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Imidacloprid, a Neonicotinoid Insecticide, Potentiates Adipogenesis in 3T3-L1 Adipocytes

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ABSTRACT: There is emerging evidence that organochlorine and organophosphorus insecticide exposure may be linked to excessive weight gain and symptoms of diabetes. However, there is a lack of knowledge for other types of insecticides with potential influence on obesity and diabetes. Thus, the purpose of this investigation was to determine the role of imidacloprid, a neonicotinoid insecticide, in lipid metabolism by use of 3T3-L1 adipocytes. Imidacloprid treatment potentiated lipid accumulation in 3T3-L1 adipocytes and significantly increased expression of a key regulator of adipocyte differentiation and key regulators of lipogenesis. These results imply the involvement of imidacloprid in altered adipogenesis, resulting in increased fat accumulation. This finding is the first report of a potential link between neonicotinoid insecticide exposure and lipid accumulation in adipocytes. Further in vivo as well as epidemiological studies will be required before we can extrapolate these findings to a potential contribution of imidacloprid in human obesity.

KEYWORDS: *Imidacloprid, neonicotinoid, lipid metabolism, obesity, adipocyte differentiation*

INTRODUCTION

During the 20th century, the exponential use of organic insecticides occurred with the help of the syntheses of numerous organic insecticides. Among them, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, known as DDT, was one of the most successful synthetic organic insecticides used to control malaria and typhus during World War II.[1] However, since toxicological effects of DDT are known, such as its effects on human health and wildlife populations, there are significant efforts to evaluate the safety as well as environmental impact of insecticides.[2] Although most developed countries have banned the use of DDT and its analogues, some of them are widely present in the environment and even food due to high hydrophobic and very stable characteristics.[2] Coincidentally, the current obesity epidemic, including the rise in childhood obesity, cannot be explained completely by dietary, social, and behavioral changes that have occurred in the last few decades.[3,4] As a potential cause of obesity and its associate pathologies, a growing body of evidence suggests the contribution of exposure to environmental chemicals, including insecticides.[5−15]

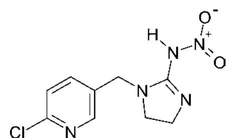

Figure 1. Structure of imidacloprid, *N*-[1-[(6-chloro-3-pyridyl)-methyl]-4,5-dihydroimidazol-2-yl]nitramide.

Insecticides are classified on the basis of their structures and modes of action; organochlorines, organophosphorus, carbamates, pyrethroids, and neonicotinoids. There is emerging evidence that organochlorine and organophosphorus insecticide exposures are linked to excessive weight gain and increased symptoms of diabetes.[5−15] Among others, neonicotinoids are the newest and fastest-growing class of insecticides.[16] Neonicotinoids are widely used in agricultural crop protection and animal health applications, representing ~24% of the global insecticide market in 2008.[16] Recent animal studies with neonicotinoids report increased oxidative stress.[17−21] Since increased oxidative stress has been linked to development of obesity and diabetes, we hypothesize that neonicotinoid exposure will have implications for these pathologies as seen with other insecticides.[22−24]

Imidacloprid (Figure 1), a potent neonicotinoid, was first introduced commercially in the early 1990s. Imidacloprid is considered to be “moderately toxic” on the basis of data from rodents: oral LD_{50} values for rats and mice are 380−650 and 130−170 mg/kg of body weight, respectively.[25] Compared to organochlorine and organophosphorus insecticides, imidacloprid is relatively water-soluble (0.5−0.6 g/L).[25,26] Nonetheless, it has been found to be persistent in the environment with approximately a 3-year half-life under aerobic conditions.[27] In addition to its agricultural application, imidacloprid is used as a common flea and tick application for pets. Thus there is great imidacloprid exposure risk at relatively high concentrations, particularly for people living with pets. Therefore, the purpose of this investigation was to determine the potential role of

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imidacloprid exposure in lipid metabolism by use of 3T3-L1 adipocytes, which are a well-characterized adipocyte model.[28]

MATERIALS AND METHODS

Materials. 3T3-L1 preadipocytes were purchased from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), methylisobutylxanthin, dexamethasone, insulin, dimethyl sulfoxide, and imidacloprid (*N*-[1-[(6-chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide) were purchased from Sigma–Aldrich Co. (St. Louis, MO). Triglyceride and protein were quantified by use of kits from Genzyme Co. (Cambridge, MA) and Bio-Rad Co. (Hercules, CA), respectively. Trizol reagent was from Invitrogen Corp. (Carlsbad, CA). Moloney murine leukemia virus (M-MLV) reverse transcriptase (high-capacity reverse transcription kit) was from Applied Biosystems (Carlsbad, CA). RIPA buffer with EDTA and EGTA supplemented 1% protease inhibitor cocktail was from Boston Bioproducts Inc. (Ashland, MA).

3T3-L1 Culture. 3T3-L1 preadipocytes were cultured as previously described with minor modifications.[29] Briefly, 3T3-L1 preadipocytes were maintained at 37 °C in DMEM containing 10% FBS to confluence. At 2 days postconfluence (designated day 0), adipocyte differentiation was induced with a mixture of methylisobutylxanthin (0.5 mM), dexamethasone (1 μM), and insulin (1 μg/mL) in DMEM containing 10% FBS. On day 2 this medium was replaced with DMEM containing 10% FBS and insulin only. On day 4 and thereafter, medium consisting of DMEM plus 10% FBS was subsequently replaced with fresh medium at 2-day intervals. Cells were treated with imidacloprid at final concentrations of 10 or 20 μM by adding stock solution of 100 mM imidacloprid in dimethyl sulfoxide starting at day 0. Control was treated with dimethyl sulfoxide only, and all treatments had dimethyl sulfoxide at a final concentration of 0.02%. Previous studies reported serum levels of organochlorine pesticides and acceptable daily intake of organophosphorus insecticides ranged from 0.2 to 60 nM or 0.0002–0.03 mg/kg of body weight.[8,10] Thus, concentrations of imidacloprid used in our study were higher than potential exposure levels. However, there are no data available for serum levels of imidacloprid and it is important to consider the potentially high direct contact exposure risk, as imidacloprid is currently used to treat pets. These concentrations of imidacloprid had no influence on cell viability measured by a 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) based assay (data not shown).[30]

At day 8, cells were washed with phosphate-buffered saline and stained with Oil Red O for 20 min, according to methods reported elsewhere.[31] Photomicrographs were taken after the cells were air-dried. The triglyceride and protein contents in the cell lysates were quantified by use of commercially available kits according to the manufacturer's protocols. The triglyceride level was expressed as the amount of triglyceride per milligram of protein.

mRNA Expression Analysis. Total RNA from cells was extracted by use of Trizol reagent under ribonuclease-free conditions. Total RNA was reverse-transcribed by use of Moloney murine leukemia virus (M-MLV) reverse transcriptase (high-capacity reverse transcription kit, Applied Biosystems). mRNA expression levels of CCAAT/enhancer-binding protein α (C/EBPα), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL), glucose transporter 4 (GLUT4), and leptin were analyzed from 3T3-L1 adipocytes. Real-time polymerase chain reaction (PCR) was performed on a StepOne Plus real time PCR instrument (Applied Biosystems, Carlsbad, CA) and Taqman probe-based gene expression analysis (Applied Biosystems, Carlsbad, CA). Respective integrated sequences for Taqman gene expression were NM_007678.3 (C/EBPα), NM_133360.2 (ACC), NM_007988.3 (FAS), NM_001039507.1 (HSL), NM_008509.2 (LPL), NM_001163689 (ATGL), NM_009204.2 (mouse GLUT4), and NM_008493.3 (leptin), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_008084.2) as an internal standard.

Immunoblotting. Cells were washed twice with cold phosphate-buffered saline, suspended in RIPA buffer with EDTA and EGTA (Boston Bioproducts Inc., Ashland, MA) supplemented with 20× protease inhibitor cocktail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and phosphatase inhibitors (1 mM Na_2VO_4 and 1 mM NaF) on ice for 30 min, and centrifuged at 12000*g* for 15 min at 4 °C. Protein concentration of the cell lysate of each sample was determined by use of DC protein assay kit (Bio-Rad, Foster City, CA) with bovine serum albumin standard. Aliquots (30 μg) of protein were separated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and transferred to an Immobilin P membrane (Millipore, Bedford, MA). The blots were blocked with Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and 5% bovine serum albumin for 1 h at 4 °C with agitation. After three washes with TBS-T, the membranes were incubated with a specific primary rabbit antiserum in TBS-T with 5% bovine serum albumin overnight at 4 °C with agitation. The membranes were washed with TBS-T three times and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h at room temperature. Detection was performed by use of an enhanced chemiluminescence solution (GE Heatlthcare, Piscataway, NJ) with an Image Station 4000MM (Carestream Health, New Haven, CT). Antibodies for PPARγ (1:400), GAPDH (1:5000), and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) were purchased from Abcam Inc. (Cambridge, MA). ACC (1:1000), phosphorylated ACC (pACC, 1:1000), AMP-activated protein kinase α (AMPKα, 1:1000), and phosphorylated AMP-activated protein kinase α (pAMPKα, 1:1000) were purchased from Cell Signaling (Berberly, MA). Glyceraldehyde-3-phosphate dehydrogenase expression was used as an internal control to normalize protein content. Blot image and results were quantified by use of Image J software.

Statistical Analyses. Data were expressed as mean ± standard error (SE) values and analyzed by the analysis of variance procedure (ANOVA) of the Statistical Analysis System (SAS Institute, Cary, NC). Significant differences between treatments were determined by Duncan's multiple-range test. Significance of differences was defined at the $P < 0.05$ level.

RESULTS AND DISCUSSION

Figure 2A shows lipid accumulation (in red) after treatment with imidacloprid during the adipocyte differentiation period. Cells treated with imidacloprid, at concentrations of 10 and 20 μM, had more lipid droplets compared to the control. This increase was quantified as total intracellular triglyceride accumulation as shown in Figure 2B. Imidacloprid treatments, at both concentrations tested, significantly increased total triglyceride accumulation in these cells, by 91% and 116%, respectively, compared to control (Figure 2B).

These results were further supported by the finding that imidacloprid treatment increased expression of CCAAT/enhancer-binding protein α (C/EBPα), which is one of the early key molecular markers of adipocyte differentiation (Figure 3A). In addition, expression of two key lipogenesis enzymes, acetyl Co-A carboxylase (ACC, Figure 3B) and fatty acid synthase (FAS, Figure 3C), were dose-dependently increased by imidacloprid treatment compared to those of control. These results show that imidacloprid increased adipocyte differentiation and lipogenesis, contributing to increased lipid accumulation.

Imidacloprid did not influence lipid mobilization in 3T3-L1 adipocytes, as observed by no effects of imidacloprid on expression of hormone-sensitive lipase (HSL, Figure 3D) and lipoprotein lipase (LPL, Figure 3E), which are key enzymes for lipolysis and lipid uptake, respectively.[32,33] Expressions of adipose triglyceride lipase (ATGL, Figure 3F), known to be correlated with increased adiposity,[34] was increased after imidacloprid treatment.

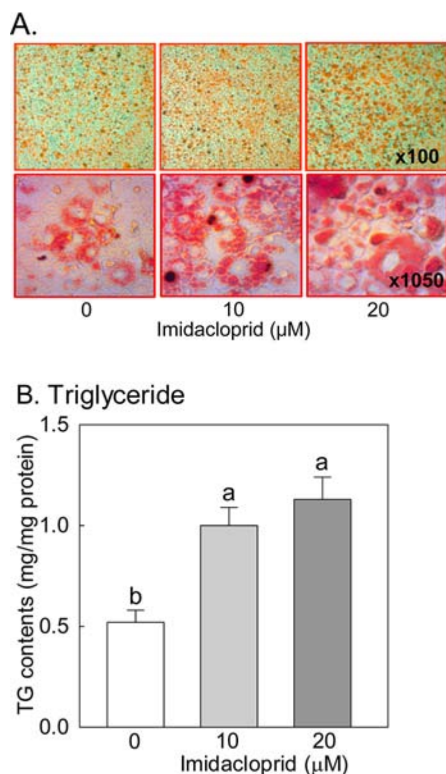

Figure 2. Imidacloprid treatment increased lipid accumulation during differentiation of 3T3-L1 adipocytes. Cells were treated with imidacloprid at 10 or 20 μM for 8 days during differentiation period. (A) Lipid droplets: Fat droplets were stained red with Oil Red O (×100 and ×1050). (B) Total triglyceride content: Intracellular triglyceride content was determined. Numbers are mean ± SE (n = 6). Means with different letters indicate significant difference at $P < 0.05$.

Expression of glucose transporter 4 (GLUT4, Figure 3G) was increased by imidacloprid treatment. This finding implies that imidacloprid may have altered glucose metabolism, which also contributes to increased lipid accumulation in this model. It was expected that leptin signaling would be increased after imidacloprid treatment since cells had more lipid accumulation (Figure 2B). However, no differences were observed in mRNA levels of leptin due to imidacloprid treatment compared to control in this model (Figure 3H). We did not measure leptin secretion in this study; however, it is known that expression of leptin is well correlated to leptin secretion in the 3T3-L1 model.[35] Lassiter and Brimijoin[15] previously reported impaired leptin production following organophosphorus insecticide treatment. Thus, the lack of effects on leptin seen in our study suggests potential impairment of leptin signaling caused by imidacloprid. This may further contribute to impaired lipid accumulation, as leptin plays an important role in regulating food intake and glucose metabolism.[36,37]

Protein expression of ACC and the phosphorylated form of ACC (pACC, the inactive form) were significantly modulated by imidacloprid (Figure 4A−C).[38] Consistently, one of the key adipogenesis markers, peroxisome proliferator-activated receptor-γ (PPARγ), was significantly increased by imidacloprid treatment (Figure 4D). In addition, expression of AMPKα and phosphorylated AMPKα (pAMPKα, the active form) were influenced by imidacloprid (Figure 4E-G). These results are further evidence that imidacloprid increased adipogenesis.

It is important to point out that our current results are limited to an in vitro model with relatively high doses of imidacloprid. Previous studies reported serum levels of organochlorine pesticides ranged from 0.2 to 60 nM.[8,10] Thus, levels of imidacloprid used in the present study are likely higher than potential exposure levels. However, it is important to consider risk from potential high exposure of imidacloprid due to direct contact, as it is currently used for household pets. The in vitro model is also limited in that we cannot determine the potential contribution of metabolism, as it is known that imidacloprid is readily absorbed and distributed to the body and excreted through urine and feces within 48 h.[25]

Previous in vivo studies suggested that imidacloprid may cause oxidative stress.[17−21] As oxidative stress is correlated with development of obesity, it is important to evaluate whether imidacloprid's role in oxidative stress contributes significantly to lipid accumulation.[23,39] There is also a report that oral administration of imidacloprid resulted in lower body weights in rats.[40] Thus, further in vivo studies of imidacloprid and its metabolites and the contribution of imidacloprid to oxidative stress are necessary to confirm its potential role in development of obesity.

In conclusion, our present finding is the first report on the influence of imidacloprid, a potent neonicotinoid, on increased adipocyte differentiation and increased lipid accumulation in adipocytes. Our present results are significant in substantiating the potential link between insecticide exposure, particularly imidacloprid, and impaired adipocyte functions. However, further in vivo as well as epidemiological studies will be required before we can extrapolate these findings to a potential contribution of imidacloprid in human obesity.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS:

ACC, acetyl-CoA carboxylase; AMPKα, AMP-activated protein kinase α; ATGL, adipose triglyceride lipase; C/EBPα, CCAAT/enhancer-binding protein α; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; FAS, fatty acid synthase; G6P, glucose-6-phosphatase; GLUT, glucose transporter; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; PPARγ, peroxisome proliferator-activated receptor-γ; TG, triglyceride

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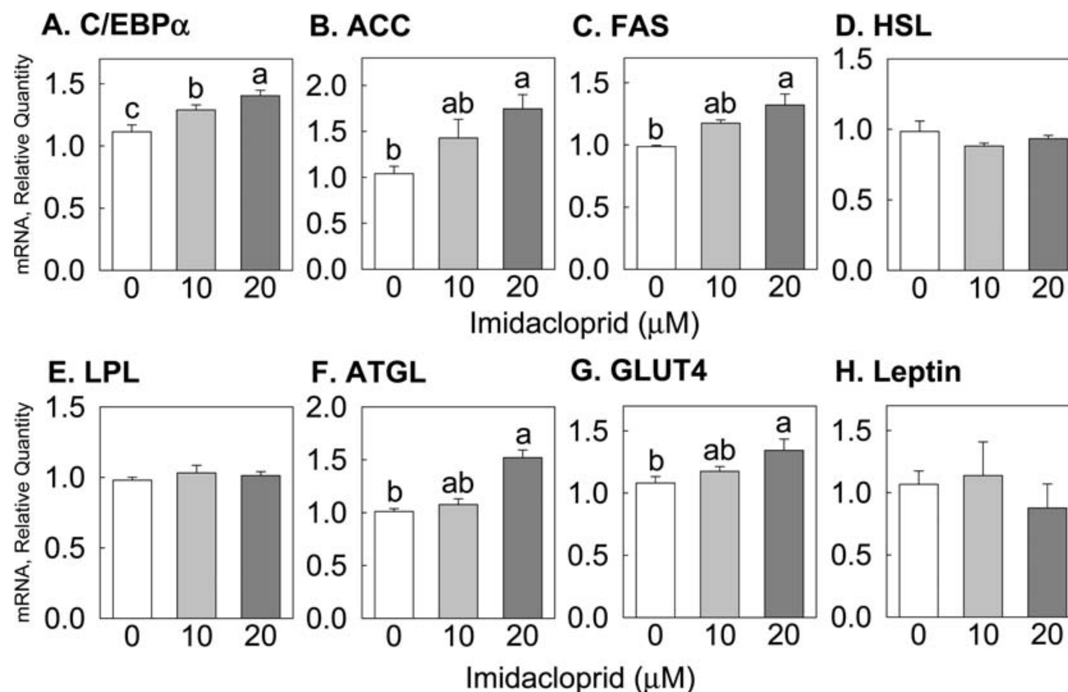

Figure 3. Effects of imidacloprid on expression of selected genes in lipid metabolism. Cells were treated with imidacloprid (10 or 20 μM) for 8 days during differentiation period. (A) CCAAT-enhancer-binding protein (C/EBPα); (B) acetyl-CoA carboxylase (ACC); (C) fatty acid synthase (FAS); (D) hormone-sensitive lipase (HSL); (E) lipoprotein lipase (LPL); (F) adipose triglyceride lipase (ATGL); (G) glucose transporter 4 (GLUT4); (H) leptin. GAPDH was used as an internal standard. Numbers are mean ± SE ($n = 6$). Means with different letters in each panel indicate significant difference at $P < 0.05$.

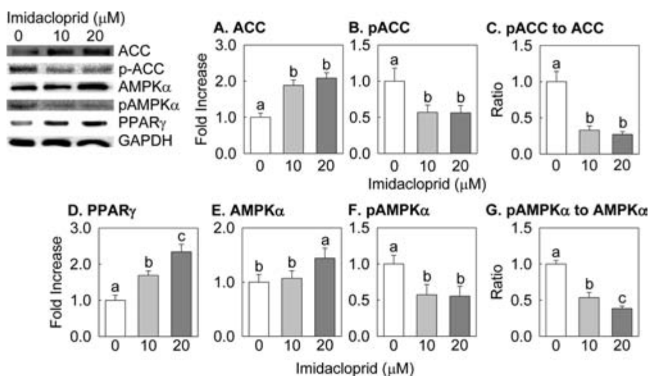

Figure 4. Effects of imidacloprid on expression of selected proteins in lipid metabolism. Cells were treated with imidacloprid (10 or 20 μM) for 8 days during differentiation period. (A) Acetyl-CoA carboxylase (ACC); (B) phosphorylated acetyl-CoA carboxylase (pACC); (C) pACC/ACC ratio; (D) peroxisome proliferator-activated receptor-γ (PPARγ); (E) AMP-activated protein kinase α (AMPKα); (F) phosphorylated AMPKα (pAMPKα); (G) pAMPKα/AMPKα ratio. GAPDH was used as an internal standard. Numbers are mean ± SE ($n = 4$). Means with different letters in each panel indicate significant difference at $P < 0.05$.

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