortic rings (n = 6). Relaxation was severely impaired (*P = .0002) in the Ob as compared with the Ln and further compromised by insulin (P = .0001) by 2-way ANOVA.**

the ED_{50} to 228 nmol/L (Fig 3). This finding suggests that PTX sensitive G_i subunits may not be normally functional in Ob vasculature. Insulin or IGF-1 preincubation did not affect either CTX or PTX action in the Ln vasculature (data not shown), suggesting that the vascular effect of these peptides are not normally dependent on G-protein interaction. However, in Ob rat aortic rings, preincubation with insulin or IGF-1 reversed the CTX-induced decrease in contractility indicating that the paradoxical contractile effects of the hormones can overcome the toxin-mediated attenuation, most probably by a non–G-protein-dependent mechanism.

Neither CTX nor PTX affected Iso-induced relaxation in vasculature from Ln animals, and there were no interactions with either insulin or IGF-1 (data not shown). However, both CTX and PTX improved relaxation in the Ob aortic rings (P = .044 and .041, respectively) (Fig 4) suggesting an impairment in cAMP generation may contribute to the lowered relaxation seen in these vessels. As in the contraction experiments, insulin negated the effects of both CTX (P = .009) and PTX (P = .014) (Fig 4), whereas IGF-1 only partially reversed those of PTX (P = .008) (data not shown). Aortic rings of Ob and Ln animals relaxed in an identical manner to graded concentrations of forskolin after similar submaximal contraction (data not shown) indicating that the response to cAMP is not deficient in Ob vasculature. Taken together, the lack of interaction between insulin/IGF-1 and CTX/PTX in both the contractility and relaxation experiments suggests that G-proteins do not play an important role in the actions of these hormones, at least in the aorta of these female animals. Densitometric quantitation of Gα subunit concentrations on Western blots (n = 3) showed no significant difference in any of the subunits studied: G_s 2,089 ± 403 in Ln versus 2,107 ± 622 in Ob; $G_{il\text{-}2}$ = 1,823 ± 552 in Ln versus 1,890 ± 731 in Ob; and G_{i3} = 2,477 ± 905 versus 2,497 ± 1,191 in Ob, in arbitrary densitometric units (Fig 5).

Glucose transport, insulin receptor binding and TKA. Glucose transport, assessed by ^{3}H-2-deoxyglucose uptake was stimulated by 23% ± 3% 20 minutes after exposure of vascular segments from Ln animals to 100 nmol/L insulin (Table 2); vasculature from Ob animals failed to respond. Specific binding of ^{125}I-Insulin to WGA-purified mesenteric artery membrane receptor preparations was higher in Ln than in Ob vascular

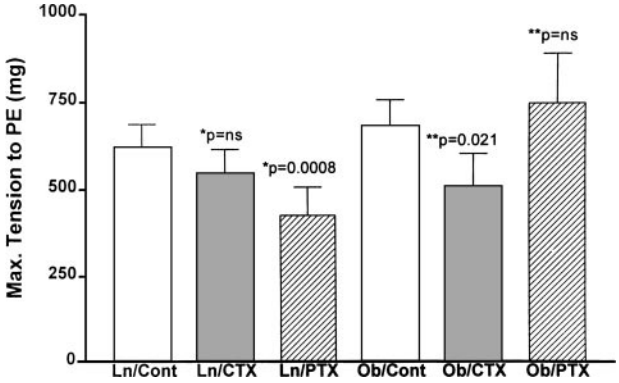

Fig 3. Effect of a 2-hour preincubation with CTX and PTX on contraction to PE in Ln and Ob (n = 10). PTX lowered maximal tension developed to PE in the Ln (*P = .02). In contrast, CTX lowered the tension development in the Ob (P = .02) (n = 10).**

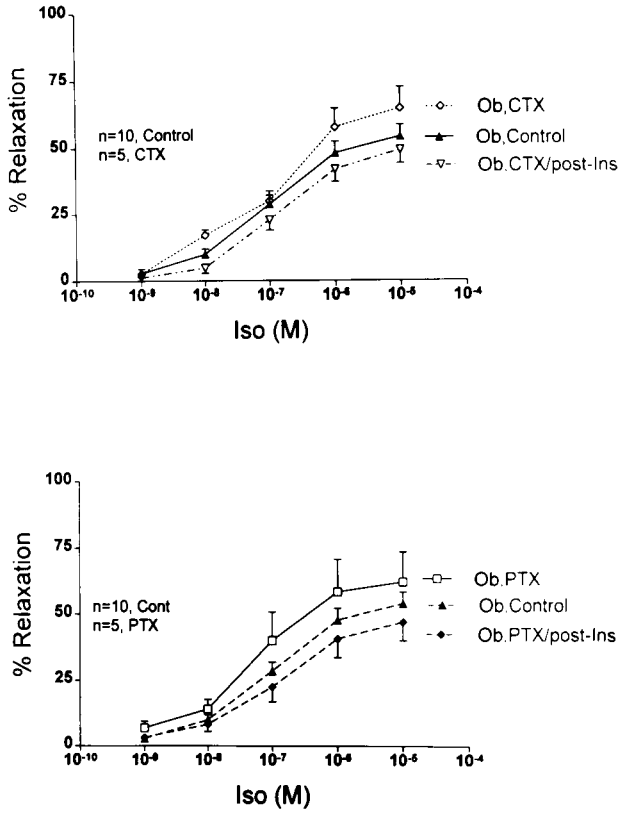

Fig 4. Effects of a 2-hour preincubation with PTX and CTX on Ob vasculature relaxation responses to Iso. Both PTX and CTX improved relaxation (*P* = .041 and .044, respectively), and this effect was abrogated by insulin (INS) (n = 6).

membranes, while TKA activity, corrected for protein concentration, in these same vascular membrane preparations was lower, but these differences were not significant (Table 2).

DISCUSSION

Although insulin resistance is known to abrogate the cardiovascular protective effects of estrogen, ours is the first report of elevated blood pressures in female insulin-resistant Ob rats. Data from this investigation indicate that, in 12-week-old female Zucker rats, elevated systolic blood pressure and fasting plasma glucose levels are accompanied by impaired aortic relaxation to Iso and resistance to the vasodilatory actions of insulin/IGF-1. This vasodilatory resistance parallels a resistance to insulin-mediated glucose transport, but cannot be explained by differences in insulin receptor binding or vascular TKA; relative abundance of various G-protein alpha subunits also does not appear to be a factor.

Table 2. Vascular Insulin Receptors and Activity in Ln and Ob Rats (n = 3)

	Ln	Ob	*P*
Glucose transport	23 ± 3%	0	.05
Mesenteric artery insulin receptors (% binding)	2.4 ± 0.7	1.8 ± 0.6	NS
Mesenteric artery receptor TKA	0.208 ± 0.04	0.238 ± 0.02	NS

Abbreviation: NS, nonsignificant.

Unlike some previous studies in males,[24,25] we did not observe an enhancement of vascular reactivity in our female Ob animals. Literature reports on Ob vascular reactivity are often contradictory, perhaps due to differences in the vessels studied, the choice of agonists, age and blood pressure status, and sex of the animals.[24-26] Insulin was shown to have no effect on norepinephrine-induced contraction in mesenteric arteries of male Ob animals.[26] Acetylcholine relaxation has been reported to be impaired[25,26] and mesenteric artery contraction/relaxation to be equal to those of male Ln controls.[27] Indeed, both insulin and IGF-1 induced a paradoxical increase in vascular reactivity in these female rats along with a further deterioration in relaxation, an observation similar to one previously reported in female stroke prone spontaneously hypertensive rats treated with a sulphonylurea for 5 months.[3]

Results of the forskolin experiments suggest that the defect mediating the impaired response to Iso in Ob animals must precede cAMP action in the signaling cascade. However, we found no differences in the abundance of G_s and $G_{i1-2,3}$ subunits in mesenteric artery membranes of Ln and Ob rats by

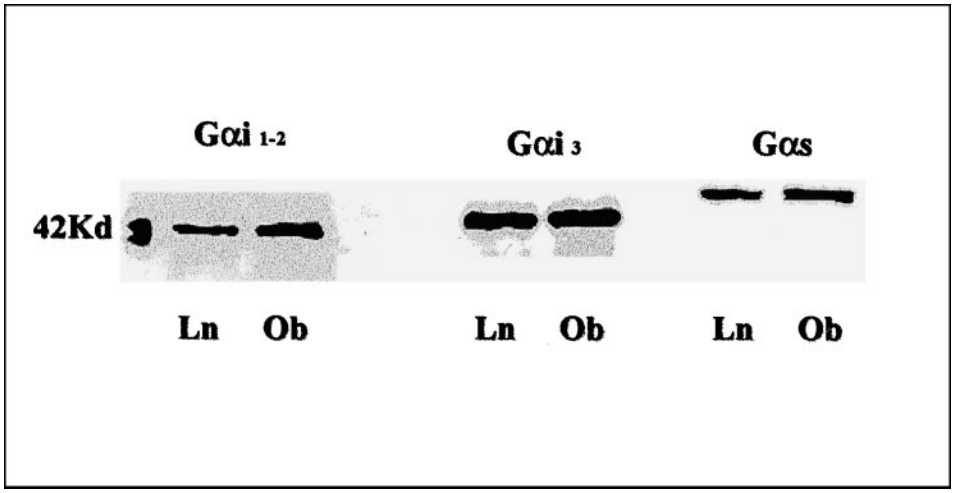

Fig 5. Representative Western blot of G-protein α subunits in solubilized mesenteric artery membrane preparations. Densitometric analysis showed no differences in abundance of any subunit tested between the Ln and the Ob (n = 3 mesenteric preparations).

Western analysis. Alterations in G-protein content have been reported in diabetes and often coincide with changes in insulin action.[28,29] In streptozotocin-induced diabetes, G_i is lower in liver, but normalized by insulin treatment; $G_{i1,2,3}$ expression is decreased in adipocytes of the Ob diabetic mouse, but only $G_{i1\text{-}2}$ are affected in the insulin-resistant db/db mouse.[30] In hypertensive models, $G_{i1\text{-}3}$ levels are increased in spontaneously hypertensive rats (SHR) aorta, while G_s and $G_{i1\text{-}3}$ are lower in the Milan hypertensive animal.[31] Reduced function of G_s was reported to be involved in the abnormal relaxation to beta adrenergic receptor agonists in the SHR.[32] Deficient cAMP generation may contribute to the impairment seen in female Ob rats, as well as treatment with either CTX or PTX improved relaxation to the Iso. However, because neither CTX nor PTX prevented the paradoxical insulin/IGF-I–induced attentuation in vascular relaxation, G-proteins probably do not participate in vascular smooth muscle resistance to the actions of these peptides.

Impairment of Iso-mediated relaxation in the female Ob rat may be related to deficiencies in endothelial cell nitric oxide (NO) generation,[33] as well as altered cAMP generation/activity at the level of the smooth muscle.[34] Insulin/IGF-1 stimulate NO production in vascular endothelial cells and G-proteins, notably of the α_i family, have been reported to be involved in the release of NO by these cells in response to various stimuli.[35,36] The experiments in this study were not designed to examine this possibility, but the lack of interaction of insulin/IGF-1 with CTX and/or PTX argue against participation of G-proteins in resistance at the level of the endothelium. In transgenic mice, decreased expression of $G_{i1\text{-}2}$ results in insulin resistance in liver and adipose tissue; this could be related neither to differences in the generation of cAMP nor to TKA of the insulin receptor. Phosphorylation of IRS-1 was decreased, but this was correlated to enhanced activity of a specific phosphatase, PTP1B.[17] An increase in phosphatase activity has been reported in Ob skeletal muscle[37]; a similar defect at the level of the vasculature could account for the insulin/IGF-I resistance seen in our study. However, lack of interaction between the peptide hormones, insulin and IGF-1, and the toxins preclude a link to a specific G-protein in the female Ob rat.

We observed a marked decrease in insulin-induced glucose uptake in vasculature from female Ob rats, in conjunction with a slight reduction in insulin binding. This is in keeping with a previous study in VSMC from male Zucker rats,[11] although the decreased response was more striking in female VSMC. Although glucose transport is usually reported to be deficient in tissues from Ob animals, insulin receptor number and TKA vary with the tissue being examined. Receptor number is generally reduced in liver, adipocytes, and skeletal muscle, but TKA is higher in liver and unchanged in heart,[38,39] but no binding or TKA data had been previously published for vascular tissue in the Ob rat to date. Overall, however, the cited literature suggests that insulin receptor binding, TKA, and glucose transport are not necessarily linearly related and that other postreceptor mechanisms must be involved. However, the greater diminution in insulin-mediated glucose transport in VSMC from females is consistent with greater resistance to the vasodilatory responses of insulin being related to greater metabolic alterations in female vasculature.

In aortic rings from female Ln rats, relaxation was not enhanced, but PE-induced contraction was not attenuated by insulin, although IGF-1 was effective in this regard. This is consistent with results obtained in a human study suggesting that insulin interacts with α_2 and β-adrenergic receptors but not α_1.[20] Disparate responses to insulin and IGF-1 and lack of additive effects between these hormones and ISO point to strain and sex-specific differences in vascular responses to vasoregulatory factors. Abnormal relaxation may be consequent to lower cAMP generation in the hypertensive female Ob rats. The mechanisms involved in the paradoxical enhancement of PE-induced contraction and exacerbation of the relaxation defect by insulin and IGF-1 do not appear to be due to G-protein deficiencies, but may be related to altered insulin and IGF-1–mediated vascular glucose uptake in female Ob rats.

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