

JPET #154633

TITLE PAGE

**An Alpha7 Nicotinic Acetylcholine Receptor-Selective Agonist Reduces
Weight Gain and Metabolic Changes in a Mouse Model of Diabetes**

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JPET #154633

RUNNING TITLE PAGE

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Non-standard Abbreviations: nAChR, nicotinic acetylcholine receptor; TNF- α , tumor necrosis factor-alpha; JAK2, janus kinase-2; STAT3, Signal transducer and activator of transcription 3; IL-6, interleukin-6; CNS, central nervous system; ACh, acetylcholine; TC-7020, 5-methyl-N-[2-(pyridin-3-ylmethyl)-1-azabicyclo [2.2.2] oct-3-yl] thiophene-2-carboxamide; Compound A, (R,E)-5-(2-pyrrolidin-3-ylvinyl)-pyrimidine; MLA, methyllycaconitine; HbA1c, glycosylated hemoglobin

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ABSTRACT

Type 2 diabetes has become a pervasive public health problem. The etiology of the disease has not been fully defined but appears to involve abnormalities in peripheral and central nervous system pathways as well as prominent inflammatory components. Because nicotinic acetylcholine receptors (nAChRs) are known to interact with anti-inflammatory pathways and have been implicated in control of appetite and body weight as well as lipid and energy metabolism, we examined their role in modulating biological parameters associated with the disease. In a model of type 2 diabetes, the homozygous leptin resistant *db/db* obese mouse, we measured the effects of a novel alpha7 nAChR-selective agonist (TC-7020) on body mass, glucose and lipid metabolism, and pro-inflammatory cytokines. Oral administration of TC-7020 reduced weight gain and food intake, reduced elevated glucose and glycated hemoglobin levels, and lowered elevated plasma levels of triglycerides and the pro-inflammatory cytokine TNF- α . These changes were reversed by the alpha7-selective antagonist methyllycaconitine, confirming the involvement of alpha7 nAChRs. Prevention of weight gain, decreased food intake and normalization of glucose levels were also blocked by the JAK2 inhibitor AG-490, suggesting that these effects involve linkage of alpha7 nAChRs to the JAK2-STAT3 signaling pathway. The results demonstrate that alpha7 nAChRs play a central role in regulating biological parameters associated with diabetes and support the potential of targeting these receptors as a new therapeutic strategy for treatment.

INTRODUCTION

In 2000 it was reported that at least 171 million people worldwide (2.8% of the population) suffered from diabetes and it has been estimated that the incidence will almost double by the year 2030 (Wild et al., 2004). The Centers for Disease Control has designated the disease an epidemic. Specific pathogenic entities contributing to diabetic risk, such as central adiposity, ectopic fat accumulation, hyperlipidemia and inflammation have been well-characterized. In general, diabetes is believed to be secondary to an insulin resistant state, which is associated with excess adiposity (Sykiotis and Papavassiliou, 2001). Insulin resistance in skeletal muscle, liver and adipose tissue impedes glucose uptake and results in the release of free fatty acids and the characteristically associated dyslipidemia. Elevations in post-prandial blood glucose levels and ultimately in fasting glucose levels result in compensatory hyperinsulinemia, a condition which is initially accompanied by islet β -cell hypertrophy and eventual failure (Sykiotis and Papavassiliou, 2001).

A key factor that underlies the development of diabetes is a characteristic systemic inflammation, marked by increases in the venous blood concentrations of C-reactive protein, interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF- α) (Bullo et al., 2003). TNF- α has been shown not only to evoke the production of other inflammatory cytokines but also to increase the activities of signaling pathways that are believed to lead to insulin resistance (Dandona et al., 2004). The central nervous system (CNS) modulates inflammation, including levels of TNF- α via the reticuloendothelial system. The vagus nerve, utilizing its major neurotransmitter acetylcholine (ACh), acts on alpha7 nicotinic acetylcholine receptors (nAChRs) of macrophages to suppress

JPET #154633

TNF- α release (Miao et al., 2003; Borovikova et al., 2000a; Borovikova et al., 2000b; Wang et al., 2003). Electrical stimulation of the vagus nerve or treatment of vagotomized animals with ACh prevents LPS-dependent increases in TNF- α release (Borovikova et al., 2000b). Conversely, vagotomy increases TNF- α serum levels and hepatic TNF- α responses (Borovikova et al., 2000b). The role of alpha7 nAChRs in cholinergic modulation of TNF- α in macrophages has been confirmed using antisense oligonucleotides to the alpha7 nAChR (Wang et al., 2003). Indeed, when the expression of this receptor is prevented, ACh loses its effect on LPS-induced TNF- α release. Furthermore, stimulation of the vagus nerve does not inhibit TNF- α release in alpha7 knockout mice (Wang et al., 2003). The key role played by alpha7 nAChRs in inflammatory processes is further supported by the observations that nicotine and alpha7 nAChR agonists are effective in models of inflammation and protective in models of sepsis and that they inhibit local leukocyte recruitment and decrease endothelial cell activation (de Jonge and Ulloa, 2007).

Obesity is also a major pre-disposing factor in the development of diabetes. Relevant to this is an extensive literature on the non-selective nAChR agonist nicotine supporting a broad involvement of both CNS and peripheral nAChRs in regulating body mass and other key metabolic pathways. It is well known that nicotine administration decreases body weight in normal rodents and human smokers and results in adaptive changes that regulate feeding behavior and energy metabolism (Fornari et al., 2007). Nicotine has also been shown to reduce the incidence of type I diabetes in mice (Mabley et al., 2002) and improve insulin sensitivity in rat adipocytes (Liu et al., 2004). Nicotine influences expression of the orexigenic peptides neuropeptide Y and Agouti-related protein in the hypothalamus as well as the expression of the metabolic protein, uncoupling

JPET #154633

protein-3 in brown adipose tissue (Fornari et al., 2007). Areas of the hypothalamus, particularly the lateral hypothalamus that regulates appetite, contain $\alpha 7$ nAChRs which have been postulated to play a key role in regulating appetite, food consumption and body mass (Jo et al., 2002).

To more precisely probe the relationship of the $\alpha 7$ nAChR to specific physiological components of diabetes we have designed and synthesized a novel agonist (TC-7020) with high selectivity for the $\alpha 7$ nAChR. The effects of this compound were studied in an animal model of type 2 diabetes, the *db/db* mouse, which expresses many of the pathological changes associated with the disease, including hyperglycemia, hyperlipidemia, increased body weight, increased TNF- α levels in adipose tissue and nephropathy (Harris et al., 2001; Hotamisligil et al., 1993; Sharma et al., 2003). The results indicate that activation of $\alpha 7$ nAChR targets by this compound significantly reverses weight gain and associated metabolic changes expressed in the leptin receptor deficient *db/db* mouse.

METHODS

Alpha7 and $\alpha 4\beta 2$ nAChR selective compounds

TC-7020 (alpha7): 5-Methyl-N-[2-(pyridin-3-ylmethyl)-1-azabicyclo [2.2.2] oct-3-yl] thiophene-2-carboxamide was prepared from commercially available quinuclidin-3-one by aldol condensation with 3-pyridinecarboxaldehyde to afford 2-[(pyridin-3-yl)methylene] quinuclidin-3-one followed by catalytic hydrogenation. The carbonyl moiety of the resulted 2-[(pyridin-3-yl)methyl] quinuclidin-3-one was converted into amino group by reductive amination. Final coupling of 3-amino-2-[(pyridin-3-yl)methyl]-1-azabicyclo[2.2.2]octane with 5-methylthiophen-2-carboxylic acid provided 5-methyl-N-[2-(pyridin-3-ylmethyl)-1-azabicyclo[2.2.2]oct-3-yl] thiophene-2-carboxamide (see Figure 1 for structure).

Compound A ($\alpha 4\beta 2$): (R,E)-5-(2-pyrrolidin-3-ylvinyl)-pyrimidine

Receptor Binding Assays

[³H]-Nicotine binding to $\alpha 4\beta 2$ nAChRs in rat cortical membrane preparations was assayed using standard methods adapted from published procedures (Lippiello and Fernandes, 1986). [³H]-MLA binding to alpha7 nAChRs was determined in hippocampal membranes as described previously (Davies et al., 1999). The IC₅₀ (concentration of the compound that produces 50% inhibition of binding) was determined by least squares non-linear regression using GraphPad Prism software (GraphPad, San Diego, CA). K_i was calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Patch Clamp Electrophysiology

Expression in Xenopus oocytes. Mature (>9 cm) female *Xenopus laevis* African toads (Nasco, Ft. Atkinson, WI) were used as a source of oocytes. After linearization and purification of cloned cDNAs, RNA transcripts were prepared *in vitro* using the appropriate mMESSAGE mMACHINE kit from Ambion Inc. (Austin, TX). Stage 5 oocytes were isolated and injected with 50 nL (5-20 ng) each of the appropriate subunit cRNAs. Recordings were made 2 to 7 days after injection.

Electrophysiology. Patch clamp electrophysiology studies of the human alpha7 nAChR using *Xenopus laevis* oocytes were performed in the laboratory of Roger Papke (University of Florida, Gainesville, FL). Experiments were conducted using the OpusXpress 6000A (Axon Instruments, Union City CA). Responses were calculated using a net charge analysis for alpha7 receptors (Papke and Porter Papke, 2002). For concentration-response relations, data derived from net charge analyses were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA), and curves were generated from the Hill equation

$$\text{Response} = I_{\max} [\text{agonist}]^n / [\text{agonist}]^n + (EC_{50})^n \quad (1)$$

where I_{\max} denotes the maximal response for a particular agonist/subunit combination, and n represents the Hill coefficient. I_{\max} , n , and the EC_{50} were all unconstrained for the fitting procedures.

Receptor Function Assays

TE671/RD and SH-SY5Y cell lines (obtained from Dr. Ron Lukas, Barrow Neurological Institute) and the SH-EP1 cell line were used to assess the functional properties of the muscle,

JPET #154633

ganglionic and $\alpha 4\beta 2$ nAChR subtypes, respectively. Cells were maintained in proliferative growth phase in Dulbecco's modified Eagle's medium (Gibco/BRL) with 10% horse serum (Gibco BRL), 5% fetal bovine serum (HyClone, Logan UT), 1 mM sodium pyruvate, 4 mM L-glutamine. Forty-eight hours prior to each experiment, cells were plated in 96 well black-walled plates (Corning) at 60,000 cells/well. On the day of the experiment, growth media was gently removed, 200 μ L FLIPR Calcium 4 Assay reagent (Molecular Devices) in assay buffer (10 mM HEPES, 2.5 mM CaCl_2 , 5.6 mM D-glucose, 0.8 mM MgSO_4 , 5.3 mM KCl, 138 mM NaCl, pH 7.4 with TRIS-base) was added to each well and plates were incubated at 37°C for 1 hour. The plates were removed from the incubator and allowed to equilibrate to room temperature for at least 30 minutes. Plates were transferred to a Flexstation fluorescence plate reader for addition of compound and monitoring of fluorescence at 485 nm. The amount of calcium flux was compared to both a positive (10 μ M nicotine) and negative control (buffer alone) to determine the percent response relative to that of nicotine. The positive control was defined as 100% response and the results of the test compounds were expressed as a percentage of the positive control.

Animals

Mice used in these studies included C57BL/6J heterozygous lean controls (referred to herein as *db+*) and leptin receptor deficient (referred to herein as *db-*) mice on a C57BL/6J background, both obtained from Jackson Laboratories (Bar Harbor, ME). Animals had *ad libitum* access to drinking water and rodent chow. Studies were conducted in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Drug Treatment

The effects of the alpha7 selective agonist (TC-7020) on body weight and food intake were measured bi-weekly from ages 3 to 10 weeks. TC-7020 was given via gavage at 1 mg/kg daily. In selected cohorts, the alpha7 antagonist methyllycaconitine (MLA) was also given concurrently via gavage at 3 mg/kg daily. The JAK2 kinase inhibitor (AG-490) was administered intraperitoneally (IP) at 1 mg/kg daily. Fasting glucose was measured once a week after food withdrawal, with a Precision XL glucometer using tail vein bleeding. HbA1c levels were measured from samples with the A1C kit from Metrika, Inc. For measurements of blood plasma analytes, a separate group of fasted mice were anesthetized by isoflurane in a rapid induction chamber and swiftly decapitated. Blood was collected in heparin and rapidly centrifuged at 4°C to remove cells and to obtain plasma, and the samples were frozen for later analyses. Plasma TNF- α concentrations were determined using ELISA assay kits from eBioscience and plasma triglyceride levels were determined using the L-Type TG H test (Wako Diagnostics), an *in vitro* assay for the quantitative determination of triglycerides in serum or plasma.

Data Analyses

All data are expressed as Mean plus or minus Standard Error of the Mean (\pm SEM). Differences among all groups were compared using a two-way analysis of variance (ANOVA). Where significant main effects were shown, post hoc analyses with Tukey's multiple comparisons test were performed to determine significant differences between treatment groups. For all analyses, an alpha level of 0.05 was considered statistically significant.

RESULTS

In Vitro Pharmacology

TC-7020 is a novel proprietary agonist that is highly selective for the alpha7 nAChR subtype, based on both binding affinity and function (Table 1). The compound binds to alpha7 nAChRs with high affinity ($K_i \sim 2$ nM in displacement studies using ^3H -methyllycaconitine (MLA) in rat hippocampal synaptosomes) and exhibits very poor affinity toward other nicotinic receptor subtypes ($K_i > 1000$ nM), including the other major subtype in brain ($\alpha 4\beta 2$). In functional studies, TC-7020 is an agonist at alpha7 nAChRs (E_{max} 69%), as evidenced by voltage clamp studies of human alpha7 nAChRs transiently expressed in *Xenopus* oocytes. TC-7020 showed minimal functional activity at either muscle ($\sim 7\%$ of nicotine's E_{max} at 100 μM) or ganglionic ($\sim 6\%$ of nicotine's E_{max} at 100 μM) nAChR receptor sub-types, as demonstrated by measuring calcium flux in SH-SY5Y cells and TE-671 cells, respectively. TC-7020 does not exhibit selectivity for any other (non-nicotinic) receptor targets (IC_{50} s > 10 μM at more than 60 targets in a broad receptor selectivity panel (Supplementary Table 1). Although there was a slight binding to hERG channels (21% @ 10 μM), a follow-up functional assay showed that the EC_{50} was > 100 μM .

Physiological Effects of Selective alpha7 nAChR Agonist

Plasma TNF- α Levels

It has been shown that the plasma concentrations of inflammatory mediators such as TNF- α are increased in the insulin resistant diabetic state, and that the reduction of the levels of TNF- α in

JPET #154633

diabetic mice correlates with increased insulin sensitivity and decreased plasma insulin and blood glucose levels (Uysal et al., 1997). Therefore, we determined the effects of the alpha7 agonist on obesity-induced levels of plasma TNF- α . TC-7020-treated and untreated lean *db+* mice showed no change in the plasma levels of TNF- α , but obese *db-* mice had elevated fasting plasma TNF- α levels. When the *db-* obese mice were treated with TC-7020, they displayed significantly decreased plasma TNF- α levels compared to their vehicle-treated controls ($p < 0.05$). However, levels did not return to those of lean controls. The decrease was blocked by the alpha7 antagonist MLA (Figure 1), implicating the involvement of alpha7 nAChRs.

Glucose Metabolism

Because weight is known to correlate with glucose metabolism and insulin sensitivity in obesity and diabetes (Williams et al., 2003) we assessed plasma glucose levels in the treated and untreated *db-* obese mice. Lean TC-7020-treated and untreated *db+* mice all showed normal glucose levels. At the end of seven weeks of treatment, fasting plasma glucose levels in the *db-* obese mice treated with the alpha7 agonist were significantly lower ($p < 0.05$) than those in the vehicle treated *db-* mice (Figure 2A). Levels did not return to those of lean controls. When the alpha7 nAChR antagonist MLA was given concurrently with TC-7020 the obese mice showed no significant decrease in plasma glucose, indicating that the effects on glucose levels are dependent either directly or indirectly on alpha7 nAChR activation. The effects on plasma glucose level also appear to be dependent on JAK2 activation, as demonstrated by the finding that the JAK2 inhibitor AG-490 prevented the TC-7020-induced decrease in plasma glucose (Figure 2B).

JPET #154633

Since total glycemic load includes both fasting and post-prandial glucose levels in the blood, a time-averaged index of glycemic load is reflected in the accumulation of advanced glycation end products, as exemplified by the quantitative glycosylation of hemoglobin, HbA1c. Lean TC-7020-treated and untreated *db+* mice all showed HbA1c levels lower than 5% (Figure 3), consistent with normal glycemic control. In contrast, obese *db-* mice showed markedly elevated HbA1c levels, and these levels were significantly lowered ($p < 0.05$) by TC-7020. These observations indicate that the $\alpha 7$ nAChR plays a central role in regulating both the fasting and post-prandial glucose levels in the blood. Consistent with this, co-administration of the $\alpha 7$ antagonist MLA suppressed the reduction in HbA1c levels induced by the $\alpha 7$ agonist TC-7020 (Figure 3).

Lipid Metabolism

The non-selective nAChR agonist nicotine has been shown to have effects on peripheral (non-neural) sites of energy metabolism, including decreased lipolysis and decreased triglyceride uptake and storage in adipose tissue (Jo et al., 2002). Therefore, to explore the involvement of $\alpha 7$ nAChRs in modulating lipid metabolism we monitored the effects of TC-7020 on plasma triglyceride levels. Lean TC-7020-treated and untreated *db+* mice all showed normal levels of triglycerides. Obese *db-* mice displayed elevated fasting triglyceride levels, consistent with a loss of insulin sensitivity in adipocytes. When the *db-* obese mice were treated with TC-7020 there was a marked reduction of elevated triglyceride levels compared to vehicle-treated obese controls ($p < 0.05$), but levels did not return to those of lean controls. The effects of TC-7020 were blocked by the $\alpha 7$ antagonist MLA (Figure 4), suggesting modulation of lipid metabolism and possibly of adipocyte insulin resistance via an $\alpha 7$ nAChR-mediated pathway.

Body Weight Gain and Food Consumption

The relatively non-selective nAChR agonist nicotine is well known to have effects on body mass, a phenomenon well illustrated by the lower average weight of smokers. These effects of nicotine have been linked to changes in feeding behavior as well as increased energy metabolism (Fornari et al., 2007), presumably mediated by nAChR subtypes that have been identified in relevant pathways in the CNS and periphery (Jo et al., 2002). Because the *db-* mouse expresses an obese phenotype we probed the role of the alpha7 nAChR subtype in regulating weight gain by monitoring the effects of the alpha7-selective agonist TC-7020 on body mass. At the end of seven weeks of treatment, between ages 3 and 10 weeks, weight gain in the vehicle control *db-* obese groups was significantly greater ($p < 0.05$) than that of lean vehicle controls (Figure 5A) as expected. By comparison, weight gain was significantly reduced ($p < 0.05$) in the alpha7 agonist-treated *db-* obese mice (Figure 5A). The daily food intake in vehicle control obese groups was significantly greater ($p < 0.05$) than that of lean vehicle controls and was significantly lower ($p < 0.05$) in the TC-7020-treated obese mice than in the obese controls (Figure 5B). The food consumption and body mass of the *db+* lean mice were unaffected by TC-7020 or MLA, confirming that the compounds were not producing toxic effects that altered food intake or weight. However, when the selective alpha7 antagonist MLA was given concurrently with TC-7020, the obese mice showed no significant differences in body weight gain or food intake compared to the obese vehicle-treated controls (Figure 5 A, B), confirming that the reduced weight gain is mediated by alpha7 nAChRs.

Previous studies have shown that alpha7 nAChRs are linked to anti-apoptotic and anti-inflammatory effects through JAK2/STAT3 signaling pathways (Marrero and Bencherif, 2008),

JPET #154633

so to determine if this pathway is also involved in the observed effects on food consumption and weight loss we utilized the JAK2 tyrosine kinase specific inhibitor AG-490. AG-490 prevented ($p < 0.05$) both the weight loss and the decreased food intake in obese *db-* mice treated with the alpha7 agonist TC-7020 (Figure 6 A, B).

Specificity of Alpha7 versus $\alpha 4\beta 2$ -Selective Ligands

To further explore the role of nAChR subtypes in modulating parameters of the metabolic syndrome we compared the effects of a full agonist with high selectivity for the CNS $\alpha 4\beta 2$ nAChR subtype (Compound A) to those of TC-7020. The in vitro profile of Compound A is summarized in Table 1. The results from in vivo studies (Table 2) confirmed the effects of the alpha7-selective compound TC-7020 on weight gain reduction, reduction of increased glucose levels, decreased glycation of hemoglobin, reduction of the pro-inflammatory cytokine TNF- α and reduction of triglyceride levels. The $\alpha 4\beta 2$ nAChR-selective compound reduced weight gain and food intake but did not elicit significant changes in any of the other parameters.

DISCUSSION

In the present studies we have explored the role of alpha7 nAChRs in regulating key biological pathways involved in type 2 diabetes and probed the potential of selective alpha7 nAChR agonists as a novel therapeutic approach to treat this condition. The results indicate that a prototypical selective alpha7 nAChR agonist can reduce the pro-inflammatory cytokine TNF- α , reduce elevated glucose levels, decrease glycated hemoglobin, reduce triglycerides and reduce food intake and weight gain in a murine model of type 2 diabetes. These effects were reversed by the alpha7 antagonist MLA. Furthermore, the JAK2 kinase specific inhibitor AG-490 also inhibited the alpha7 agonist-induced weight loss, decreased food intake and reduction of glucose levels. The findings indicate that alpha7 nAChRs play an important role in regulating the biological parameters associated with type 2 diabetes and that this regulation involves JAK2/STAT3 signaling pathways.

Although we did not identify the anatomical localization of the alpha7 nAChRs involved, it is likely that both central and peripheral components contribute to the effects seen. Areas of the hypothalamus, particularly the lateral hypothalamus that regulates appetite, contain both alpha7 and $\alpha 4\beta 2$ nAChRs (Jo et al., 2002). Evidence suggests that activation of presynaptic alpha7 nAChRs on GABAergic terminals in the lateral hypothalamus decreases appetite by inhibiting the activity of MCH (melanin-concentrating hormone) neurons (Jo et al., 2005). It is possible that the decreases we observed in food consumption and weight gain involve activation of CNS alpha7 nAChRs since TC-7020 is readily accessible to the brain (oral bioavailability in rats 33%; brain:plasma ratio = 0.4 at 4h post-dose) and the effects of TC-7020 were blocked by the alpha7

JPET #154633

antagonist MLA, which has also been shown to cross the blood-brain barrier (Turek et al., 1995). It is interesting to note that the effects of TC-7020 were also blocked by the JAK2 inhibitor AG-490 since the metabolic feedback on adiposity which is mediated by leptin receptors in the hypothalamus also involves receptor-JAK2 interactions (Ahima et al., 2006). This raises the intriguing possibility that in the *db-* obese mice, which lack an active leptin receptor, the $\alpha 7$ nAChR may substitute for the leptin receptor in the activation of JAK2. This would re-establish the feedback loop normally activated by adipose-derived leptin that signals a decreased need for food intake. Consistent with this is the observation that nicotine directly increases leptin signaling in rat hypothalamus (Li and Kane, 2003).

Interestingly, the $\alpha 4\beta 2$ -selective ligand Compound A only affected food intake and weight gain but not glucose levels, TNF- α , HbA1c or triglyceride levels. This nAChR subtype is known to be expressed in brain areas such as the lateral hypothalamus that are involved in the control of feeding behavior (Jo et al., 2002) but is not widely expressed in peripheral tissues. This suggests that the additional effects of the $\alpha 7$ agonist TC-7020 on TNF- α and the other metabolic parameters may involve a peripheral component. Based on a growing body of evidence it is now believed that low-grade chronic inflammation is associated with the onset of insulin resistance and type 2 diabetes (Tilg and Moschen, 2008). This chronic inflammation is in turn characterized by an increased number of macrophages in adipose tissue together with the production of inflammatory cytokines, including TNF- α (Zeyda and Stulnig, 2007). Increases in TNF- α and concomitant insulin resistance of adipocytes can lead to a cascade of events including mitochondrial damage, increased lipolysis and redistribution of fatty acids to ectopic triglyceride deposits (Maassen, 2008; Ruan and Lodish, 2003). Relevant to the present findings, previous

JPET #154633

studies suggest a direct link between alpha7 nAChRs and regulation of TNF- α in adipocytes whereby activation of alpha7 nAChRs reduces TNF- α protein levels (Liu et al., 2004). This may partly explain the normalization of TNF- α levels by the selective alpha7 agonist TC-7020.

The effects of TC-7020 on TNF- α are also consistent with the previously reported involvement of peripheral alpha7 nAChRs in the cholinergic anti-inflammatory pathway (de Jonge and Ulloa, 2007). This pathway involves the vagus nerve, which utilizes acetylcholine to activate alpha7 nAChRs on macrophages, leading to decreased production of inflammatory cytokines, including TNF- α by these cells (Gallowitsch-Puerta and Tracey, 2005). Previous studies that examined the effects of nicotine on LPS-treated and control peritoneal macrophages have shown that nicotine treatment leads to phosphorylation of STAT3 and that this nicotine-mediated effect is blocked by the alpha7-selective antagonists α -bungarotoxin and MLA and by AG-490, a selective inhibitor of JAK2 phosphorylation (de Jonge et al., 2005). Taken together, these data support the interaction of JAK2 and alpha7 nAChRs in macrophages and reveal the critical role played by STAT3 in mediating peripheral cholinergic anti-inflammatory effects. The present results extend these findings to demonstrate the relevance of alpha7 nAChR interactions with JAK2 in modulating the biological parameters associated with the development of type 2 diabetes, including increased food intake, weight gain and dyslipidemia. In this regard, alpha7-mediated regulation of macrophage-derived inflammatory factors in adipose tissue may play a prominent role.

The effects of TC-7020 on blood glucose and glycated hemoglobin levels are somewhat more difficult to interpret. On the one hand, it may simply be a consequence of weight loss, which is

JPET #154633

known to improve glucose metabolism and insulin sensitivity in obesity and diabetes (Williams et al., 2003). However, this does not explain why the $\alpha 4\beta 2$ -selective compound (Compound A) also reduced food consumption and weight gain but did not affect any of the metabolic parameters, including glucose levels. Another possible explanation is that increased insulin sensitivity in adipose and other tissues by $\alpha 7$ -mediated reduction of pro-inflammatory components facilitates more efficient uptake and metabolism of glucose by cells. Additional studies will be required to probe the mechanistic basis of these findings.

Although our studies provide insights into the molecular pathways recruited during the development of type 2 diabetes, the relative participation and contribution of central cholinergic pathways and peripheral mechanisms remain to be fully elucidated. Likewise, there is still an open debate on the causal relationship of insulin-resistance and inflammation observed in type 2 diabetes, i.e. is inflammation responsible for the associated insulin resistance or does insulin resistance lead to pro-inflammatory cascades. Although the present studies did not address this specific question, a reversal of pro-inflammatory cytokines prior to decreases in weight gain would be indicative of a causal linkage between them. Conversely, $\alpha 7$ selective drugs could have a primary effect on food intake leading to decreased weight gain and this could result in normalization of insulin resistance and decreased inflammatory effects. It is hoped that future studies will give us a better understanding of these mechanisms and ultimately lead to the development of therapies that target specific nAChR receptor subtypes and downstream signaling pathways as a novel approach to the management of type 2 diabetes.

JPET #154633

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JPET #154633

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JPET #154633

FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Structure of alpha7 nAChR selective agonist TC-7020

Figure 2. Effects of the alpha7 nAChR agonist TC-7020 and the alpha7 nAChR antagonist MLA on plasma TNF- α levels

Both lean (*db+*) and obese (*db-*) mice were treated for 7 weeks either with vehicle (V) or TC-7020 (TC). In some experiments obese mice were treated with TC-7020 plus the alpha7 antagonist methyllycaconitine (MLA). TC-7020 (1 mg/kg) and MLA (3 mg/kg) were administered daily via oral gavage. Results represent the mean \pm SEM of eight treated mice and are expressed as plasma TNF- α levels in pg/mL. * $p < 0.05$, significantly different from lean vehicle controls; # $p < 0.05$, significantly different from obese vehicle controls; † $p > 0.05$, not significantly different from obese vehicle controls.

Figure 3. Effects of the alpha7 nAChR agonist TC-7020, the alpha7 nAChR antagonist MLA and the JAK2 antagonist AG-490 on blood glucose levels

Both lean (*db+*) and obese (*db-*) mice were treated for 7 weeks either with vehicle (V) or TC-7020 (TC). In some experiments obese mice were treated with TC-7020 plus the alpha7 antagonist methyllycaconitine (MLA) or the JAK2 inhibitor AG-490 (AG). TC-7020 (1 mg/kg) and MLA (3 mg/kg) were administered daily via oral gavage. AG-490 was administered daily, i.p. (1 mg/kg). Results represent the mean \pm SEM of eight treated mice and are expressed as fasting blood glucose levels at week 7 in mg/dL. * $p < 0.05$, significantly different from lean

vehicle controls; # $p < 0.05$, significantly different from obese vehicle controls; † $p > 0.05$, not significantly different from obese vehicle controls.

Figure 4. Effects of the alpha7 nAChR agonist TC-7020 and the alpha7 nAChR antagonist MLA on glycated hemoglobin levels

Both lean (*db+*) and obese (*db-*) mice were treated for 7 weeks either with vehicle (V) or TC-7020 (TC). In some experiments obese mice were treated with TC-7020 plus the alpha7 antagonist methyllycaconitine (MLA). TC-7020 (1 mg/kg) and MLA (3 mg/kg) were administered daily via oral gavage. Results represent the mean +/- SEM of eight treated mice and are expressed as fasting % HbA1c levels at week 7. * $p < 0.05$, significantly different from lean vehicle controls; # $p < 0.05$, significantly different from obese vehicle controls; † $p > 0.05$, not significantly different from obese vehicle controls.

Figure 5. Effects of the alpha7 nAChR agonist TC-7020 and the alpha7 nAChR antagonist MLA on plasma triglycerides

Both lean (*db+*) and obese (*db-*) mice were treated for 7 weeks either with vehicle (V) or TC-7020 (TC). In some experiments obese mice were treated with TC-7020 plus the alpha7 antagonist methyllycaconitine (MLA). TC-7020 (1 mg/kg) and MLA (3 mg/kg) were administered daily via oral gavage. Results represent the mean +/- SEM of eight treated mice and are expressed as plasma triglyceride levels in mg/dL. * $p < 0.05$, significantly different from lean vehicle controls; # $p < 0.05$, significantly different from obese vehicle controls; † $p > 0.05$, not significantly different from obese vehicle controls.

JPET #154633

Figure 6. Effects of the alpha7 nAChR agonist TC-7020 and alpha7 nAChR antagonist MLA on body mass and food consumption

Both lean (*db+*) and obese (*db-*) mice were treated for 7 weeks either with vehicle (V) or TC-7020 (TC). In some experiments obese mice were treated with TC-7020 plus the alpha7 antagonist methyllycaconitine (MLA). TC-7020 (1 mg/kg) and MLA (3 mg/kg) were administered daily via oral gavage. Results represent the mean +/- SEM of eight treated mice and are expressed as their body mass in grams (panels A) or average food consumption in grams/day (panel B) at week 7. * $p < 0.05$, significantly different from lean vehicle controls; # $p < 0.05$, significantly different from obese vehicle controls; † $p > 0.05$, not significantly different from obese vehicle controls.

Figure 7. Effects of the alpha7 nAChR agonist TC-7020 and JAK2 Inhibitor AG-490 on body mass and food consumption

Both lean (*db+*) and obese (*db-*) mice were treated for 7 weeks either with vehicle (V) or TC-7020 (TC). In some experiments obese mice were treated with TC-7020 plus the JAK2 inhibitor AG-490 (AG). TC-7020 (1 mg/kg) was administered daily via oral gavage. AG-490 was administered daily, i.p. (1mg/kg). Results represent the mean +/- SEM of eight treated mice and are expressed as their body mass in grams (panel A) or average food consumption in grams/day (panel B) at week 7. * $p < 0.05$, significantly different from lean vehicle controls; # $p < 0.05$, significantly different from obese vehicle controls; † $p > 0.05$, not significantly different from obese vehicle controls.

TABLE 1. *In vitro* Pharmacological Profile of TC-7020 and $\alpha 4\beta 2$ -selective Compound A.

Values for oocytes represent the mean of duplicate determinations. Values for all other parameters represent the Mean \pm SEM of at least 3 triplicate determinations.

nAChR Subtype	Source	Parameter	TC-7020	Compound A
Alpha7	Rat Hippocampus	Ki (nM)	2.0 \pm 0.3	5500 \pm 1900
	Oocytes (voltage clamp)	EC50 (nM) Emax (% ACh)	ND 69 \pm 4 *	ND
$\alpha 4\beta 2$	Rat Cortex	Ki (nM) **	4166 \pm 1851	27 \pm 11
	Human (SH-EP1 cells)	EC50 (nM) Ca ⁺⁺ flux Emax (% nicotine)	ND 1.3 \pm 0.8	600 120 \pm 10
Muscle	Human (TE-671 cells)	Ca ⁺⁺ flux (% nicotine @ 100 μ M)	6.5 \pm 4.0	12 \pm 4 ***
Ganglion	Human (SH-SY5Y cells)	Ca ⁺⁺ flux (% nicotine @ 100 μ M)	5.8 \pm 1.2	32 \pm 12 ****

* Value determined for racemate

** The values are consistent with the percent inhibition at 10 μ M for the Nicotinic, Neuronal (α -Bungarotoxin insensitive) receptor (Supplementary Table 1)

*** Rb⁺ flux

**** @ 200 μ M

ND = not determined

TABLE 2. Comparison of the effects of alpha7 and $\alpha 4\beta 2$ selective compounds on body mass, food intake and plasma parameters. Obese (*db-*) mice were administered vehicle (Control), TC-7020 (1 mg/kg) or Compound A (3 mg/kg) once daily by oral gavage for 14 days. Results represent the Mean \pm SEM (n=8; * p<0.05).

Parameter	Control	TC-7020	Control	Compound A ($\alpha 4\beta 2$)
Food Intake (grams/day)	6.1 \pm 0.5	3.9 \pm 0.4*	6.0 \pm 0.9	2.9 \pm 0.6*
Body Mass (grams)	65 \pm 9	45 \pm 7*	55 \pm 9	44 \pm 6*
Blood Glucose (mg/dL)	401 \pm 11	268 \pm 14*	344 \pm 19	284 \pm 15
Plasma Triglycerides (mg/dL)	380 \pm 50	140 \pm 25*	388 \pm 21	411 \pm 12
TNF- α (pg/mL)	42 \pm 5	19 \pm 2*	43 \pm 6	52 \pm 2

Figure 1

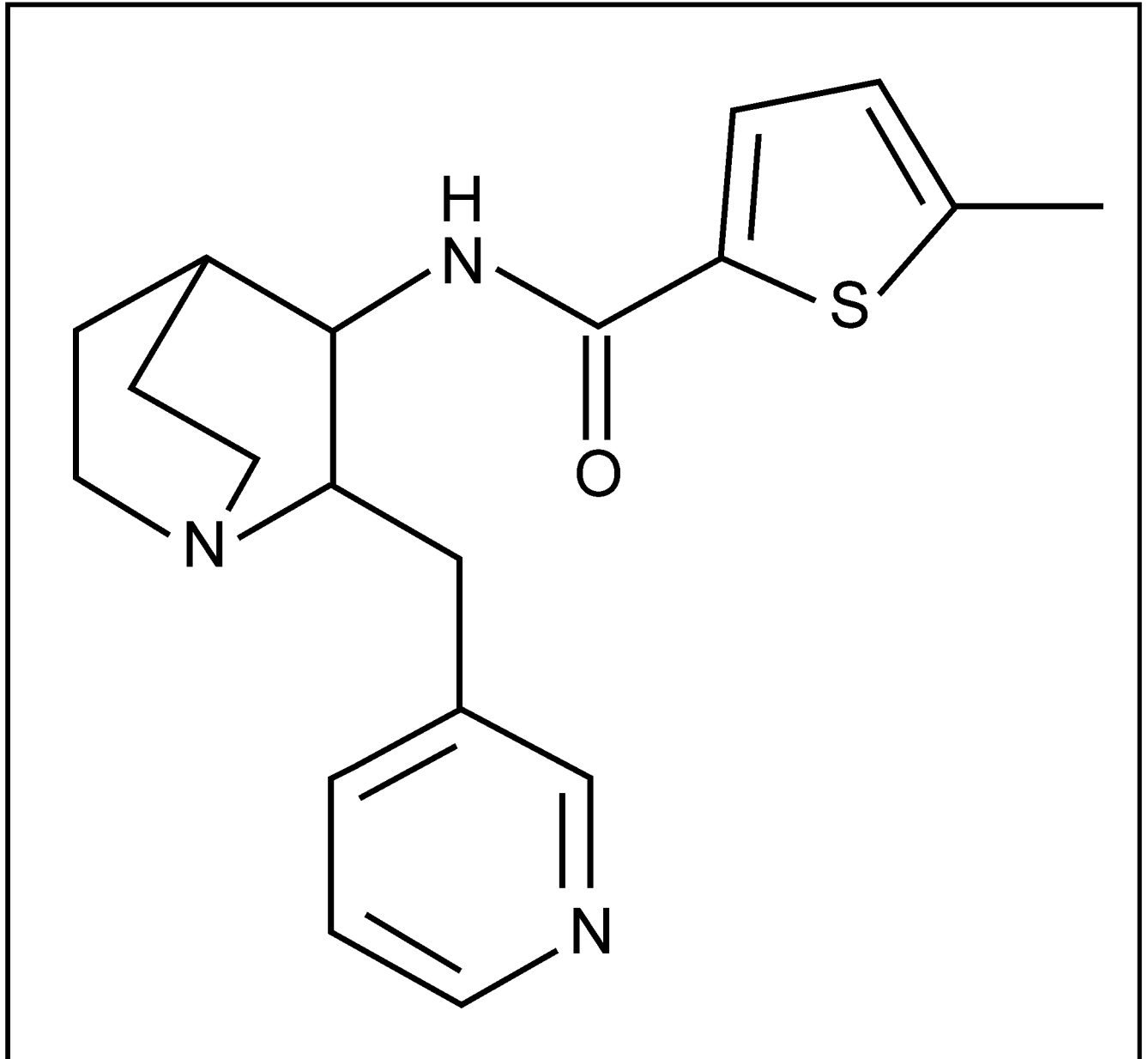


Figure 2

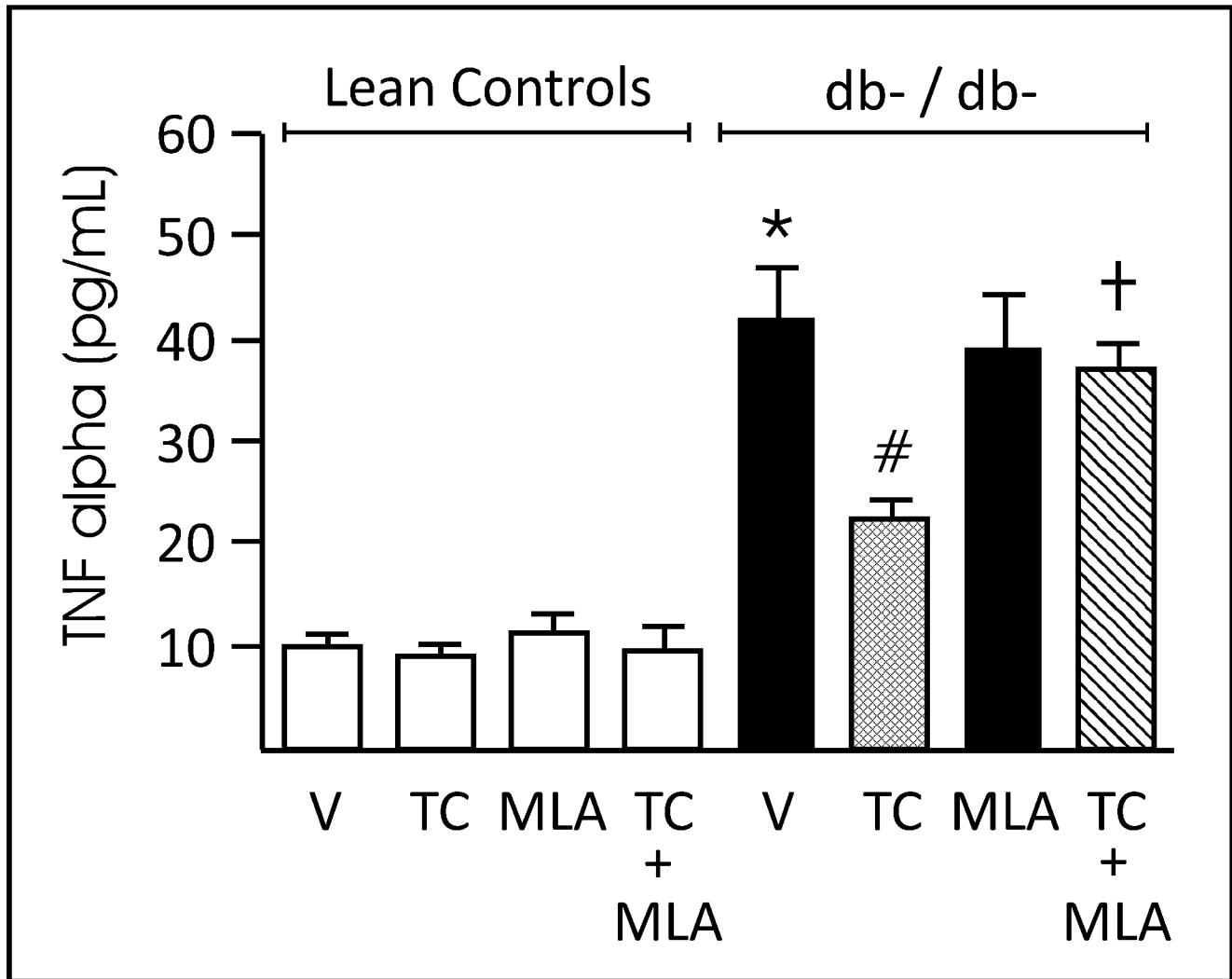


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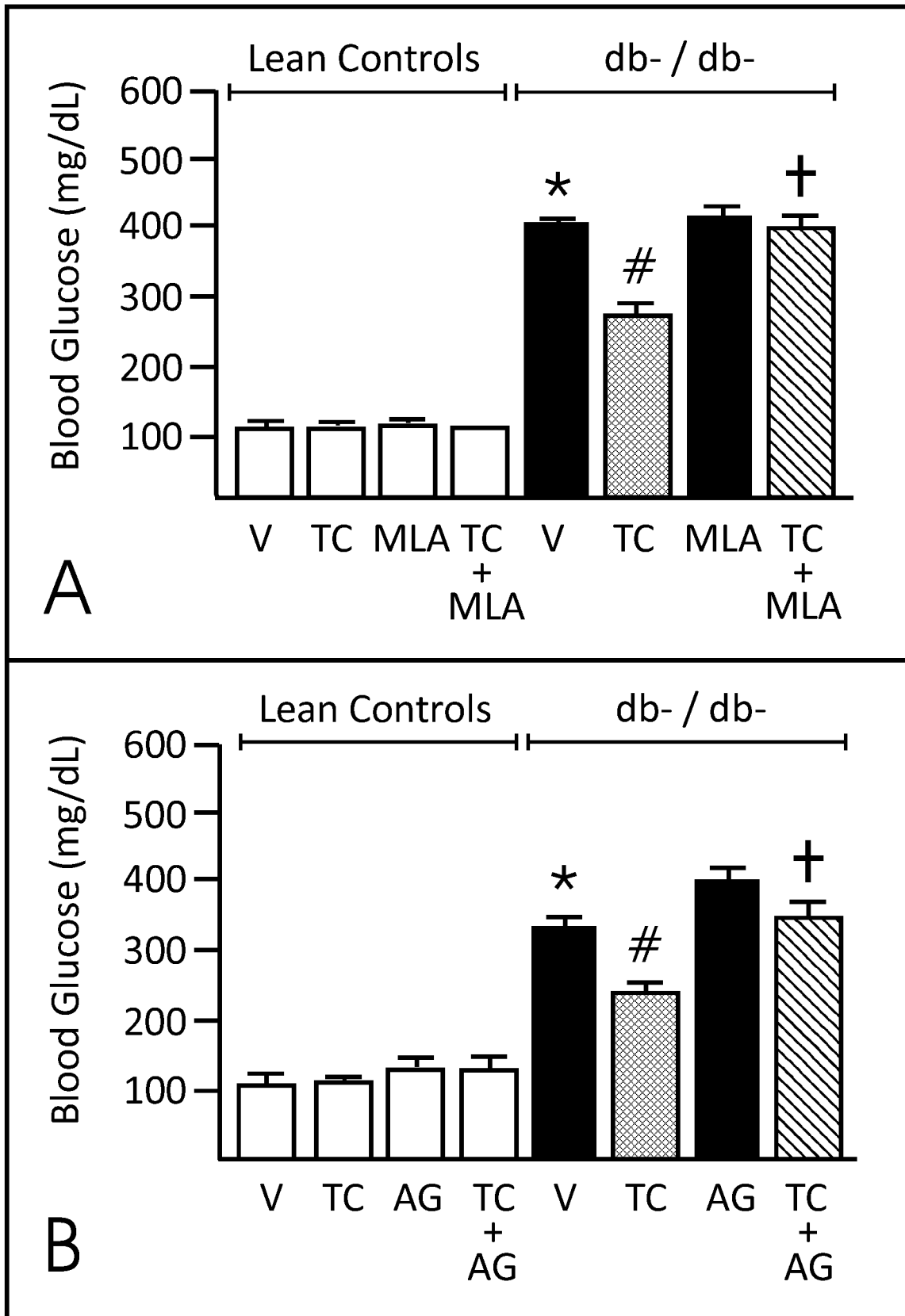


Figure 4

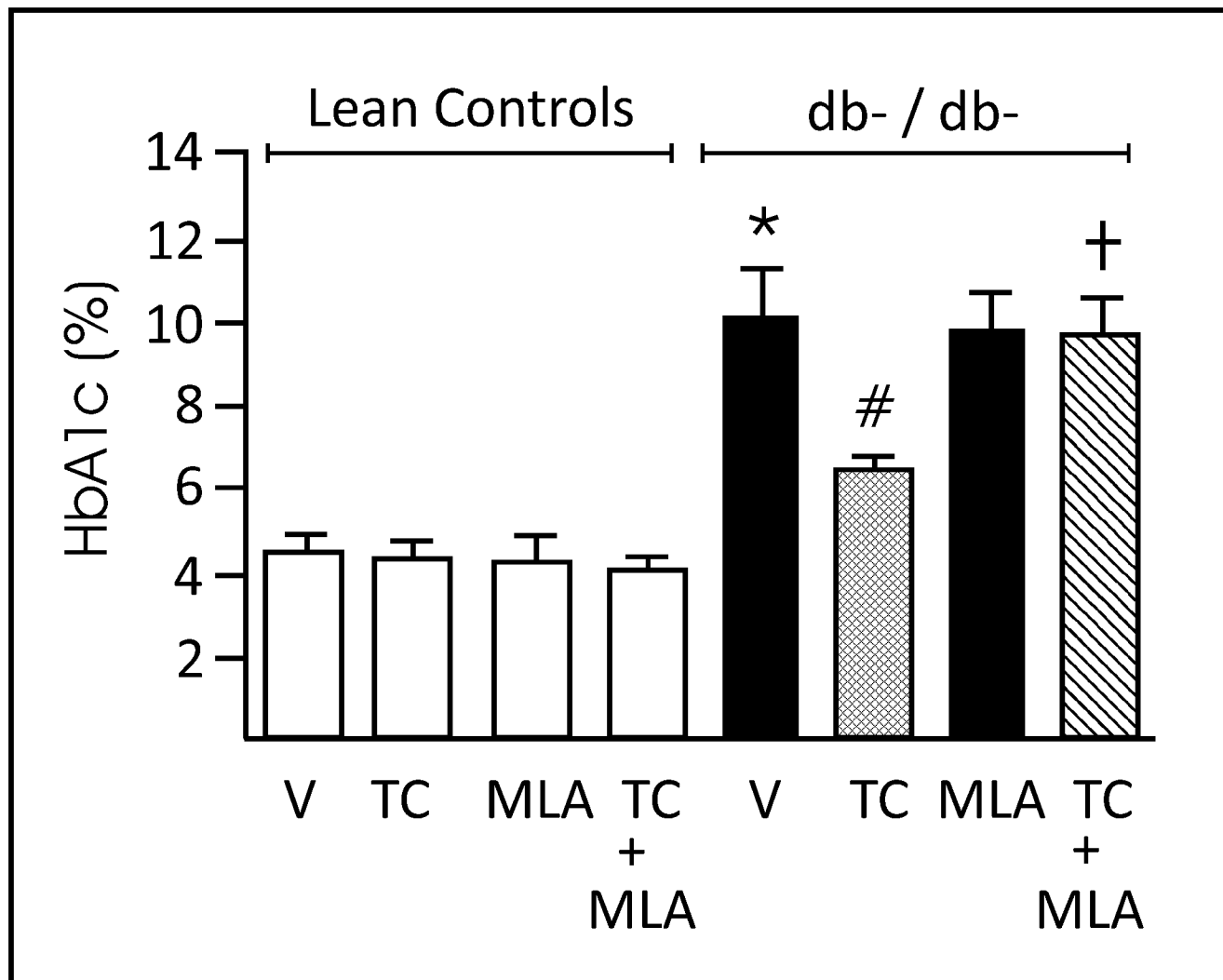


Figure 5

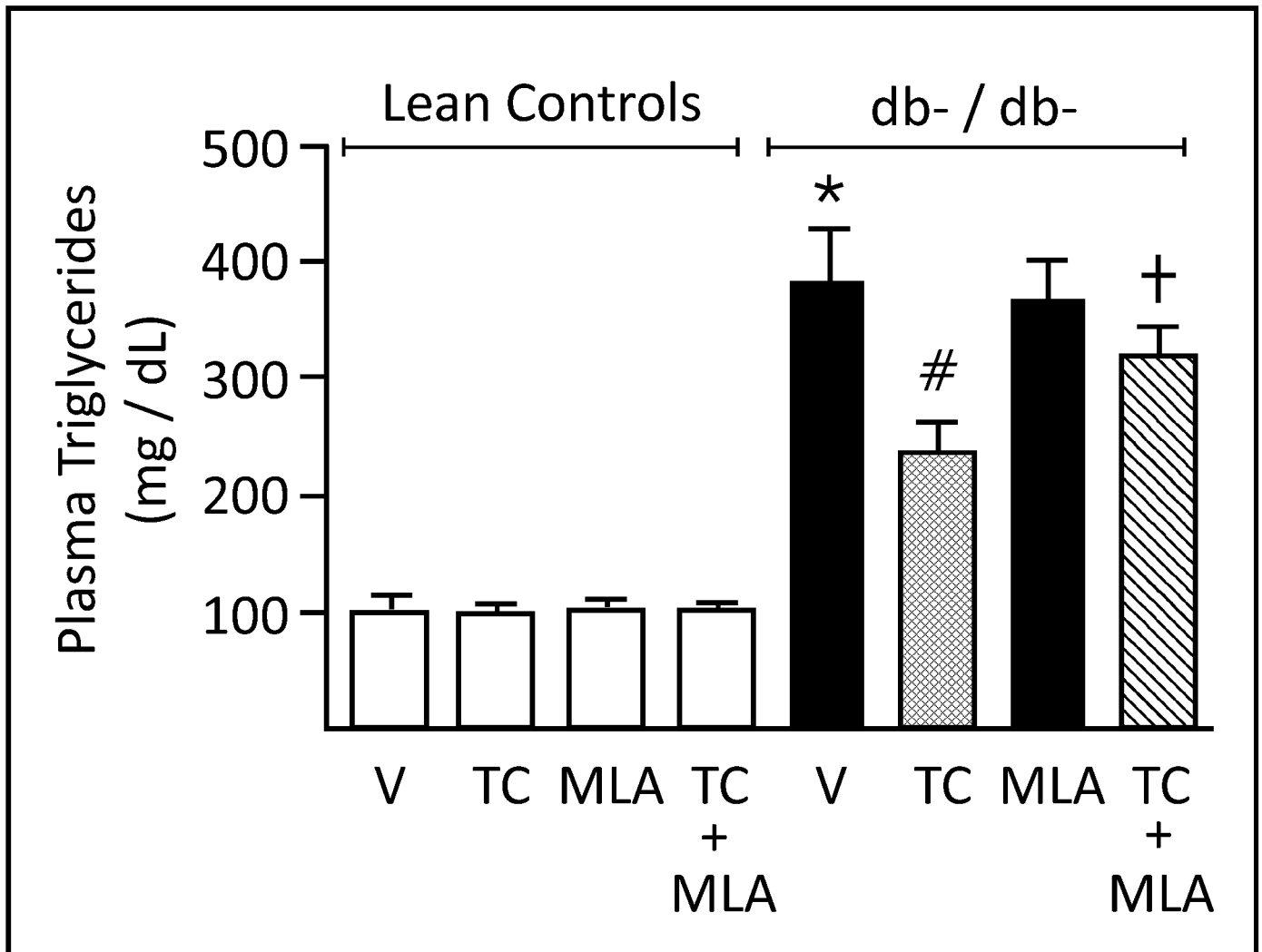


Figure 6

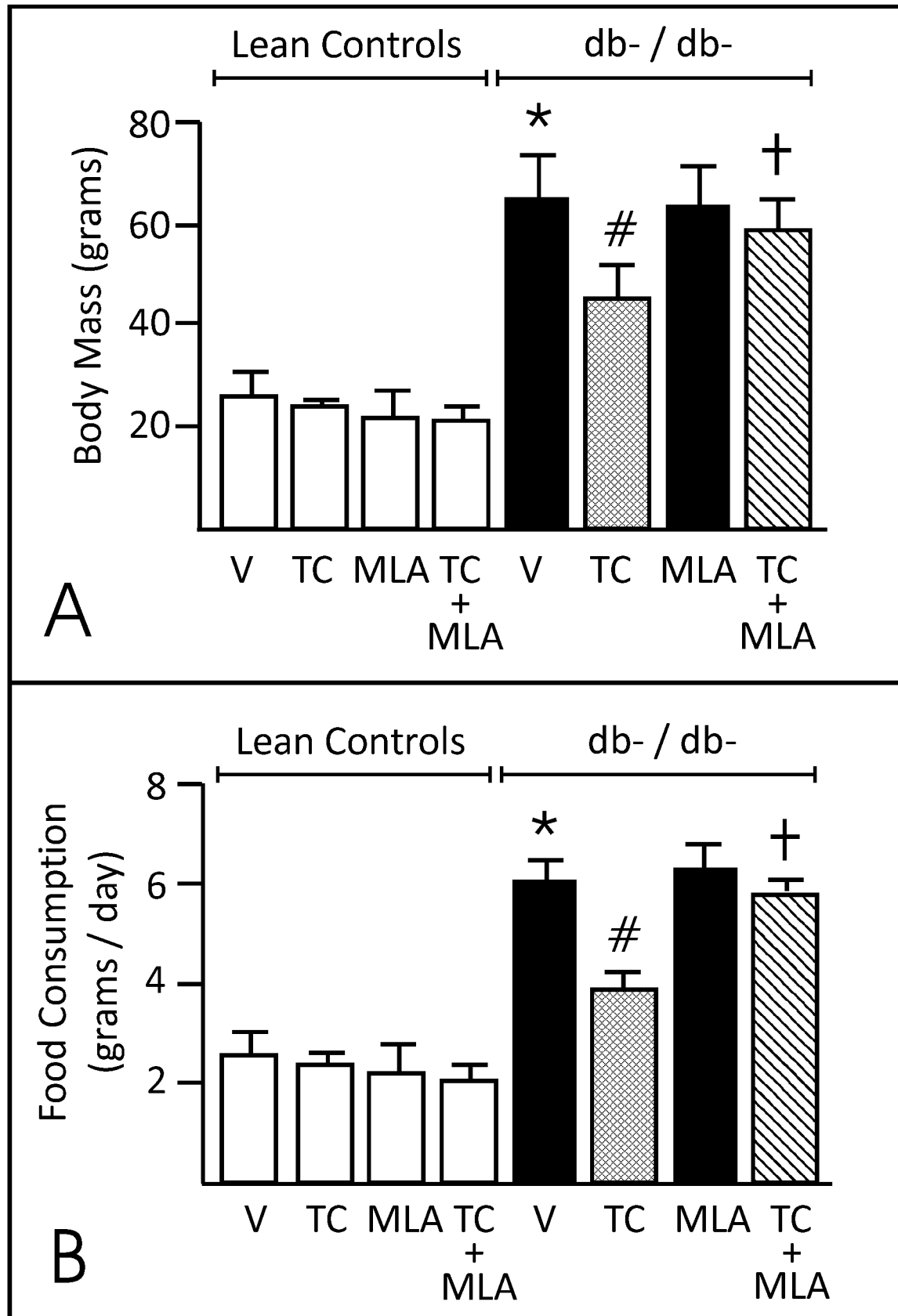


Figure 7

