INTRODUCTION

In the past few decades there has been a dramatic increase in the incidence rates of type 2 diabetes as well as obesity in US (Ahmad and Crandall, 2010). This trend cannot be explained completely by dietary, social and behavioral changes that have occurred during the same time (Biro and Wien, 2010). Simultaneously, emerging evidence suggests that persistent organic pollutants, including insecticides, are positively linked to altered glucose homeostasis (Boada et al., 2007; Montgomery et al., 2008; Son et al., 2010; Rezg et al., 2010; Lee et al., 2011). There is currently limited knowledge on the underlying biochemical mechanisms by which insecticides alter glucose homeostasis. Those include Slotkin et al. (2005) and Lassiter et al. (2008) reporting exposure to organophosphorus insecticides (parathion and chlorpyrifos) during the developmental period disrupted glucose homeostasis in rats (Slotkin et al., 2005; Lassiter et al., 2008). However, other insecticides have not been tested for their potential contribution with regard to glucose homeostasis.

Among the major classes of insecticides, neonicotinoids are the newest and largest single insecticide class on the market currently, representing about 27% global insecticides used in 2010 (Jeschke et al., 2011). Neonicotinoids are not only used for agricultural crop protection but also for controlling fleas and ticks for household pets, which implies significant exposure risk for humans. Neonicotinoids are broad-spectrum insecticides, acting on nicotinic acetylcholine receptors providing a selective toxicological profile due to a higher affinity for insect nicotinic acetylcholine receptors compared to the vertebrate equivalents (Matsuda et al., 2001; Bal et al., 2010; Li et al., 2011). Previously the correlation between nicotinic acetylcholine receptor, particularly α7 subtype, and improved insulin sensitivity was reported (Xu et al., 2012). However, there are no studies addressing the potential contribution of neonicotinoids on diabetes incidence in humans. Therefore, the purpose of this study was to determine the potential role of neonicotinoid insecticides, including imidacloprid, a neonicotinoid insecticide, on glucose metabolism.
cide in glucose metabolism. We particularly selected imidaclorpid for the current study since it is the most widely used neonicotinoid insecticide, accounting for approximately 41.5% of neonicotinoid use (Jeschke et al., 2011). Up to 75% of insulin stimulated glucose uptake occurs in muscle, however, adipocytes and hepatocytes also play significant roles in glucose uptake and synthesis, respectively (Klip and Paquet, 1990; Abel et al., 2001; Saltiel and Kahn, 2001). Thus, we used all three types, 3T3-L1 adipocytes, C2C12 myotubes, and HepG2 hepatocytes for the current experiments.

MATERIALS AND METHODS

Materials

3T3-L1 preadipocytes (mouse), C2C12 myoblasts (mouse), and HepG2 hepatoma (human) were purchased from American Type Culture Collection (Manassas, VA, USA). Dubelco’s modified Eagle’s medium (DMEM), Kaighn’s Modification of Ham’s F-12 (F-12K), fetal bovine serum (FBS), methylisobutylxanthin, dexamethasone, insulin, dimethyl sulfoxide, imidacloprid (N-[1-[(6-chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide), and protease inhibitor cocktail were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). RIPA Buffer with EDTA and EGTA was from Boston Bioproducts Inc. (Ashland, MA, USA). Horse serum was purchased from Invitrogen (Grand Island, NY, USA).

Cell culture

3T3-L1 preadipocytes were cultured as previously described (Park et al., 2013). HepG2 hepatoma and C2C12 myoblast cells were maintained at 37°C in DMEM containing 10% FBS. 3T3-L1 adipocytes were treated with imidacloprid for 6 days starting from day 0. At 80% confluence (designated as day 0), HepG2 cells were treated with imidacloprid containing media (DMEM with 10% FBS) for 4 days, and C2C12 cells were differentiated into myotubes using media that contained 3% horse serum and differentiated for 6 days along with imidacloprid. Cells were treated with imidacloprid at final concentrations of 10 or 20 μM by adding stock solution of 100 mM imidacloprid in dimethyl sulfoxide. Control was treated with dimethyl sulfoxide only and all treatments had dimethyl sulfoxide at a final concentration of 0.02%. These concentrations of imidacloprid had no effects on cell viability measured by a 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) based assay in all cell culture models used (data not shown) (Gerlier and Thomasset, 1986).

Measurement of glucose uptake

Glucose uptake was measured by using fluorescent D-glucose analog (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose, 2-NBDG) as previously described (Zou et al., 2005). After treatment of imidacloprid (6 days for 3T3-L1 & C2C12 and 4 days for HepG2), media were changed to F-12K (7 mM D-glucose) with 10% fetal bovine serum, and the culture was continued two more days. The cells were then cultured in serum-free F-12K medium (7 mM D-glucose) for 12 hr. Glucose uptake following insulin treatment was measured by adding 100 μg/ml of 2-NBGD into serum-free F12K medium (33 mM D-glucose) with or without 100 nM bovine insulin for 15 min. During insulin-resistant induction and glucose uptake determination, the cells were treated with 0 (control), 10 or 20 μM imidacloprid. Glucose uptake was compared based on insulin induced glucose uptake of insulin-resistant induced cells grown without imidacloprid.

Immunoblotting

Cell protein was harvested after cells were treated with 100 nM bovine insulin for 15 min and immunoblotting was conducted as previously described (Park et al., 2013). Antibodies for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5,000), and horseradish peroxidase conjugated goat Anti-rabbit IgG (1:5,000) were purchased from Abcam Inc. (Cambridge, MA, USA). Protein kinase B (AKT, 1:1,000), phosphorylated AKT (pAKT, 1:1,000), and phosphorylated p70-S6 kinase (pS6K, 1:1,000) were purchased from Cell Signaling (Berberly, MA, USA). GAPDH expression was used as an internal control to normalize protein content. Blot image and results were quantified using Image J software.

Statistical analyses

Data were expressed as mean ± S.E. values and analyzed using the analysis of variance procedure (ANOVA) of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Significant differences between treatments were determined using Duncan’s multiple-range test. Significance of differences was defined at the P < 0.05 level.

RESULTS AND DISCUSSION

Fig. 1 shows the influence of imidacloprid on glucose uptakes in all three types of cell models used. Treatment of imidacloprid, at 10 or 20 μM, did not influence the non-insulin stimulated glucose uptake in adipocytes, myotubes, or hepatocytes. However, when cells were stimulated with insulin, imidacloprid significantly decreased glu-
cose uptake in all three models.

We further analyzed the influence of imidacloprid on protein kinase B (AKT), which is one of the major signaling molecules of glucose homeostasis (Summers et al., 1998; Bryant et al., 2002). As expected insulin treatment significantly increased phosphorylation of AKT in all cell types tested (Fig. 2). Imidacloprid treatments at 10 or 20 μM did not alter overall AKT levels (Fig. 2). However, the activation of AKT (phosphorylated AKT, pAKT) was significantly reduced by imidacloprid treatments, except when 10 μM of imidacloprid was used in myotubes (Fig. 2). These results suggest that imidacloprid potentially impaired downstream targets of AKT, such as glucose transporter 4 (GLUT4) translocation in myotubes and adipocytes, or glycolysis and glycogen synthesis in hepatocytes (Summers et al., 1998; Bryant et al., 2002; Engelman et al., 2006). Treatment with imidacloprid significantly reduced insulin stimulated activation of S6K, a downstream target of AKT, further suggesting AKT-mediated impairment of insulin signaling by imidacloprid in these models (Um et al., 2004; Manning, 2004; Harrington et al., 2004). It is not clear why pAKT at 10 μM in myotubes was not influenced by imidacloprid. As seen in reduced pS6K under the same conditions and reduced pAKT at 20 μM in myotubes, imidacloprid may have adversely influenced glucose uptake via an AKT mediated mechanism in this model. Alternatively, there is an additional regulatory mechanism by which imidacloprid regulates insulin-stimulated glucose uptake.

Activation of a 160 kDa substrate of AKT (AS160) is a crucial step of glucose transporter 4 (GLUT4) trafficking (Sano et al., 2003), where this is often impaired in type 2 diabetic subjects (Karlsson et al., 2005). Thus the observation that imidacloprid impairs this signaling step suggests the exposure to this insecticide may contribute to development of type 2 diabetes. One potential mechanism of imidacloprid may be impairing insulin receptor substrate-1 (IRS-1) activation via calcium-dependent mechanisms. This is based on the fact that [i] imidacloprid is reported to increase oxidative stress, [ii] it increases intracellular calcium similar to other agonists of nicotinic acetylcholine receptors, and [iii] both of these effects are known to be associated with insulin resistance by impairing IRS-1 activation (Draznin, 1993; Zemel et al., 1995; Li et al., 2000; Kimura-Kuroda et al., 2012; Cui et al., 2013). It is further supported in a report by Tomizawa and Casida (2002) that desnitro-imidacloprid, an imidacloprid metabolite, activates nicotinic receptors via an intracellular calcium dependent mechanism (Tomizawa and Casida, 2002). Alternatively, impaired function of phosphatase and tension homolog (PTEN) or SH2-containing inositol phosphatase 2 (SHIP2) is correlated with type 2 diabetes (Krasilnikov, 2000; Butler et al., 2002; Baumgartener, 2003; Taniguchi et al., 2006). Thus it is possible that imidacloprid modulates phosphoinositide 3-kinase (PI3K), PTEN, or SHIP2, all of which are known to regulate cellular levels of phosphatidylinositol-3,4,5-triphosphate (PIP3), the key upstream regulator of AKT (Wu et al., 1998; Cantley and Neel, 1999; Wan and Helman, 2003; Engelman et al., 2006). Further mechanistic studies to determine the molecular target of imidacloprid as well as its metabolites with regard to altered insulin signaling would be needed in the future.

Previously it was reported that nicotine improves insu-

**Fig. 1.** Measurement of insulin stimulated glucose uptake following imidacloprid treatment. C2C12 and 3T3-L1 cells were treated with imidacloprid for 6 days starting at differentiation. HepG2 cells were treated for 4 days at 80% of confluency. Glucose uptake was determined with fluorescent D-glucose analog, 2-NBDG, after treatment w/o (○) or w/ (□) insulin (100 nM) for 15 min. Numbers are mean ± S.E. (n = 6). Means with different letters are significantly different at P < 0.05.
lin sensitivity via PI3K/AKT cascade mechanism by stimulating nicotinic acetylcholine receptor (Xu et al., 2012). This is inconsistent with our current results, as imidacloprid impaired insulin sensitivity. This discrepancy suggests that effects of imidacloprid on glucose homeostasis as we have seen here may be independent of its effects on nicotinic acetylcholine receptor.

Impaired hepatic glucose uptake was also reported in type 2 diabetes (Iozzo et al., 2003). A major glucose transporter in the liver is glucose transporter 2, which is not stimulated by insulin (Elsas and Longo, 1992). However, a moderate increase of glucose uptake due to insulin in HepG2 cells may be the result of increased hepatic glycogen synthesis, not via GLUT4. It is known that hepatic glycogen synthesis is controlled by AKT by affecting glycogen synthase kinase 3β and glycolysis (hexokinase and phosphofructokinase) (Manning and Cantley, 2007). Thus insulin stimulates AKT to stimulate glycogen synthesis and may result in increased glucose uptake. Reduction of glucose uptake by imidacloprid in hepatocytes compared to control in our results may suggest decreased intracellular clearance of glucose through AKT pathway (Fig. 2).

Imidacloprid is a potent and also the most used neonicotinoid insecticide (Jeschke et al., 2011). Compared to high lipophilic organochlorine and organophosphorus insecticides, imidacloprid is relatively water-soluble (International Programme on Chemical Safety, 2001; Flores-Cespedes et al., 2012). Imidacloprid has about a 3-year half-life in the environment and thus still poses significant exposure risk due to environmental residue in addition to its household applications. Currently, there are no reports on serum levels of imidacloprid from animals or humans. Based on the reports that serum levels of organochlorine pesticides were between 0.2 nM and 60 nM (Son et al., 2010; Rezg et al., 2010), it is likely that the doses used in the current studies are relatively...
high. However, since imidacloprid is commonly used in household products for pets, higher exposure levels may be possible due to direct contact with the products containing imidacloprid. It is also important to note that current models are limited with regard to metabolic aspects of imidacloprid since it is reported that imidacloprid is easily absorbed, distributed, and excreted from the body (International Programme on Chemical Safety, 2001). Thus the significance of the current findings needs to be carefully extrapolated for human perspective.

In conclusion, our current finding is the first on the role of imidacloprid in the development of impaired glucose metabolism in myotubes, adipocytes, and hepatocytes. It is particularly important to note the impairment of glucose uptake by imidacloprid in myotubes, since the majority of glucose uptake occurs in muscle (Klip and Paquet, 1990; Abel et al., 2001). Along with our recent report of increased adipogenesis due to imidacloprid treatment in 3T3-L1 adipocytes (Park et al., 2013), the current results suggest the significance of imidacloprid in the development of obesity and its related diseases such as type 2 diabetes. Further mechanistic studies in animal models as well as epidemiological evaluation and potential exposure risk for imidacloprid will be necessary prior to concluding the significance of imidacloprid exposure and the incidence of type 2 diabetes.

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