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Therapeutic Actions of an Insulin Receptor Activator and a Novel PPARγ Agonist In the SHROB Rat Model of Metabolic Syndrome X

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Abbreviations: SHROB: spontaneously hypertensive obese rats; SHR: spontaneously hypertensive rats; DMAQ-B1: demethylasterriquinone B-1, PPEIA: 2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid

Abstract: Insulin resistance clusters with hyperlipidemia, impaired glucose tolerance, and hypertension as metabolic Syndrome X. We tested a low molecular weight insulin receptor activator (demethylasterriquinone B-1, DMAQ-B1), and a novel indole PPARγ agonist, (2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid; PPEIA) in spontaneously hypertensive obese rats (SHROB), a genetic model of Syndrome X. Agents were given orally for 19d. SHROB showed fasting normoglycemia, but impaired glucose tolerance following an oral load as shown by increased glucose area under the curve (AUC) (20,700 mg*min/mL vs 8100 in lean SHR). Insulin resistance was indicated by 20-fold excess fasting insulin and increased insulin AUC (6300 ng*min/mL vs 990 in SHR). DMAQ-B1 did not affect glucose tolerance (glucose AUC = 21,300), but reduced fasting insulin 2-fold and reduced insulin AUC (insulin AUC = 4300). PPEIA normalized glucose tolerance (glucose AUC = 9100) and reduced insulin AUC (to 3180) without affecting fasting insulin. PPEIA also increased food intake, fat mass and body weight gain (81±12g vs 45±8 g in untreated controls), while DMAQ-B1 had no effect on body weight but reduced subscapular fat mass. PPEIA but not DMAQ-B1 reduced blood pressure. In skeletal muscle, insulin-stimulated phosphorylation of the insulin receptor, and IRS-1 associated PI 3-kinase activity were decreased by 40-55% in SHROB relative to lean SHR. PPEIA, but not DMAQ-B1 enhanced both insulin actions. SHROB also showed severe hypertriglyceridemia (355±42 mg/dL vs 65±3 in SHR) attenuated by both agents (DMAQ-B1: 228±18; PPEIA: 79±3). Both these novel antidiabetic agents attenuate insulin resistance and hypertriglyceridemia associated with metabolic syndrome, but via distinct mechanisms.

Introduction

Metabolic syndrome X consists of insulin resistance as a primary defect associated with compensatory hyperinsulinemia, impaired glucose tolerance, dyslipidemia, and hypertension (Zavaroni et al., 1994). Abdominal obesity is often present. This clinical syndrome is often a harbinger of type 2 diabetes and atherosclerotic heart disease. Early interventions might prevent the progression of this syndrome to potentially lethal disease states.

A leading genetic model of metabolic Syndrome X is the SHROB or Koletsky rat (Velliquette and Ernsberger, 2003b;Ernsberger et al., 1999b;Friedman et al., 1997). The SHROB rat carries a non-sense mutation in the leptin receptor (*fa*^{K}), propagated on a spontaneously hypertensive background(Ernsberger et al., 1999b) . The SHROB shows multiple metabolic phenotypes including abdominal obesity, spontaneous hypertension, hyperinsulinemia and hyperlipidemia without fasting hyperglycemia (Koletsky and Ernsberger, 1992;Ernsberger et al., 1993;Ernsberger et al., 1994;Koletsky et al., 1995). These features closely resemble those found in the human metabolic Syndrome X.

The insulin resistance in Syndrome X likely arises through multiple mechanisms. On the cellular level, impaired tyrosine phosphorylation of the insulin receptor and insulin receptor substrate protein 1 (IRS-1) has been reported previously in skeletal muscle of sub-populations of insulin resistant subjects (Brozinick, Jr. et al., 2003;Pratipanawatr et al., 2001;Friedman et al., 1999) and in the SHROB (Friedman et al., 1997). IRS-1 tyrosine phosphorylation is required to fully activate the enzyme PI 3-kinase, a required step in the pathway for insulin mediated glucose transport in skeletal msucle (Petersen and Shulman, 2002). Most available data support the hypothesis that failure to activate PI 3-kinase occurs in insulin resistant states mainly due to reduced

phosphorylation of IRS-1 on tyrosine residues (Esposito et al., 2001). Agents that improve insulin signaling at the level of the insulin receptor, IRS-1 or PI 3-kinase, particularly in skeletal muscle, may therefore have high potential to target insulin resistance associated with Syndrome X.

Thiazolidinediones are the best known of a new generation of antidiabetic agents which act through activation of peroxisome proliferator-activated receptor gamma (PPARγ) (Berger and Moller, 2002). PPARγ agonists have multiple insulin sensitizing actions. The primary site of action is in adipose tissue, since this is the primary site of PPARγ receptor expression and mice lacking adipose tissue do not show antidiabetic effects from PPARγ agonist treatment (Chao et al., 2000). PPARγ agonists promote the differentiation of preadipocytes to adipocytes and increase cell number while decreasing average cell size. Actions in the liver are suggested by changes in hepatic gene expression following treatment with PPARγ agonists (Dana et al., 2001;Singh et al., 2001) , and by the preservation of lipid lowering effects of these agents in mice lacking adipose tissue (Chao et al., 2000). In the present study, we tested the action of a novel PPARγ agonist on an animal model of syndrome X. Since these agents have been shown to delay the onset of type 2 diabetes in susceptible individuals, investigation of the actions of PPARγ agonists on the insulin resistant prediabetic state is warranted. Because the predominant class of PPARγ agonists, the thiazolidinediones, may have additional effects not mediated by PPARγ receptors (Brunmair et al., 2001), we elected to test a novel indole compound which is a high-affinity PPARγ agonist (Berger et al., 1999).

A small molecule that mimics many of the actions of insulin has been described (Air et al., 2002;Roper et al., 2002;Ding et al., 2002;Li et al., 2001;Salituro et al.,

2001;Qureshi et al., 2000;Zhang et al., 1999). This benzoquinone, DMAQ-B1, directly activates the intrinsic tyrosine kinase activity of the insulin receptor (Li et al., 2001;Salituro et al., 2001;Qureshi et al., 2000;Zhang et al., 1999). This orally active compound has the potential to augment insulin action in murine models of Type 2 diabetes. Insulin-resistant diabetic mice bearing the db/db and ob/ob genotype show reduced levels of plasma glucose, improved glucose tolerance, and reduced food intake and weight loss. They also showed improved insulin sensitivity beyond that caused by weight loss (Air et al., 2002). In the present study, we sought to compare the effectiveness of DMAQ-B1 in a model of metabolic syndrome X, an insulin resistant prediabetic state marked by extreme hyperinsulinemia. We hypothesized that DMAQ-B1 may partially lower endogenous insulin requirements, thereby reducing the burden on the pancreas imposed by excessive insulin production.

MATERIALS AND METHODS

Materials. DMAQ-B1 and PPEIA were obtained from Merck Research Laboratories (Rahway, New Jersey). Other drugs and reagents were obtained form Sigma-Aldrich Chemicals (St. Louis, MO).

Animals. The SHROB strain was developed at Case Western Reserve University School of Medicine and has been maintained as a closed colony (SHROB/Kol) since 1970 and propagated by continuous brother-sister mating for over 30 years. SHROB (N =24) were housed in individual plastic cages in a separate limited-access room of the animal facility. Both male (N =12) and female (N =12) animals were used, and divided evenly between groups. The initial age at the start of the study was 88 ± 4 days. The animals were sorted into control and treated groups, matched on the basis of age, sex, body weight and blood pressure. Littermates were separated into different groups. A group of lean SHR littermates (N=8) were studied for comparison. Rats had free access to tap water at all times.

Drug treatments. Drugs were incorporated into rat chow (Teklad) in order to provide the appropriate dose in mg/kg. Based on food intake data in SHROB, chow provided 750 ppm of a novel indole-acetic acid PPARγ agonist, PPEIA (2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid), in order to provide a dose of approximately 50 mg/kg/d. The chow for the DMAQ-B1 group incorporated 600 ppm of drug in order to provide a dose of approximately 40 mg/kg/d. Control SHROB and lean SHR littermates received normal chow. Animals had free access to chow and tap water at all times, and food intake and body weight was measured every other day.

Oral glucose tolerance tests were carried out at the end of the treatment period as previously described (Velliquette and Ernsberger, 2003a;Velliquette and Ernsberger, 2003b;Ernsberger et al., 1999a). All rats were fasted for 18 h and administered a 50% glucose solution in water by gavage at a dose of 6 g/kg body weight. Blood samples (0.4 ml) were collected under local anesthesia (bucaine) applied from the tail into heparinized capillary tubes at 0, 30, 60, 90, 120, and 180 and 240 min. Glucose was measured in whole blood by colorimetric glucose oxidase assay (One-Touch, Lifescan, Milpitas, CA). The remaining blood sample was chilled on ice and centrifuged for 20 min at 5,000 x g at 4^{o}C, and the plasma frozen at -70 ^{o}C until assayed for insulin, triglycerides and free fatty acids. An insulin radioimmunoassay kit was used with rat insulin standards and antibodies directed against rat insulin (Linco, St. Louis, IL). Assays were conducted in duplicate and the intra-assay coefficient of variation was less than 5%. Blood cholesterol, triglyceride and free fatty acid levels were assayed in blood obtained at the time of sacrifice after an overnight fast by using colorimetric assays (Sigma Diagnostics, St. Louis, MO; Wako Chemicals, Richmond, VA).

Blood pressure measurements in conscious animals Systolic blood pressure and heart rate were measured by tail cuff methods with photoelectric pulse detection as previously described (Ernsberger et al., 1996).

Insulin Receptor and IRS-associated PI 3-kinase. To observe the effects of the compounds on skeletal muscle insulin signaling, insulin challenge tests were carried out at the end of the study as described previously (Ernsberger et al., 1999a;Friedman et al., 1998). Rats were allowed 48h recovery from glucose tolerance tests prior to the insulin challenge test. Insulin (10U/kg body weight) was administered to rats anesthetized with ketamine and acepromazine via the portal vein. Hindlimb muscles

were removed before and 5 minutes after insulin injection and rapidly frozen in liquid nitrogen. The frozen samples were pulverized in liquid nitrogen and homogenized immediately under denaturing conditions using a Polytron PTA 20S generator at maximum speed for 30 seconds in ice-cold 10X volume of homogenization buffer (50 mM HEPES, pH 7.5, 100 mM Na_2HP0_4, 100 mM NaF, 10 mM EDTA, and 10 mM Na_3VO_4, phenylmethylsulphonylfluoride (1 mM), plus aprotinin (0.1 mg/ml), leupeptin (10 µg/ml), and 1% Triton-X 100. The homogenate was incubated for 30 min at 4^oC, and then centrifuged at 75,000 x g for 30 min to remove insoluble material. The supernatant was collected and assayed for protein concentration (Bradford dye assay, Bio-Rad Chemicals, Hercules, CA). The level of insulin receptor and IRS-1 tyrosine phosphorylation was determined after immunoprecipitation with anti-phosphotyrosine antibodies, followed Western blot analysis of immunoprecipitated and control samples with SDS-PAGE using anti-insulin receptor or IRS-1 antibody as described previously (Ernsberger et al., 1999a;Friedman et al., 1998). The levels of total insulin receptor and IRS-1 were determined by Western blot analysis using specific antibodies (Upstate Biochemical, Saranac Lake, NY), also as described previously. The results were expressed as arbitrary units above basal levels prior to insulin injection, after correction for total insulin receptor or IRS-1 levels.

IRS-1-associated PI 3-kinase activity was measured as recently described (Shao et al., 2002) in muscle extracts after immunoprecipitation with IRS-1 overnight at 4^oC (400 µg muscle protein/4 µg IRS-1 antibody), followed by overnight incubation with Protein-A conjuaged to sepharose. The immunoprecipitation complex was pelleted at 14,000 x g for 10 min, followed by three washes in phosphate-buffered saline containing 1% Nonidet P-40, two washes in 0.5 M $LiCl_2$/100 mM Tris-HCl, pH 7.6, and two washes

in 10 mM Tris-HCl, pH 7.4, containing 100 mM NaCl and 1 mM EDTA. The pellets were re-suspended in 50 µL of the final wash buffer, and 10 µL of 100 mM $MgCl_2$ was added along with 10 µL of a PI assay mix containing: 0.5 mg/mL phosphatidylinositol and 1 mM EGTA in 10 mM Tris buffer. The tube was mixed by sonication for 20 sec. To start the PI 3-kinase reaction, we added 10 µL of an ATP mix containing: 100 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 0.55 mM ATP, and 10 µCi of [^{32}P]-ATP. After 10 min at room temperature, the reaction was stopped with 20 µl of 8 N HCl. After 5 min, 160 µl of $CHCl_3$:MeOH (1:1) was added. Organic and aqueous phases were separated by centrifugation and the lower organic phase was removed, lyophylized to dryness and resuspended in 15 µl of ethanol. 5 ul of the product was then loaded on to a silica gel thin-layered chromatography (TLC) plate pre-coated in 1% potassium oxalate. The lipids were resolved in $CHCl_3$:methanol:H_20:NH_4OH (60:47:11:2), then the plate was dried and the bands visualized by autoradiography. The images were quantified using a Kodak Dynamic Phosphoimager and results (in duplicate) expressed as percent stimulation over basal (arbitrary units) relative to control samples not exposed to insulin.

Statistical Analysis All values are given as means ± standard error. Repeated measures analysis of variance was used for multiple-timepoint data. Group means were compared by using t-tests for simple comparisons or the Newman-Keuls test for post-hoc analyses. All analyses were carried out using Prism (GraphPad Software, San Diego, CA).

RESULTS

Body Weight. The progression of body weight during the treatment period is shown in Figure 1. At baseline, the SHROB groups were all about 80% overweight compared to their lean SHR littermates. The obese treatment groups did not differ in starting weight. All three obese groups and the lean group gained weight during the treatment period. SHROB treated with the PPARγ agonist gained more weight over the course of the study (78 ± 11 g) than SHROB controls (42 ± 8 g, $P < 0.05$). Treatment with DMAQ-B1 did not affect weight gain (33 ± 7 g). Lean SHR gained considerably less weight (13 ± 6 g).

Food Intake. Consumption of food in g/d is shown in Figure 2. At baseline and throughout the study, food intake in all of the SHROB groups was nearly double that of lean SHR littermates ($P < 0.05$ for each comparison). Despite the increased rate of weight gain in the SHROB treated with the PPARγ agonist PPEIA, noted above, overall food intake did not differ significantly between groups ($P > 0.05$, ANOVA with repeated measures). The total cumulative food consumption was not significantly greater for the PPARγ agonist group (639 ± 48 g) than for the control SHROB (569 ± 36 g) but was greater than those treated with DMAQ-B1 (527 ± 26 g, $P < 0.05$). Although the two drugs were administered in the food, the dose of PPARγ agonist and DMAQ-B1 did not differ significantly from the target dose calculated as mg/kg body weight.

Body fat distribution. Each major fat depot weighed considerably more in each SHROB group than in lean SHR (Table 1). The subscapular depot, a dorsal subcutaneous accumulation of adipose tissue, was not even apparent in SHR but prominent in SHROB. Relative to untreated SHROB, obese animals treated with

DMAQ-B1 showed reduced adipose mass in the gonadal and subscapular deposits. Conversely, obese animals treated with the PPARγ agonist showed increased mass of the retroperitoneal and subscapular deposits. Thus, DMAQ-B1 tended to reduce adipose mass whereas the PPARγ agonist tended to increase it.

Glucose tolerance. Figure 3 shows the results of the oral glucose tolerance test in SHROB with and without antidiabetic treatment, relative to control SHR. Fasting glucose did not differ between groups. In response to glucose challenge, control SHROB showed a more sustained increase in plasma glucose, with significantly higher glucose values at 30, 60 and 120 min compared to SHR ($P < 0.05$, Newman-Keuls test after repeated measures ANOVA), suggestive of relative glucose intolerance. Treatment with DMAQ-B1 had no effect on glucose tolerance in SHROB, whereas the PPARγ agonist normalized glucose tolerance such that glucose levels did not differ from untreated lean SHR controls.

Fasting insulin and response to a glucose load. Fasting insulin levels were elevated 45-fold in untreated SHROB relative to SHR, indicating profound insulin resistance. Treatment with DMAQ-B1 reduced fasting insulin by nearly one-half ($P < 0.05$, t-test). In contrast, the PPARγ agonist had no effect.

The plasma insulin response during the oral glucose tolerance test showed parallel responses in all groups, except that insulin reached its peak at 60 min in SHR but not until 240 min in the SHROB groups. Treatment with DMAQ-B1 reduced insulin AUC (4300 vs. Control of 6300 ng*min/mL). The PPARγ agonist PPEIA also reduced insulin area AUC (3180 ng*min/mL). Thus, glucose transits during the challenge were maintained at lower levels of insulin in both treated groups.

Insulin Receptor Signaling. In order to confirm the presence of insulin resistance at the cellular level in our model, skeletal muscle samples from SHROB and their SHR littermates were compared before and after insulin stimulation in vivo. Insulin-stimulated tyrosine phosphorylation of the insulin receptor, and IRS-1 associated PI 3-kinase activity were both significantly decreased by 40-55% in control untreated SHROB relative to control SHR (Figures 4 and 5).

In order to assess the effects of PPEIA and DMAQ, insulin resistance at the cellular level was examined in treated and control SHROB. Treatment with PPEIA, but not DMAQ-B1 significantly enhanced both these actions of insulin by up to 2 fold in SHROB relative to untreated controls, with no change in basal levels of tyrosine phosphorylation.

In order to account for a possible change in the levels of insulin receptor protein, blots were probed with anti-insulin receptor antibody (Figure 6). Insulin receptor protein was reduced by about 50% in untreated SHROB relative to SHR. However, neither treatment significantly improved the level of insulin receptor protein expression. These data suggest the reduced insulin receptor tyrosine phosphorylation and PI 3-kinase activity in SHROB may be primarily due to reduced insulin receptor protein levels. However, the improvement in IR and IRS-1 associated PI 3-kinase activity after PPEIA treatment appears to be greater than can be accounted for by changes in the insulin receptor.

Plasma lipids. Control SHROB rats displayed marked hypertriglyceridemia compared to lean littermates tested in parallel (Figure 7). Random daytime non-fasted samples were not different from samples obtained after an 18h fast. Treatment with

DMAQ-B1 or the PPARγ agonist PPEIA both significantly reduced plasma triglycerides. In the case of PPEIA, triglycerides were reduced to a level equal to lean SHR.

Plasma free fatty acids (FFA) were not significantly elevated in control SHROB relative to lean SHR littermates. Overnight fasted levels were higher than those obtained in daytime without fasting. Treatment with DMAQ-B1 had no effect, but the PPARγ agonist PPEIA significantly and consistently reduced plasma FFA.

Blood pressure. The level of hypertension was similar in the SHROB compared to lean SHR littermates (Figure 7). Treatment with DMAQ-B1 had no effect, but the PPARγ agonist PPEIA significantly reduced systolic blood pressure by roughly 45 mmHg.

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DISCUSSION

The present study showed that both the insulin mimetic DMAQ-B1 and the PPARγ agonist PPEIA had beneficial effects in an animal model of insulin resistance syndrome. Although both agents have potent antidiabetic actions in hyperglycemic models, in this pre-diabetic model the effects of the two novel agents were divergent. PPEIA showed pleitropic effects impacting nearly every aspect of metabolic syndrome we examined, while the actions of DMAQ-B1 were narrower in scope.

The primary effect of DMAQ-B1 appeared to be on the amount of insulin required to maintain normoglycemia. The SHROB rat shows a severe fasting hyperinsulinemia characterized by a 45-fold elevation of plasma insulin with unchanged fasting plasma glucose. Treatment with DMAQ-B1 halved the level of fasting insulin, possibly indicating improved insulin sensitivity. The reduction of fasting insulin was not sufficient to normalize insulin receptor protein in SHROB skeletal muscle, but insulin receptors were no longer down-regulated relative to control lean SHR. The compound also lowered the amount of insulin required to maintain normal glucose during a glucose tolerance test. These data suggest that DMAQ-B1 given chronically to SHROB was able to supplant a portion of the pancreatic output of insulin.

The effects of DMAQ-B1 were distinct from those PPEIA. DMAQ-B1 failed to increase body weight and selectively reduced fat depot mass whereas PPEIA increased fat depot mass. DMAQ-B1 but not PPEIA reduced fasting insulin. In contrast, DMAQ-B1 did not ameliorate glucose intolerance whereas PPEIA nearly restored normal tolerance. DMAQ-B1 and PPEIA both reduced triglycerides, but only PPEIA reduced free fatty acids. PPEIA had a dramatic blood pressure lowering effect, driving systolic

blood pressure from nearly 200 to 140 mmHg. Other PPARγ agonists have been reported to lower blood pressure in SHR (Schiffrin et al., 2003), but PPEIA appears notably effective in this regard. Thus, it is likely that DMAQ-B1 acts independently of PPARγ, consistent with in vitro studies indicating direct actions on the insulin receptor.

One might speculate that by reducing the demands on the pancreas in a highly insulin resistant state, treatment with DMAQ-B1 or a similar agent might delay the deterioration of pancreatic insulin output that occurs in human type 2 diabetics thought to be the result of pancreatic exhaustion. As an added benefit, DMAQ-B1 significantly reduced plasma triglycerides. This agent also reduced subcutaneous and intra-abdominal fat deposits, despite a lack of change in body weight. Although our fat distribution data were limited in scope and relied upon crude methods, DMAQ-B1 might conceivably have significant effects on body composition. Earlier studies also showed reduced body fat content and suppression of adipocyte hypertrophy by the insulin mimetic agent in mice fed high fat diet (Air et al., 2002).

The present data are not entirely consistent with previous reports of the actions of DMAQ-B1 in obese animal models (Air et al., 2002;Salituro et al., 2001;Liu et al., 2000;Zhang et al., 1999). Prior reports showed a lowering of plasma glucose levels, improvement of glucose tolerance, and improved insulin secretory profile. Notably, the models used in these previous reports were hyperglycemic and the SHROB is normoglycemic. Thus, the metabolic actions of DMAQ-B1 may be amplified in animals with existing hyperglycemia. DMAQ-B1 also reduced food intake and weight loss in rats when dosed centrally via intracerebroventricular injection and in mice which were fed with a high fat diet (Air et al., 2002;Strowski et al., 2004). We found no change in food intake or body weight in SHROB rats, but a significant reduction in the size of two

adipose depots. The lack of effect on food intake in this experimental paradigm could be attributed to the fact that the SHROB rats have a naturally occurring knockout of the leptin receptor. Thus, the effects of DMAQ-B1 on food intake and body weight may depend upon intact leptin signaling.

From the present data the mechanism for the decrease in fasting insulin following DMAQ-B1 treatment is unclear. DMAQ-B1 treatment was not associated with an improvement in insulin receptor signaling to PI 3-kinase in response to exogenous insulin. These results suggest the insulin-sparing effect might not result from insulin sensitizing effects on the insulin signaling cascade in the SHROB model of insulin resistance. DMAQ-B1 itself has been shown to have a direct effect on phosphorylation of the insulin receptor and IRS-1 (Ding et al., 2002). In-vitro studies suggest that this compound may interact with the inactive receptor kinase domain of the insulin receptor, thereby changing its confirmation and partially relieving the auto-inhibition allowing ATP to access the active site of the receptor and mimicking insulin (Qureshi et al., 2000)(Qureshi, 2000). This agent also potentiated insulin receptor tyrosine kinase activity by 30% in liver extracts from normal animals given the compound acutely (Qureshi et al., 2000), and in skeletal muscle of a high-fat fed mildly diabetic animal model (Strowski et al., 2004). It is not clear why the compound was unable to potentiate the effect of insulin on PI 3-kinase in the SHROB model of insulin resistance. However, in the present study, the compound was administered chronically in the chow, and food was removed for 18h prior to the glucose tolerance and insulin challenge tests, thereby allowing clearance of DMAQ-B1 by the time of testing. Despite acute withdrawal of the drug, both fasting and glucose stimulated hyperinsulinemia secretion were significantly improved, suggesting a persistent and possibly indirect mechanism for lowering insulin.

The direct effect of the compound on the pancreas is to actually increase insulin secretion (Roper et al., 2002). An insulin-sparing effect might result from insulin-mimetic actions on the otherwise reduced insulin receptor tyrosine kinase activity characteristic of the SHROB rat, or DMAQ-B1 might have an additional insulin sensitizing effect. Unlike the PPARγ agonist, we found that chronic treatment with DMAQ-B1 had no significant effect on the maximal insulin-induced insulin receptor tyrosine phosphorylation or IRS-1 associated PI 3-kinase activity in skeletal muscle *in-vivo*. Our use of a relatively high dose of insulin (10 U/kg) might have obscured any effect of DMAQ-B1. Previous studies have shown that DMAQ-B1 alone acutely increases tyrosine phosphorylation of the insulin receptor and IRS-1 in rat primary adipocytes (Ding et al., 2002). This agent also potentiated insulin receptor tyrosine kinase activity by 30% in liver extracts from normal animals given the compound acutely (Qureshi et al., 2000). In the present study, the compound was administered chronically in the chow, and food was removed overnight prior to the glucose tolerance and insulin challenge tests, thereby allowing clearance of DMAQ-B1 by the time of testing. Despite acute withdrawal of DMAQ-B1, both fasting and glucose stimulated hyperinsulinemia secretion were significantly improved, suggesting a persistent and possibly indirect insulin sensitizing action. The lack of effect on insulin signaling in skeletal muscle may reflect limited tissue penetration of this hydrophilic drug.

The PPARγ agonist PPEIA had a remarkable impact on multiple components of metabolic syndrome X. Glucose intolerance was completely corrected, the exaggerated insulin response to glucose challenge was attenuated, hypertriglyceridemia was normalized, free fatty acids were reduced, and hypertension was nearly eliminated. All of these beneficial effects occurred despite a significant weight gain and an

enlargement of intrabdomenal and subcutaneous fat deposits. Levels of circulating Insulin and skeletal muscle insulin receptors were not changed, however, insulin-stimulated insulin receptor and PI 3-kinase activity was significantly increased in skeletal muscle, suggesting PPEIA is effective at reversing the mechanism for insulin resistance in the SHROB model. These findings support the utility of PPARγ agonists in insulin resistance syndrome prior to the development of fasting hyperglycemia. In combination with other agents that might limit expansion of adipose depots, the PPARγ agonists may be of even greater value.

Thiazolidinediones have previously been reported to preferentially increase fat cell number in specific depots of the rat: gonadal > retroperitoneal > subcutaneous (de Souza et al., 2001). This resulted in preferential increases in abdominal depots relative to subcutaneous depots, accompanied by a major decrease in average cell size. Reduced adipocyte size after PPARγ agonist treatment may contribute to reduced insulin resistance and improved glucose uptake and storage, as well as indirect effects on skeletal muscle by lowering circulating FFA and triglycerides. In the present study, we did not observe preferential increases in abdominal depots relative to subcutaneous depots with the novel non-thiazolidinedione PPARγ agonist PPEIA. Further studies are required to determine whether this difference is the result of differential actions of this particular PPARγ agonist, or a differential response of this particular animal model to this entire class of agents. However, prior data suggest potential advantages of PPEIA over thiazolidinedione PPARγ agonists (Berger et al., 2003).

In conclusion, both a low molecular weight insulin mimetic and a novel PPARγ agonist had beneficial effects in an animal model of insulin resistance syndrome. Both

agents represent possible approaches to retard the progression of insulin resistance to type 2 diabetes by decreasing plasma lipids and improving fasting insulin levels.

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Footnotes

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Figure Legends

Figure 1. Effects of an insulin mimetic and a PPARγ agonist on body weight in SHROB. Shown is the mean ± SE of body weight in g over the course of 18d of treatment. Untreated lean SHR are shown for comparison. N = 8 for each group. Body weights were relatively stable but increased in the group receiving PPARγ agonist.

Figure 2. Effects of an insulin mimetic and a PPARγ agonist on food intake in SHROB. Shown is the mean ± SE of food consumption (Teklad rat chow) in g/d. All SHROB groups consumed more than lean SHR littermates, but treatment groups did not differ significantly.

Figure 3. Glucose and insulin responses to an oral glucose load. Shown is mean ± SE for glucose in whole blood (Panel A) and plasma immunoreactive insulin (Panel B). Rats were fasted for 18h prior to administration of a glucose load (6g/kg) by gavage.

Figure 4. Representative immunoblots and quantification of insulin receptor tyrosine phosphorylation in skeletal muscle before and after insulin treatment *in-vivo*. Shown at top are representative pairs of lanes with and without insulin for each of the four groups, with two rats per group. Bars represent the mean ± S.E.; of 4-6 samples per group. Rats were fasted overnight, anesthetized, and a biopsy of the gastrocnemius removed and frozen in the basal state. Insulin was injected into the portal vein (10U/kg body weight). Samples from the opposite hindlimb were removed 5 min after insulin treatment. Proteins were immunoprecipitated with an anti-phosphotyrosine antibody followed by immunoblotting. The results are expressed as arbitrary units above basal prior to insulin injection. *$p<0.05$ relative to control SHROB,

Figure 5. IRS-1 associated PI 3-kinase activity in skeletal muscle before and after insulin treatment *in-vivo*. IRS-1 associated PI 3-kinase activity was determined in

protein extracts after immunoprecipitation with anti-IRS-1 as described in the Methods. The results are expressed as arbitrary units above basal prior to insulin injection. Data are means ± S.E.; *p<0.05 relative to control SHROB, *n* = 4-6 samples per group.

Figure 6. Insulin receptor immunoreactivity in skeletal muscle in SHROB treated with an insulin mimetic or a PPARγ agonist relative to untreated control SHROB and SHR. Aliquots of skeletal muscle protein were analyzed by Western blot with primary antibodies directed against the beta subunit of the insulin receptor. Samples from each group were run in parallel on each blot and optical density was expressed as a ratio to untreated SHROB controls. *p<0.05 relative to control SHROB, *n* = 4-6 samples per group.

Figure 7. Plasma lipid and blood pressure in SHROB treated with an insulin mimetic or a PPARγ agonist relative to untreated control SHROB and SHR. Tail blood samples were taken either in the fasting state prior to the oral glucose tolerance test, or two days prior in the morning (08:00 to 10:00) without a prior fast. Systolic blood pressures were measured by tail cuff just prior to the non-fasting blood sampling.

Table 1: Effects of an insulin mimetic and a novel PPARγ agonist on adipose depot mass (in g)

	Retroperitoneal	Mesenteric	Gonadal	Subscapular
Control SHR	2.9 ± 0.2	1.8 ± 0.2	4.4 ± 1	<1
Control SHROB	18 ± 1	17 ± 1	22 ± 1.8	22 ± 1
PPEIA	26 ± 2*	16 ± 1	19 ± 0.9	28 ± 2*
DMAQ-B1	21 ± 1	15 ± 1	13 ± 0.7*	16 ± 1*

Legend to Table 1
*Significantly different from untreated control, P < 0.05 by t-test.

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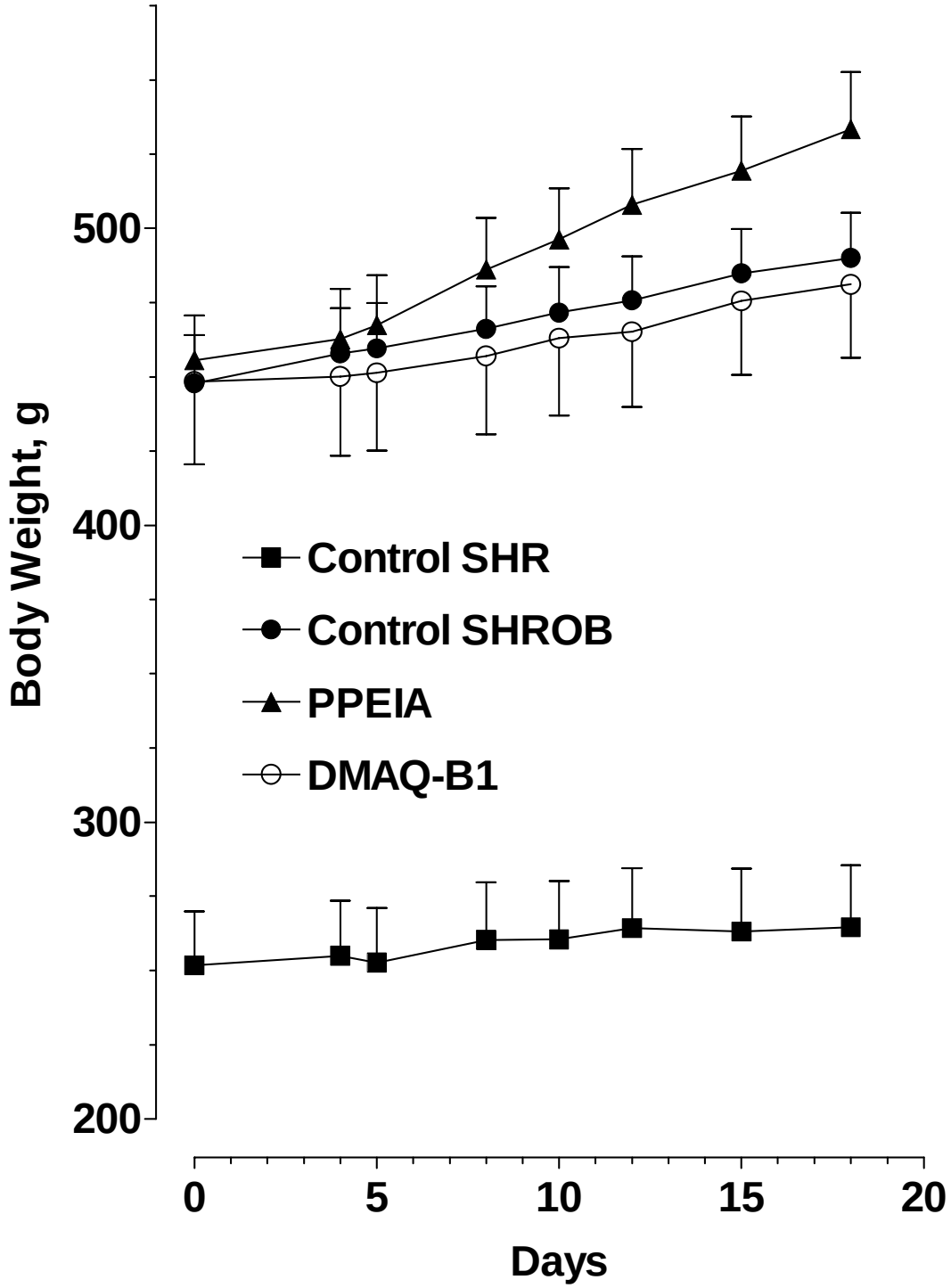

Figure 1

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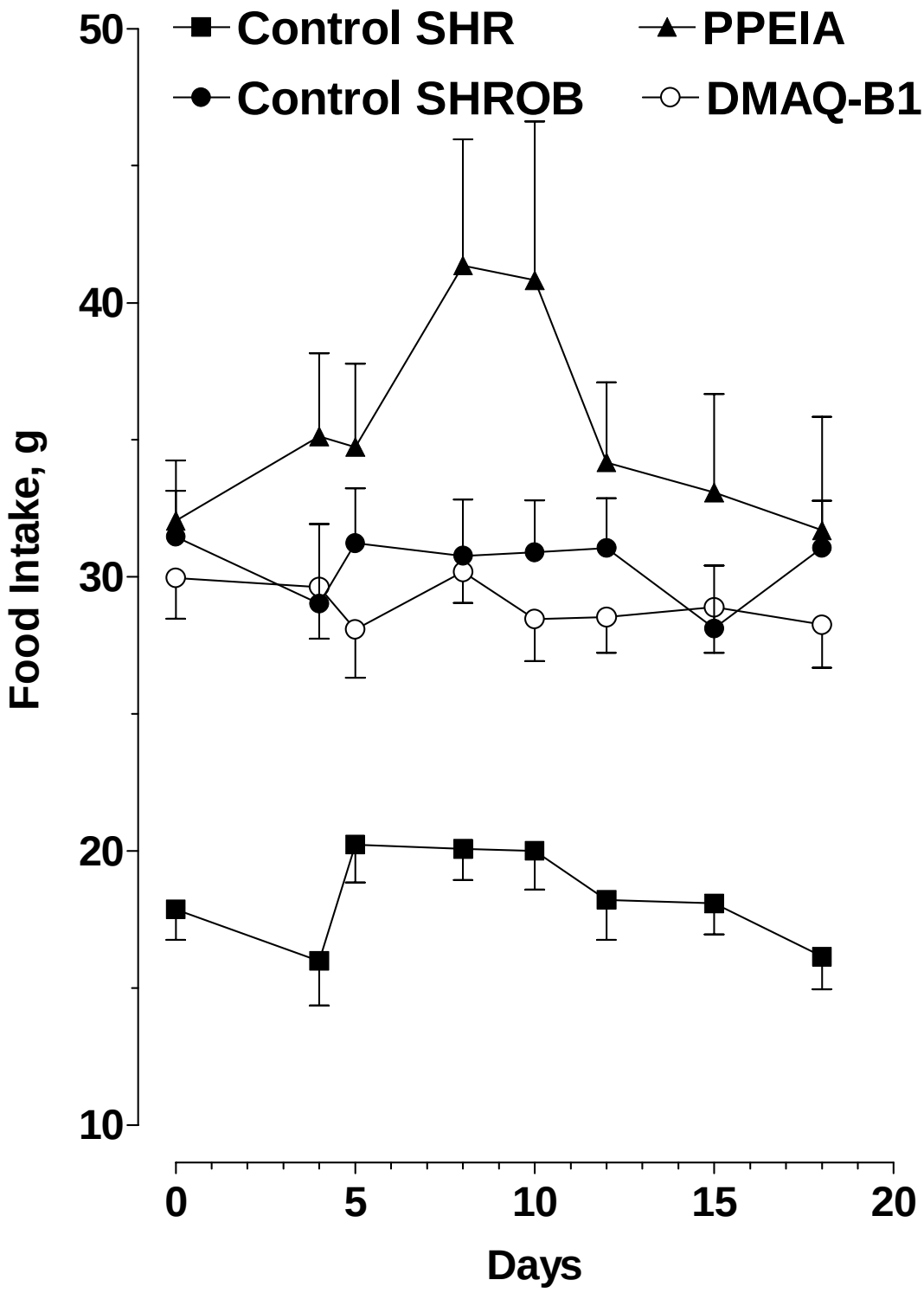

Figure 2

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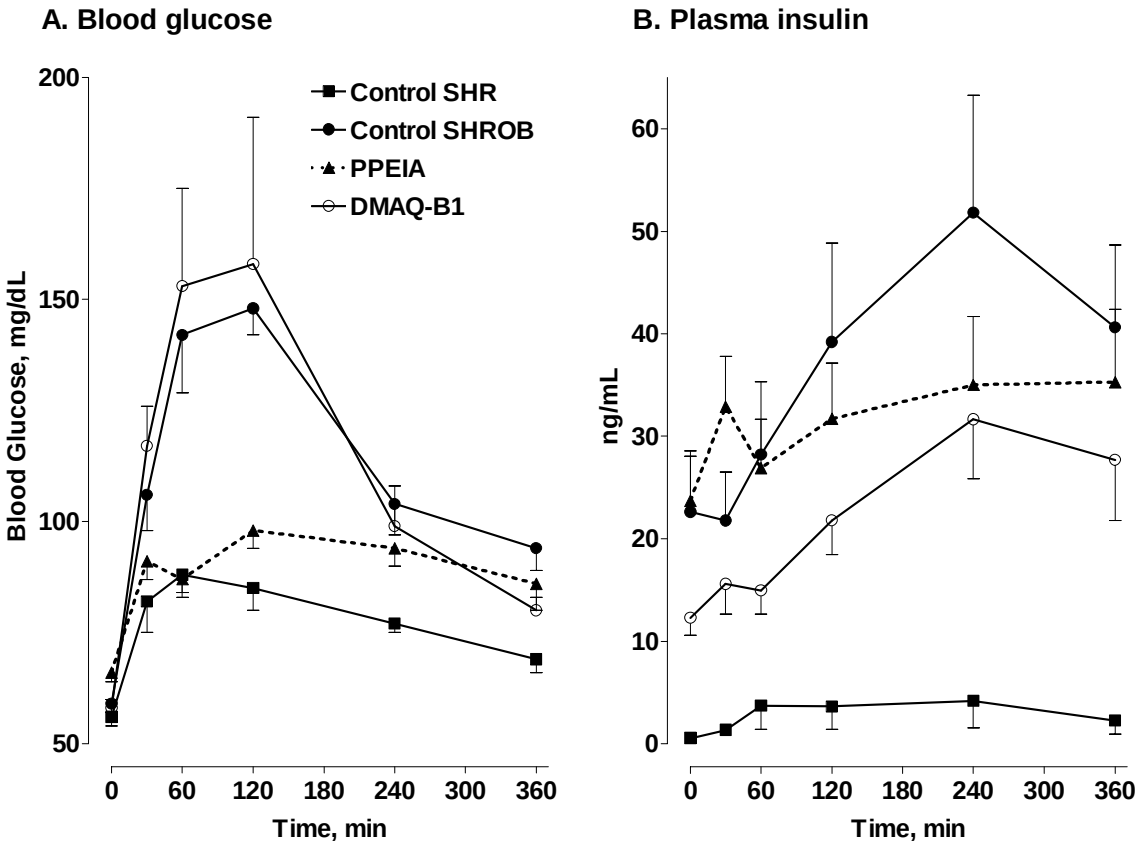

Figure 3

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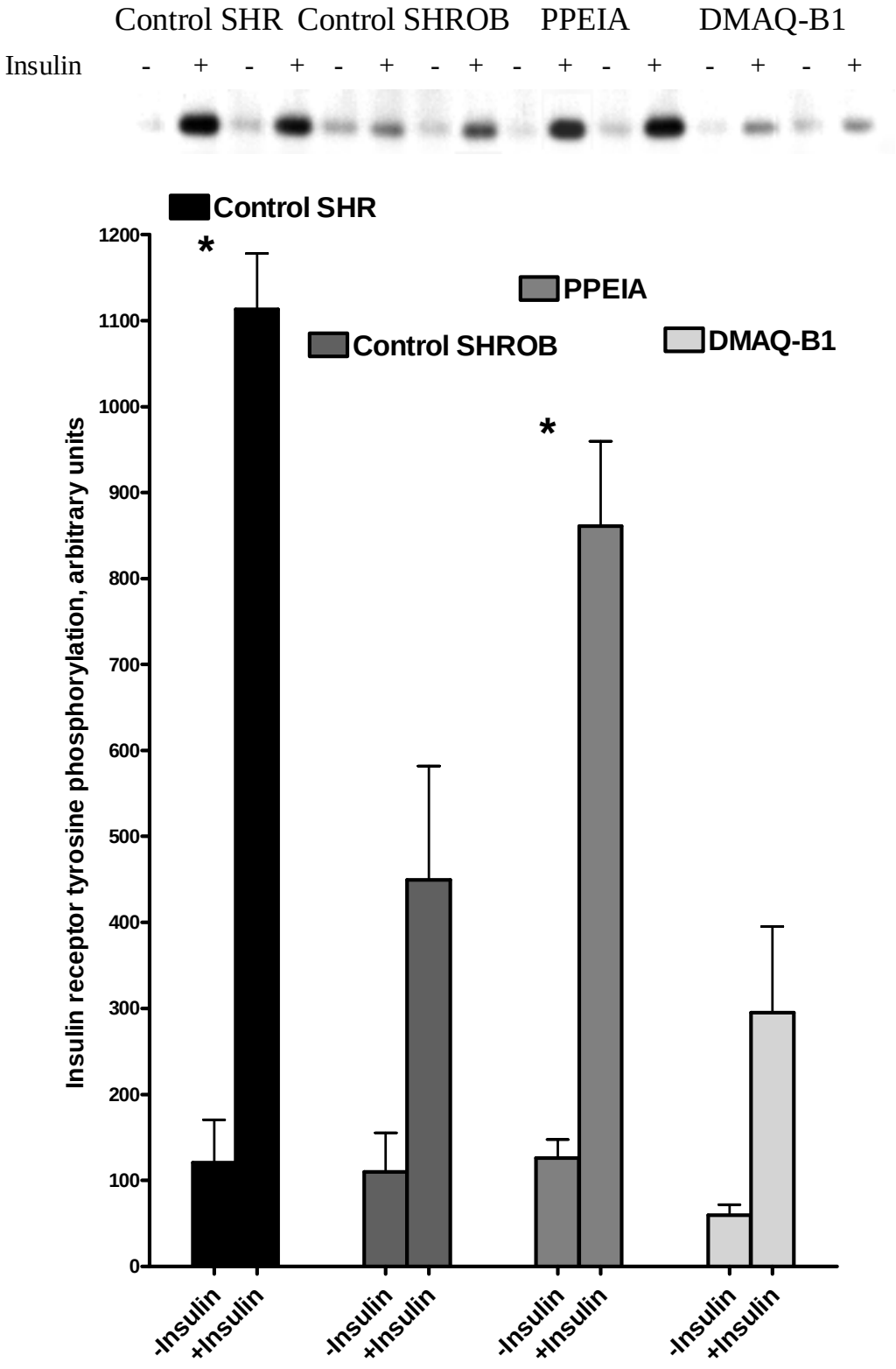

Figure 4

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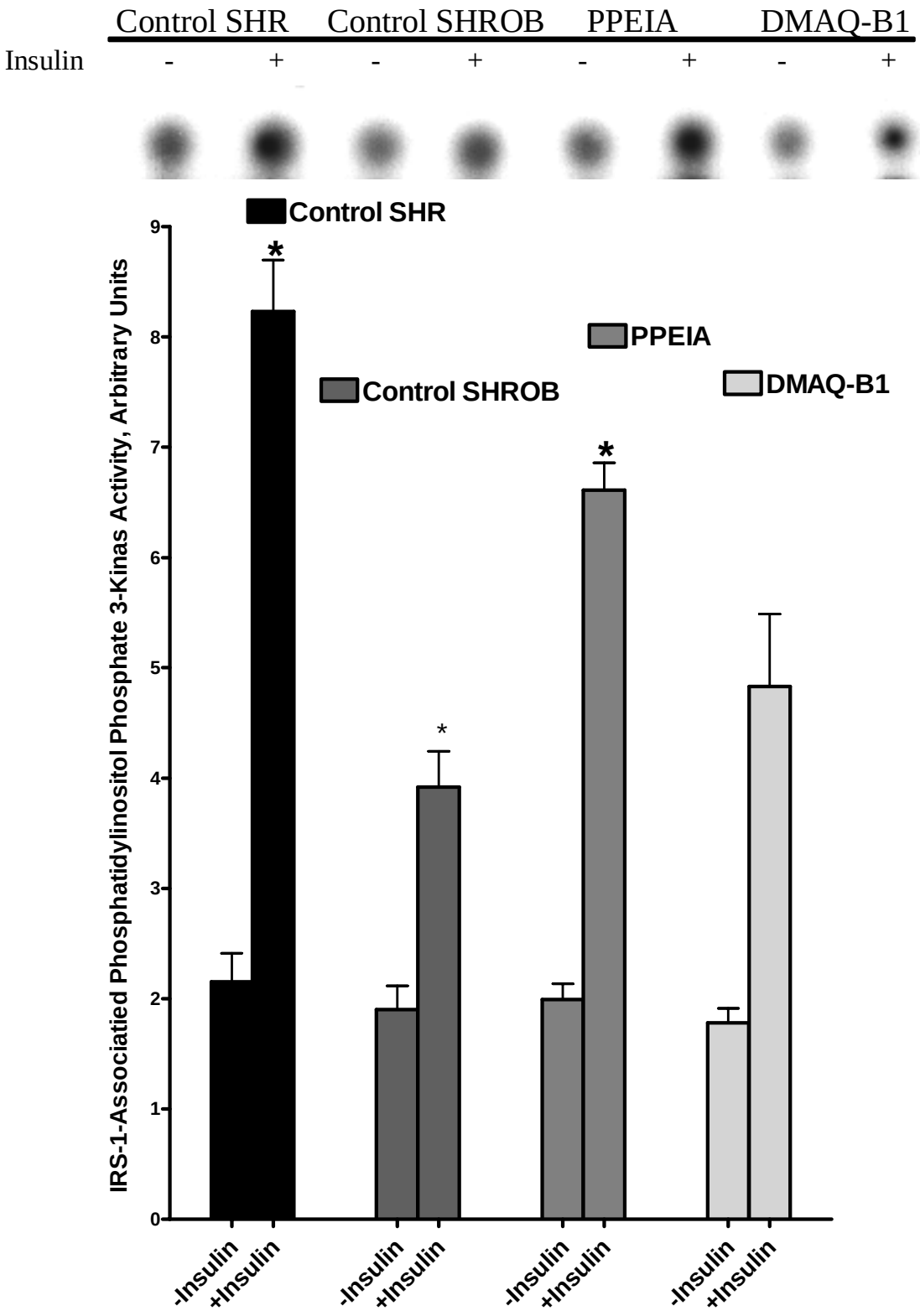

Figure 5

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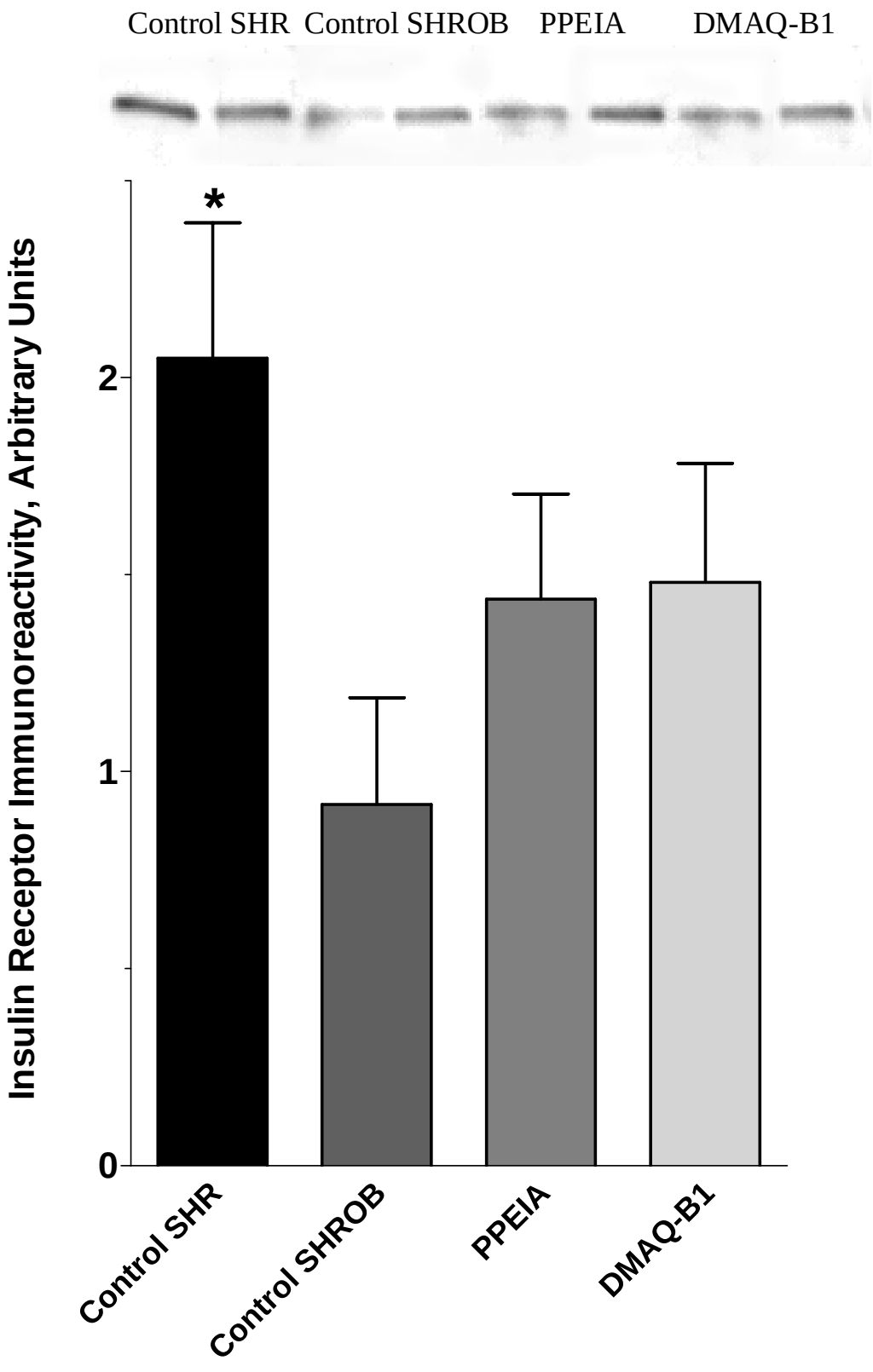

Figure 6

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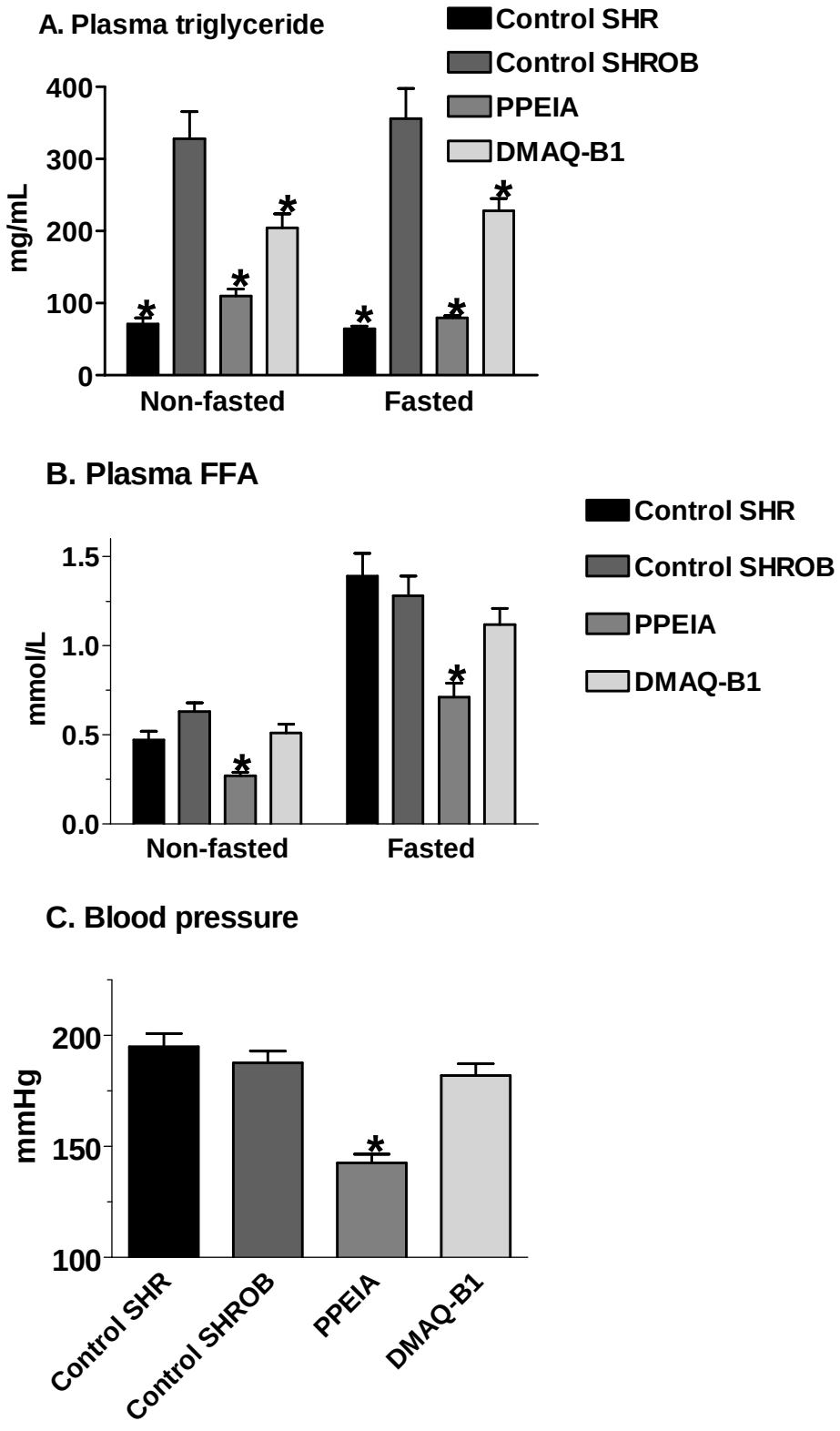

Figure 7